Pontifícia Universidade Católica do Rio Grande do Sul
Faculdade de Biociências
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PAPEL DO RECEPTOR P2X7 E DA ENZIMA CD39/NTPDASE1 NA RESPOSTA À RADIOTERAPIA EM GLIOMAS

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Orientadora: Profª Dr Fernanda Bueno Morrone

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RESUMO

Os gliomas representam a classe mais comum de tumores malignos do sistema nervoso central, sendo o tumor cerebral mais agressivo e letal entre os tumores cerebrais primários. Dentre os tratamentos, a radiação é uma das terapias mais utilizadas, porém a radiorresistência intrínseca destes tumores continua a ser um problema crítico na gestão de destes pacientes. Atualmente, sabe-se que o efeito da radiação se estende além da citotoxicidade direta causada nas células tumorais. A radioterapia parece induzir uma morte celular imunogênica, que entre as características está a liberação de ATP. O ATP pode causar citotoxicidade através do receptor P2X7 e também atua como um sinal de dano celular ativando o sistema imune. O ATP pode ser hidrolisado por enzimas do sistema purinérgico, dentre elas a ectonucleotidase CD39/NTPDase1, à adenosina, que tem um efeito aposto ao ATP, causando imunossupressão. A secreção de ATP induzida pela radioterapia e a capacidade deste nucleotídeo em modular a resposta imune, levantou a hipótese da participação da sinalização purinérgica na resposta de células tumorais à radiação e na resposta imune induzida pela radioterapia. Portanto, neste estudo visou-se investigar: i) o papel da ectonucleotidase CD39/NTPDase1 na resposta imune induzida pela radioterapia em gliomas, ii) a importância da via ATP-receptor P2X7 na resposta de gliomas à radioterapia. Através de camundongos *knockout* para a enzima CD39/NTPDase1, observamos que a deleção desta enzima combinada com a radioterapia reduziu significativamente as células imunossupressoras Tregs no tumor e no baço, atenuou a infiltração de células mieloides supressoras causada pela radiação, e aumentou a expressão de CCR7 em células dentríticas e macrófagos localizados no baço, indicando a presença células apresentadoras de antígeno recém-mobilizadas e disponíveis para se diferenciarem em células imunes efetoras que sustentam uma resposta imune mais prolongada mediada por células T antígeno específicas. Deste modo, mostrou-se que o bloqueio da atividade da CD39/NTPDase1 pode controlar mecanismos imunossupressores gerados pelo tumor e promete melhorar a resposta à radioterapia. Além disso, neste estudo observou-se que a radioterapia ativa o receptor P2X7 e através do silenciamento deste receptor na linhagem de glioma GL261, demonstramos que a radioterapia foi pouco eficiente *in vivo*, quando comparado com camundongos injetados com a GL261 WT, que expressa constitutivamente o receptor P2X7. Também demonstramos que pacientes com glioma que expressaram mais o receptor P2X7, apresentaram uma melhor resposta a radioterapia, revelando a importância da expressão deste receptor em células de glioma como um marcador útil para analisar a sensibilidade tumoral à radioterapia e para uma resposta bem-sucedida à radioterapia. Em suma, nossos dados lançam luz sobre a sinalização purinérgica para a modulação da resposta à radioterapia em gliomas.

Palavras-chave: ATP, gliomas, CD39/NTPDase1, receptor P2X7, sistema purinérgico, radioterapia

ABSTRACT

Gliomas represent the most common class of malignant tumors of the central nervous system being the most aggressive, and lethal brain tumors in primary brain tumors. Among the treatments, radiation is one of the most used therapies, but the intrinsic radioresistance of these tumors remains a critical problem in the management of these patients. Currently it is known that the effect of radiation extends beyond the directly cytotoxicity caused in tumor cells. Radiation therapy appears to induce an immunogenic cell death that among the features is ATP release. The ATP can cause cytotoxicity via P2X7 receptor and also acts as a sign of damage activating the immune system. The ATP can be hydrolyzed by enzymes of the purinergic system, among them the ectonucleotidase CD39, to adenosine, which has an opposite effect to ATP, causing immunosuppression. The radiation-induced ATP release and the ability of this nucleotide in modulate immune responses raised the hypothesis about the purinergic signaling participation in the tumor and immune cells response to radiation. Therefore, this study investigated: i) the role of ectonucleotidase CD39/NTPDase1 in the radiation-induced immune response in gliomas, ii) the importance of ATP-P2X7 receptor in the gliomas response to radiotherapy. Using knockout mice for CD39/NTPDase1, we observed that the deletion of this enzyme combined with radiotherapy significantly reduced the immunosuppressive cells Tregs in the tumor and spleen, attenuated the infiltration of myeloid derived suppressor cells caused by radiation and increased CCR7 expression in splenic dendritic cells and macrophages, indicating the presence of freshly mobilized antigen presenting cells available to differentiate in immune-effector cells that sustain a more prolonged antigen-specific T-cell–mediated immune response. Thereby, showing that blocking the activity of CD39/NTPDase1 can control immunosuppressive mechanisms generated by the tumor and promises to improve the radiotherapy response. Furthermore, in this study we observed that radiation actives the P2X7 receptor and by silencing this receptor on the GL261 glioma cell line, we have shown that radiotherapy is less efficient *in vivo* when compared with mice injected with GL261 WT cells, which constitutively express the P2X7 receptor. We also showed that patients with glioma that overexpress the P2X7 receptor, showed a better response to radiotherapy, revealing the importance of the expression of this receptor on glioma cells as a useful marker to analyze the tumor sensitivity to radiation and a successful radiotherapy response. In summary, our data shed light on the purinergic signaling for modulating the radiotherapy response in gliomas.

Key-words: ATP, gliomas, CD39/NTPDase1, P2X7 receptor, purinergic signaling, radiotherapy

LISTA DE ABREVIATURAS

7AAD – 7-amino actinomycin D

APCs – Células apresentadoras de antígeno

Arg1– Arginase 1

ATCC - American Type Culture Collection

ADP – Adenosina difosfato

AMP – Adenosina monofosfato

ATP – Adenosina trifosfato

BBG – Brilliant Blue G

CCL2 – Quimiocina (C-C motif) ligante 2

CCL3 – Quimiocina (C-C motif) ligante 3

CCL5 – Quimiocina (C-C motif) ligante 5

CCR7 – C-C quimiocina receptor tipo 7

CD39KO – CD39 knockout

DC – Células dendríticas

DMEM – Meio Dulbecco's modified Eagle's

EDTA – Ethylenediamine tetraacetic acid

EGFR – Fator de crescimento epidérmico

EHNA – Erythro-9-(2-hydroxy-3-nonyl) adenine inhibitor

FACS – Fluorescence-Activated Cell Sorting

FBS – Soro Fetal Bovino

GITR – Glucocorticoid-induced tumour necrosis factor receptor

GBM – Glioblastoma

HBSS – Solução de sais balanceados de hank

HIF-1α – Fator induzido por hipoxia-1α

HMGB1 – High mobility group box 1 protein

IACUC – Institutional Animal Care and Use Committee

ICD – Morte celular imunogênica

IHC – Imunohistoquímica

IL – Interleucina

IL-17 – Interleucina 17

IL-17R – Receptor de interleucina 17

iNos – Óxido nítrico sintase induzível

IFN-γ – Interferon γ

kDa – Kilodaltons

MAPK – P roteína quinase ativada por mitogeno

MDSC – Células supressoras derivadas de origem mieloide

MHC – Complexo de histocompatibilidade

MMP – Metaloproteinases

mRNA – RNA mensageiro

NCI – Instituto Nacional do Câncer

NF-kB – Fator transcricional nuclear kappa B

NTPDases – Nucleoside trifosfato difosfohidrolases

OMS – Organização Mundial da Saúde

NK – Natural Killer

ROS – Espécies reativas de oxigênio

RT – Radioterapia

RT-qPCR – Reação em cadeia da polimerase da transcrição reversa em tempo real

SNC – Sistema Nervoso Central

STAT3 – Fator de transcrição transdutor de sinais e ativador de transcrição 3

TAM – Macrófagos associados ao tumor

TLC – Cromatografia de camada fina

TNF-α – Fator de necrose tumoral α

Tregs – Células T regulatórias

UDP – Uridina trifosfato

UTP – Uridina difosfato

VEGF – Fator de crescimento endotelial vascular

WT – Tipo-selvagem

SUMÁRIO

1 INTRODUÇÃO

1.1 GLIOMAS

Segundo a Organização Mundial da Saúde (OMS) a incidência de câncer em nível mundial dobrou entre 1975 e 2000 [1]. Apesar de mais de 150 anos de esforços da medicina moderna no tratamento do câncer, esta doença continua a tirar milhões de vidas por ano em todo o mundo, e se pode observar pouco progresso, especialmente para pacientes com câncer metastático [2].

Os gliomas representam a classe mais comum de tumores malignos do sistema nervoso central (SNC) [3], sendo o tumor cerebral mais agressivo [4-7] e letal entre os tumores cerebrais primários [8]. A média de idade no momento do diagnóstico é de 64 anos e é 1.5 vezes mais comum em homens do que mulheres e duas vezes mais comum em brancos do que em negros [9-11]. A incidência aumentou ligeiramente ao longo dos últimos 20 anos principalmente devido à melhora no diagnóstico radiológico, e especialmente em idosos. Clinicamente, os pacientes com glioblastoma (GBM) podem apresentar cefaléia, déficits neurológicos focais, confusão, perda de memória, alterações de personalidade e/ou convulsões [11-12].

Quanto aos fatores de risco, a única causa ambiental exógena firmemente estabelecida é a exposição à radiação [11, 13]. Dados apoiam a plausibilidade biológica de fatores de risco ambientais como produtos químicos neuro-carcinogênicos, tais como compostos N-nitroso, espécies reativas de oxigênio e nitrogênio, vários produtos químicos utilizados industrialmente [13], como cloreto de vinilo ou pesticidas, trabalho com produção ou refinamento de petróleo, empregos em fábrica de borracha sintética [11], e hidrocarbonetos aromáticos policíclicos. Embora as pessoas possam estar expostas a muitos desses produtos químicos através da dieta, ocupação e hábitos pessoais, estudos da epidemiologia de gliomas em humanos têm gerado resultados inconsistentes ou nulos para esses fatores de risco [13]. Além de fatores de risco ambientais, alguns estudos associam o glioblastoma com o vírus humano herpesvirus-6 (HHV-6) [14-15]. Um subgrupo pequeno dos pacientes com glioma está associado com síndromes hereditárias. Todos os outros pacientes com glioma representam casos esporádicos. Fatores adicionais, tais como a exposição a campos eletromagnéticos residenciais, formaldeído, irradiação de diagnóstico e telefones celulares não foram comprovados como indutores do GBM

[11]. Dada à escassez de estudos conclusivos que examinaram fatores de risco ambientais para os glioma é prematuro concluir que os riscos ambientais não existam [13].

A OMS classifica os tumores cerebrais de acordo com sua agressividade, graus I e II ou gliomas de baixo grau são pouco evoluídos e considerados tumores benignos, e graus III e IV ou gliomas de alto grau, são considerados tumores malignos [3]. Os gliomas de origem astrocitária (astrocitomas) são classificados em astrocitoma pilocítico (grau I), astrocitoma difuso (grau II), astrocitoma anaplásico (grau III) e glioblastoma (GBM) (grau IV). Os tumores derivados de oligodendrócitos incluem grau II (oligodendroglioma ou oligoastrocitoma) e grau III (oligodendroglioma ou oligoastrocitoma anaplásico) [16-17]. Neste trabalho utilizamos linhagens de GBM, portanto nosso estudo foi embasado neste tipo tumoral.

O GMB se localiza preferencialmente nos hemisférios cerebrais, com maior freqüência nos lobos temporais e frontais, seguidos pelos lobos parietais e occipitais [9]. A transformação maligna das células gliais ocorre em várias etapas de alterações genéticas [12] e estes tumores são provavelmente compostos por um grupo heterogêneo de células, incluindo células tumorais diferenciadas e células progenitoras neurais [6]. O GBM desenvolve-se de duas maneiras: 1) Como um tumor primário (também chamado de "*de novo*") que progride rapidamente sem evidência clínica ou histológica de uma lesão precursora menos maligna. Este tumor afeta principalmente idosos e ocorre na maioria dos casos (90%) [12, 18]. 2) Como um tumor secundário que é oriundo da progressão de um astrocitoma difuso ou de astrocitoma anaplásico, e se manifesta em pacientes jovens [12, 18].

As características patológicas do GBM são exemplificadas por proliferação celular descontrolada, infiltração difusa, intensa resistência à apoptose, instabilidade genômica [12], células gigantes, pleomorfismo celular e nuclear [19]. Pode-se distinguir patológicamente o GBM de outros tumores cerebrais de baixo grau pela presença necrose e hiperplasia microvascular (angiogênese especializada) [12, 20]. Além disso, nos gliomas os focos necróticos são normalmente cercados por regiões "pseudopaliçadas" – que são zonas hipercelulares alinhandas em torno de focos irregulares de necrose tumoral –, esta é uma característica quase constante de glioblastomas e, portanto, as regiões "pseudopaliçadas" foram incorporadas na definição patológica deste tumor agressivo, distinguindo-o dos astrocitomas de baixo grau [21].

O tratamento do glioma é definido pelo grau de malignidade, pelo tipo de tumor, e pela idade dos pacientes [22]. O padrão atual de tratamento para pacientes com GBM recentemente diagnosticado consiste em cirúrgia máxima (idealmente, ressecção total bruta: > 95% da lesão) [3], seguida de radioterapia e quimioterapia com o agente alquilante temozolomida, de forma concomitante (radioquimioterapia), dentro de 30 dias após a cirurgia [17, 23-24], seguido por exames de imagem para monitorar a progressão tumoral pós-tratamento [25].

Os avanços recentes no padrão de tratamento dos tumores cerebrais com o uso das terapias citadas anteriormente levaram a uma melhora na sobrevida dos pacientes com GBM de uma média de 10 a 12 meses [5-6, 8] para 15 meses, após o diagnóstico [6, 22, 24]. Porém, por ser um tumor infiltrativo, o prognóstico ainda permanece incerto devido à presença de células tumorais residuais altamente invasivas [7, 26], resultando em um diagnóstico maior de pseudoprogressão [25], onde células tumorais invasoras escapam da remoção cirúrgica, bem como, da exposição à irradiação e da quimioterapia [12].

1.2 RADIOTERAPIA E GLIOMAS

A radioterapia tem sido amplamente utilizada para tratar pacientes com vários tipos de tumores primários e metastáticos [27], apresentando graus variáveis de sucesso. As estimativas atuais indicam que, de fato, pelo menos 50% de todos os pacientes com câncer foram ou vão ser expostos à irradiação ionizante [28-29].

A apoptose induzida pela radiação pode ocorrer por meio de mecanismos TP53-dependentes e independentes. As vias de transdução de sinal, que resultam em apoptose induzida por radiação envolvem o núcleo e o citoplasma, com alterações no transporte mitocondrial de elétrons, e na liberação do citocromo c das mitocôndrias, iniciando a clivagem de caspases, que finaliza na ativação de nucleases responsáveis pela digestão do DNA internucleossomal [30]. A radioterapia envolve também a produção de espécies reativas de oxigênio (ROS) como um intermediário chave na toxicidade tumor. Quando o nível de ROS sobe além de um limite tolerável dentro da célula, a rede de sinalização se altera e o processo de apoptose começa levando a toxicidade. ROS representa uma ameaça para várias moléculas celulares vitais, incluindo DNA, membrana celular, lipídios, proteínas e mitocôndrias. A mitocôndria é o

principal local de produção de radicais livres e, talvez, o principal alvo de dano induzido pela radiação, juntamente com membrana celular [31].

Desde que estudos randomizados na década de 1970 mostraram os benefícios clínicos da radioterapia em gliomas malignos, a cirurgia é seguida de radioterapia (total de 60 Gy de radiação fracionada, durante seis semanas) no glioma e nas margens do tumor, o que melhora claramente as taxas de sobrevivência [25, 32]. Porém, infelizmente, praticamente em todos os pacientes, algumas células radiorresistentes persistem em sites primários e retomam seu crescimento, com um mau prognóstico para os pacientes, fazendo com que estes sofram recidiva do tumor, apesar da irradiação agressiva, enfatizando a natureza radiorresistente do GBM [32-34]. As células tumorais podem escapar dos efeitos letais de irradiação por hipóxia, desregulação do ciclo celular ou da apoptose, ou por aumentar a sua capacidade de reparar os danos induzidos ao DNA, resultando na recorrência da doença [35].

Embora a radioterapia seja amplamente utilizada para o tratamento de pacientes com GBM, a radiorresistência intrínseca destes tumores continua a ser um problema crítico na gestão de tais pacientes [32]. A identificação dos mecanismos moleculares subjacentes à radiorresistência do GBM é essencial para o desenvolvimento de regimes de tratamento de radioterapia mais eficazes para este tipo tumoral [33] [34]. Espera-se que os estudos sobre a expressão de genes em grande escala possam contribuir para esclarecer o mecanismo de resistência celular subjacente à radioterapia. De fato, relatórios sobre a modulação da expressão gênica na resposta a radioterapia em linhagens celulares de tumores, como leucemia, linfoma, câncer cervical e pulmonar, têm indicado uma lista de genes responsivos à radiação. A caracterização de diferenças no perfil de expressão de genes em células tumorais irradiadas *in vitro* podem proporcionar informação acerca dos mecanismos moleculares envolvidos na radiorresistência [32].

1.3 SISTEMA IMUNE E GLIOMAS

A presença de leucócitos no microambiente tumoral, observado no século 19 por Rudolf Virchow, providenciou a primeira indicação de uma possível ligação entre inflamação e câncer. No entanto, foi somente na última década que se evidenciou claramente que a inflamação desempenha um papel crítico na tumorigênese [36]. O microambiente ao redor do tumor é

composto por uma combinação variável de células tumorais, células do sistema imunológico, células endoteliais e matriz extracelular [37]. Estas diversas células comunicam umas com as outras por meio de contato direto ou pela produção de citocinas e quimiocinas para controlar a forma e o crescimento do tumor. É a expressão de vários mediadores e moduladores imunes, bem como a abundância e estado de ativação de diferentes tipos de células no microambiente tumoral que determinam em que direção o equilíbrio é inclinado – se irá causar uma inflamação pró-tumoral ou uma imunidade antitumoral [36]. Entretanto, as células malignas parecem "burlar" o sistema imunológico e usá-lo a seu favor no processo de proliferação, sobrevivência e migração da neoplasia, causando uma imunossupressão [38-39]. A inflamação pró-tumoral e a imunidade antitumoral coexistem em diferentes pontos ao longo do percurso da progressão tumoral e as condições do microambiente tumoral determinam o equilíbrio entre os dois [36].

Os tumores utilizam diversas estratégias para evadiar a resposta imune. Dentre essas estratégias esta o aumento do nível de células imunosupressoras no tumor como as células supressoras derivadas de origem mieloide (MDSC) que inibem a função de linfócitos T efetores. Macrófagos associados ao tumor (TAM) também auxiliam na invasão e metástase tumoral, e as células B regulatórias locais podem produzir respostas humorais pró-tumorais. Além disso, gliomas apresentam mecanismos de evasão únicos. Estes mecanismos incluem o aumento dos níveis de células imunossupressoras T regulatórias (Tregs), a diminuição dos níveis de células T $CD8^+$ efetoras no microambiente do glioma, a ausência ou menor expressão de moléculas co-estimulatórias no cérebro, a expressão de moléculas co-estimulatórias, como a B7-H1, nos gliomas e a presença de células-tronco de glioma imunusupressoras que persistem apesar das respostas imunes antitumorais do hospedeiro [40].

Além disso, as células tumorais são capazes de induzir a produção de mediadores inflamatórios que agem a seu favor, direta ou indiretamente, ativando o endotélio vascular e recrutando leucócitos para o tumor, liberando fatores angiogênicos, mitogênicos, enzimas proteolíticas e fatores quimiotáticos. Esses processos promovem o recrutamento de mais células inflamatórias e estimula a angiogênese, sustentando, dessa forma, o crescimento do tumor e facilitando metástases [41]. Portanto, células mediadoras das respostas imunes são necessárias tanto para a progressão do tumor quanto para a eliminação deste [42]. No ambiente tumoral, os mediadores da resposta inflamatória e seus receptores representam importantes constituintes do

ambiente tumoral [41, 43]. A produção de citocinas atua como um meio de comunicação no microambiente tumoral. Células do sistema imunológico são a principal fonte de citocinas, mas muitas outras células humanas também são capazes de produzi-las. Citocinas podem ser classificadas nas seguintes categorias: interleucinas (IL), fatores de crescimento, fatores estimuladores de colônias, interferons (IFN) (α, β e γ), fator de necrose tumoral (TNF) e quimiocinas. Estas citocinas podem ter tanto atividade pró ou anti-inflamatória, dependendo do microambiente [37]. A interação entre citocinas e seus receptores resulta na formação de uma rede de interação no local do tumor, que é primariamente responsável pela progressão de tumores em geral, mas também pela disseminação de respostas imunes antitumorais e pela indução da rejeição tumoral [37].

A citocina interleucina-17 (IL-17, também conhecida como IL-17A) pertence a uma família de seis membros (IL-17A, IL-17B, IL-17C, IL-17D, IL-17E e 17F) [44-45]. A IL-17A, o membro mais conhecido desta família, foi identificada pela primeira vez em 1995 [46]. O produto humano do gene da IL-17A é uma glicoproteína homodimérica de 155 aminoácidos com um peso molecular de 35 kDa [44-45]. Esta citocina tem sido alvo de grande interesse devido à descoberta de que a produção de IL-17 caracteriza um novo subconjunto de células T *helper* $CD4^+$ (células Th17) [39]. Estudos realizados com células humanas indicam que outros tipos de células, além da Th17, também podem ser fonte da IL-17 sob certas condições, como as células CD8, T-γδ, natural killer invariante (NKi) [44, 47] e osteoclastos [39]. A IL-17 é caracterizada como uma citocina pró-inflamatória [44, 48], envolvida na imunidade inata e adaptativa [44, 46-47], que induz à liberação secundária de quimiocinas pró-inflamatórias e fatores de crescimento na maioria das principais células mesenquimais para o recrutamento e acúmulo de neutrófilos no sítio inflamatório [44].

Estruturalmente, a IL-17A não tem nenhuma sequencia similar com qualquer outras citocina conhecida ou outra proteína de mamífero. O seu receptor, IL-17R, não está relacionado com qualquer um dos outros receptores de citocinas conhecidos e não possui domínios reconhecíveis. Assim, a família de citocinas IL-17 parece representar um sistema de sinalização ligante-receptor distinto [46]. O receptor para IL-17A (IL-17R) é uma proteína transmembrana de passagem única de aproximadamente 130 kDa. Este receptor se expressa em todos os tecidos examinados até o momento [45, 48] e após a ligação da IL-17 ao seu receptor sabe-se que esta é

capaz de ativar o fator transcricional nuclear kappa B (NF-kB), que leva à expressão coordenada de vários genes que codificam proteínas envolvidas na síntese de mediadores inflamatórios e ainda na ampliação e perpetuação da resposta inflamatória [46, 48]. Além disso, as vias p38-MAPK (proteína quinase ativada por mitogeno), PI3K-AKT e ERK também parecem participar na produção de mediadores inflamatórios induzida pela IL-17 [49].

Os alvos celulares da IL-17 no microambiente do tumoral são leucócitos, células vasculares e endoteliais, células do estroma e células do próprio tumor [39]. Porém, a ação da IL-17 no câncer ainda é bem controversa. Resultados em relação a uma atividade pró ou antitumoral desta citocina não são unânimes. Porém, esses resultados heterogênios podem ser, em parte, devido a dose da IL-17 em cada um dos estudos. A concentração de IL-17 exógena pode ser diferente da concentração da IL-17 endógena, que é produzida pelas células Th17 e outras. Portanto, provavelmente a atividade biológica da IL-17 exógena em relação à endógena não é idêntica. Outra questão a ser destacada na ação da IL-17 em tumores é a imunidade do hospedeiro [39]. Estudos mostram que a resposta à IL-17 é diferente em camundongos imunodeficientes [50-51], quando comparada a camundongos imunocompetentes [39]. A IL-17 se mostrou elevada em vários tipos de câncer, mas como esta citocina pode contribuir para o crescimento do tumor é ainda pouco claro [52]. Uma hipótese é que a IL-17 induz a produção de IL-6 em células tumorais e células estromais associadas ao tumor que, por sua vez ativa o fator de transcrição transdutor de sinais e ativador de transcrição 3 (STAT3), um fator de transcrição oncogênico que aumenta a expressão de genes pró-sobrevivência e pró-angiogênicos. Porém, os efeitos da IL-17 sobre o crescimento tumoral podem ser altamente dependente do contexto [39]. Pesquisas atuais mostram que o aumento da IL-17 no microambiente tumoral, por mecanismos dependentes de células T, inibe o crescimento das células tumorais e aumenta a sobrevida em modelos murinos de câncer pancreático e hematopoiético [53-54]. Reforçando esta idéia, um grupo mostrou que o efeito antitumoral de células T infundidas em tumores (cólon, mama e sarcoma) foi perdido quando elas deixaram de expressar IL-17 [2] e ainda outro estudo demonstrou que a expressão induzida de IL-17 em melanomas suprime a progressão do tumor [39]. Esses estudos sustentam um papel antitumoral da IL-17 quando esta citocina está presente no microambiente tumoral. Opostamente, um estudo mostrou que a IL-17 endógena promove a formação do tumor induzido por *Bacteroides fragilis* e o crescimento tumoral em um modelo de

tumor transplantado em camundongos [39]. Outro estudo que que avaliou a ação da IL-17 produzida por tumores murinos (fibrosarcoma e adenocarcinoma de cólon, fracamente imunogênicos), como forma de avaliar a sua função biológica; mostrou que estes tumores, transfectados com IL-17, cresceram mais. Este estudo ainda caracterizou a IL-17 como mediadora de angiogênese, estimulando a migração de células do endotelias vasculares e regulando a produção de uma variedade de fatores pró-angiogênicos [50]. Nesta mesma linha, outros estudos também mostraram que a IL-17 exógena pode promover o crescimento do tumor por induzir vascularização do tumoral, particularmente em camundongos *nude* (imuno-deficientes). No entanto, o efeito global da IL-17 sobre o desenvolvimento e crescimento do tumor pode ser diferente em hospedeiros imuno-competentes, como mostrado pelos efeitos antitumorais potentes mediados pela IL-17 em camundongos imuno-competentes [39].

Recentemente, confirmou-se a presença da IL-17A em glioma experimental em camundongo e em amostras tumorais humanas [55]. Além disso, o RNAm do receptor da IL-17 se mostrou constitutivamente expresso em células de GBM e esta citocina estimulou a expressão de IkBa e a secreção de IL-6 e IL-8 nas células deste tumor [48]. Um estudo de Hu e colaboradores (2011) mostrou que não há diferença significativa no volume de células Th17 e na concentração de IL-17 sérica (apresentando uma concentração entre 5-10 pg/ml) de pacientes com glioma e doadores saudáveis [56]. Outro trabalho mostrou que *in vitro* a IL-17 por si só não exercem efeito sobre a proliferação de uma linhagem de glioma de camundongo (GL261) e *in vivo* gliomas cresceram igualmente bem em camundongos do tipo-selvagem e deficientes para IL-17 [57].

1.4 SINALIZAÇÃO PURINÉRGICA

A sinalização purinérgica, onde a adenosina 5'-trifosfato (ATP) e adenosina atuam como moléculas sinalizadoras extracelulares, foi proposta pela primeira vez em 1972 por Geoffrey Burnstock. Mais tarde, os receptores de purinas e pirimidinas foram clonados e caracterizados [58]. As purinas (ATP, ADP e adenosina) e pirimidinas (UTP e UDP) extracelulares são moléculas sinalizadoras que apresentam diversos efeitos sobre muitos processos biológicos, incluindo inflamação [59], proliferação, diferenciação e morte [60-62]. Esses nucleotídeos extracelulares podem ser hidrolisados por uma variedade de enzimas, denominadas

ectonucleotidases [63]. Várias ectonucleotidases foram clonadas a partir de diferentes tecidos, incluindo o cérebro, e devido a sua ação são formados ADP, AMP e adenosina a partir do ATP [64].

A degradação extracelular e inativação do ATP acontece por meio de uma cascata de enzimas da família NTPDase, nucleosídio trifosfato difosfohidrolase (EC 3.6.1.5). Oito genes diferentes codificam os membros da família de proteínas NTPDase – NTPDase 1 a 8. Quatro subtipos de NTPDases (NTPDase 1/CD39, NTPDase 2, NTPDase 3, NTPDase 8) são localizadas na superfície celular com um sítio catalítico virado para o meio extracelular. As NTPDases 5 e 6 exibem uma localização intracelular e sofrem secreção após expressão heteróloga. As NTPDases 4 e 7 são inteiramente localizadas no espaço intracelular. Notavelmente, as taxas de hidrólise para nucleosídeos difosfatos varia consideravelmente entre os subtipos. A NTPDase1 hidrolisa ATP e ADP igualmente bem, mas hidrolisa o ATP quase diretamente para AMP com a produção de apenas pequenas quantidades ADP. Em contraste, o ADP é liberado mediante a hidrólise de ATP pela NTPDase2, que então se acumula e é lentamente desfosforilado a AMP. Já a NTPDase3 e NTPDase8 têm uma preferência maior pelo ATP do que pelo ADP como substrato [65-66]. A cascata de hidrólise iniciada pelas NTPDases, gerando nucleosídeos-5'-monofosfato, como o AMP; pode ser terminada pela ação da ecto-5'-nucleotidase (E5'NT/CD73; EC 3.1.3.5), mas também pela família das E-NPP (ectonucleotídeo pirofosfatase/fosfodiesterase), que hidrolisam uma grande variedade de substratos, entre eles purinas e pirimidinas; e pela FAL (fosfatase alcalina) [26]. A ecto-5'-nucleotidase/CD73 reside largamente na glia (astrócitos, oligodendrocitos e microglia) [64]. Juntas, a ecto-5'-nucleotidase e a adenosina desaminase (ADA; EC 3.5.4.4), outra ectoenzima que está envolvida nas vias das purinas e que converte adenosina em inosina, regularam estritamente as concentrações de adenosina [65].

Além disso, os nucleotídeos e nucleosídeos extracelulares ativam a ampla família de receptores purinérgicos [67]. Em tecidos saudáveis, o ATP é quase exclusivamente localizado intracelularmente, enquanto que sua concentração extracelular em condições basais é insignificante (entre 10-100 nM) [68]. Existem duas principais famílias de receptores purinérgicos: receptores de adenosina ou P1 e os receptores P2, que têm alta afinidade por nucleosídeos di- e trifosfatados (ATP, ADP, UTP, e UDP) (Figura 1). Os receptores P1/adenosina são acoplados à proteína G e se subdividem em 4 subtipos, A_1, A_{2A} (principal

receptor de adenosina expresso por células T), A_{2B}, e A_3 [63, 67, 69-70]. Os receptores metabotrópicos P2Y são também acoplados à proteína G e já foram identificados oito membros dessa família (P2Y $_{1,2,3,4,6,11,12,13}$ e $_{14}$) [59, 70]. Esses receptores podem ativar a fosfolipase C (consequentemente liberando cálcio intracelular) ou, afetar a adenilil ciclase e alterar os níveis de cAMP [71]. Os receptores purinérgicos P2X são ionotrópicos e estão expressos em todas as células vivas e tecidos de vertebrados [67, 72], induzem o influxo de sódio e cálcio, ativando cascatas de sinalização intracelulares dependentes de cálcio, e o efluxo de potássio [60, 70, 73].

O receptor P2X7 possui várias características que o distinguem de outros membros da família dos receptores P2X [72-73]. A ativação do receptor homomérico requer concentrações de ATP que são ± 10-100 vezes maiores do que as necessárias para ativar os outros receptores P2X e que causariam a dessensibilização dos outros receptores do tipo P2X, enquanto que o receptor P2X7 não apresenta dessensibilização [67, 72, 74]. Além disso, este receptor possui uma cauda carboxi-terminal maior do que os outros P2X [73]. Baixas concentrações de ATP ativam o canal iônico do receptor P2X7 que se torna permeável aos íons de pequeno porte, enquanto que, quando exposto a altas concentrações de ATP ou, durante um longo período, pode ter seu canal iônico convertido em um grande poro transmembrana não-seletivo que permite a passagem não só de cátions, mas também de moléculas pequenas de até 900 dáltons, que em última análise, causa a morte celular [70, 73]. A ativação do receptor P2X7 é fundamentalmente diferente da observada para outros canais iônicos, pois se iniciam vários processos celulares adicionais. Estes incluem alterações na morfologia da célula, liberação de IL-1β, ativação do fator transcricional NF-κB, ativação da fosfolipase D, apoptose [72] mediada por caspases e/ou necrose [75] – a morte celular induzida pela ativação do receptor P2X7 é altamente dependente do tipo celular [76]. Portanto, a idéia de um receptor de membrana celular ligado a apoptose é um alvo atraente para a terapia tumoral [70] e vários estudos relatam que a expressão do receptor P2X7 está superativada em condições patológicas [60].

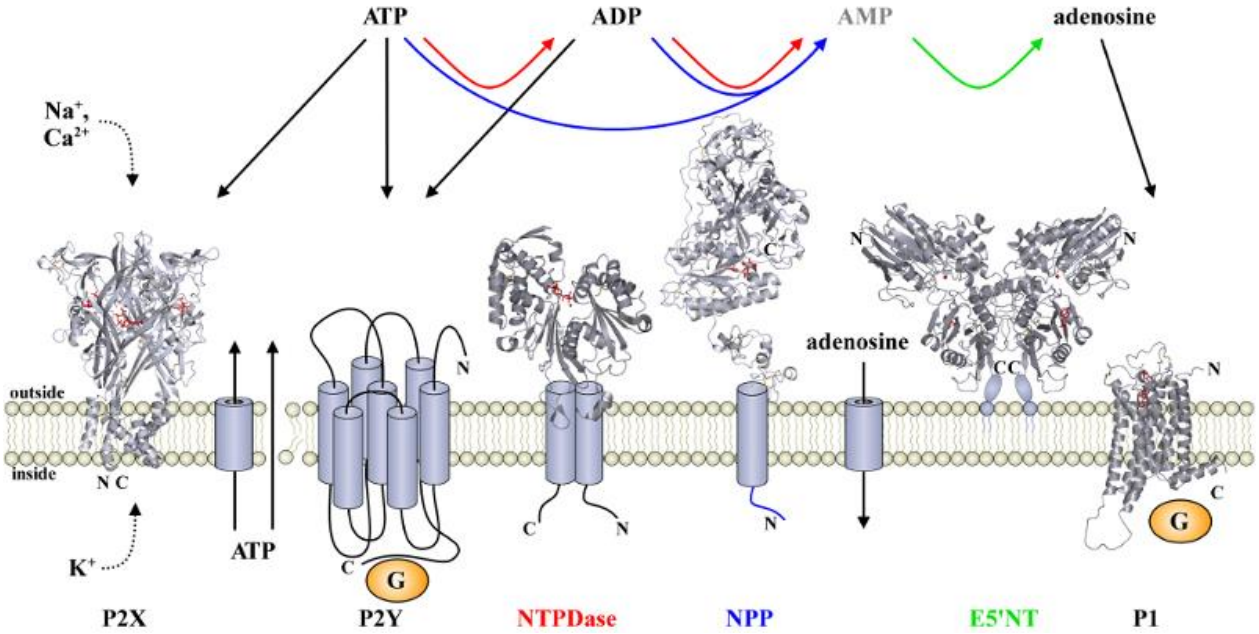

Figura 1. Sistema Purinérgico. O ATP é convertido em adenosina 5'-difosfato (ADP), adenosina 5'-monofosfato (AMP) e adenosina pelas enzimas nucleosídeo trifosfato difosfoidrolases (NTPDases), ecto-nucleotideo pirofosfatase/fosfodiesterase (NPP) e ecto-5'-nucleotidase (E5'NT). O ATP pode se ligar aos receptores purinérgicos P2X (canais iônicos) e P2Y (associado à proteína G) e a adenosina aos receptores P1. Adaptado de H. Zimmermann, M. Zebisch, N. Sträter (2012) [77]

1.4.1 Via ATP-receptor P2X7 na Proliferação de Gliomas

A sinalização purinérgica está relacionada com a patogênese de diversos tipos de câncer como o câncer colorretal, leucemia, câncer de esôfago, câncer de pulmão, câncer de próstata, câncer de bexiga, retinoblastoma, neuroblastoma, glioma e melanoma [58, 78-80]. Dentro do tumor o ATP pode ser liberado para o espaço extracelular, a partir de áreas de necrose e hemorragia presentes no GBM [79], por danos causados pela ressecção cirúrgica do tumor e a partir de células que sofrem morte celular imunogênica [81]. O cérebro possui receptores purinérgicos, e consequentemente, apresenta sensibilidade ao ATP e a seus análogos [67]. Portanto, células de glioma tanto secretam como respondem ao ATP extracelular [82].

Embora seja geralmente reconhecido que o tratamento com ATP ou análogos de ATP tem um forte efeito citotóxico em vários tumores, é também claro que as doses baixas de ATP (como ocorre, por exemplo, durante a liberação espontânea deste nucleotideo a partir de praticamente todos os tipos de células) tem um efeito de promoção do crescimento tumoral [83].

O desfecho do tratamento com ATP depende de dois fatores fundamentais: (i) expressão dos receptores purinérgico P1 e P2 no tumor e nas células inflamatórias que se infiltram, e (ii) nível de expressão das enzimas que hidrolizam nucleotídeos (CD39 e CD73) [84]. Esta observação reforça a necessidade de uma caracterização aprofundada dos receptores P2 e das ectoenzimas expressas pelas células tumorais, a fim de conhecer plenamente o potencial da sinalização purinérgica no tratamento de tumores [83].

Em 1992, Rathbone e colaboradores mostraram que a adenosina e o ATP estimulavam a proliferação celular de astrócitos de galinha e células de astrocitoma humano. O efeito da adenosina provavelmente foi via receptor A2; enquanto que o efeito proliferativo de baixas concentrações de ATP foi abolido por DPMX, um antgonista de receptor P1, e o efeito proliferativo de altas concentrações de ATP pareceu ter sido via receptores P2 [85]. Posteriormente, Bradley e colaboradores (2001) mostraram que o tratamento com ATP ou adenosina (100 μM) inibiu a proliferação de células de astrocitoma humano 1321N1 após 5 dias de tratamento, mas não causaram morte celular. O efeito do ATP pareceu ser mediado por um ou mais produtos de degradação do ATP, porque o tratamento de células com o ATP não hidrolisado (ATPγS) não causou a redução do número de células. Além disso, a ausência de receptores P2 nestas células faz com que seja improvável que o ATP estivesse interagindo com estes receptores para induzir uma resposta. O fato de que o NBTG, bloqueador do transporte de nucleosídeos como a adenosina, significativamente (embora de forma incompleta) reverter o efeito do ATP sugere que o ATP está afetando a proliferação via sua hidrólise e subsequente absorção de adenosina. O efeito pareceu ser através de um mecanismo independente do receptor P1, pois a inclusão do antagonista não seletivo de receptor P1 (8SPT) não teve nenhum efeito sobre a resposta mediada [86].

Já em 2003, Morrone e colaboradores mostraram dados semelhantes aos de Rathbone e colaboradores. O tratamento com ATP (100 μM) e adenosina (100 μM) em diferentes linhagens de glioma humano (U138, U251 e U87) resultou em um intenso aumento da incorporação de timidina, do número de células contadas e da progressão do ciclo celular. Importantemente, foi observado que o ATP adicionado no início do experimento foi degradado lentamente, e estava quase ausente no meio após 24 h. Portanto, no decorrer do experimento, as células experimentaram uma concentração mista dos produtos de degradação do ATP, o que faz com

que a caracterização farmacológica do(s) receptor(es) envolvido(s) seja um desafio. Entre os agonistas utilizados, é interessante notar que ATPγS e 2MeSATP, que são menos hidrolisados pela célula, não tiveram qualquer efeito sobre a incorporação de timidina, indicando novamente que os produtos de degradação do ATP desempenham um papel nos efeitos biológicos mediados por este nucleotídeo. De acordo, os dados mostraram que a adenosina e a inosina, moléculas produzidas a partir da degradação do ATP, induziram um forte aumento na incorporação de timidina [79].

Ainda em 2003, Wink e colaboradores compararam a degradação de nucleotídeos extracelulares (ATP, ADP e AMP) por linhagens de glioma (U87, U138, U251, U373 e C6) com astróscitos normais e observaram que estas células de glioma apresentavam uma baixa hidrólise de ATP e em contraste uma alta atividade da enzima CD73, que degrada AMP a adenosina, quando comparado com astrócitos. Indicando que essas células de glioma causam um acúmulo de ATP e adenosina/inosina [87], moléculas que parecem auxiliar na proliferação dessas células tumorais.

Em 2004, Jacques-Silva e colaboradores mostraram que a estimulação de receptores purinérgicos com ATP (100 μM) e adenosina (100 μM) em células U138 de glioma humano induziu à proliferação celular mediada por PI3K/AKT, ERK e PKC [88].

Morrone e colaboradores (2005) mostraram que células de glioma (U138 e C6) apresentaram resistência à morte induzida pelo ATP em altas concentrações (5 mM) e pelo BzATP (ativador dos receptores P2X que exibe 5-30 vezes mais potência no receptor P2X7; 100 μM), quando comparados culturas organotípicas. Elevadas concentrações de ATP (5 mM) induziram a morte celular de culturas organotípicas, mas não das linhagens de glioma após 24 horas. Os dados indicaram que o ATP liberado na região tumoral pode induzir a morte das células do tecido normal circundante do tumor, podendo abrir espaço para o rápido crescimento e invasão tumoral [62]. Além disso, em 2006 Morrone e colaboradores investigaram o efeito da depleção do ATP no crescimento de gliomas co-injetando células de glioma C6 com apirase, uma enzima que degrada ATP, em ratos. O resultado foi uma redução significativa no tamanho dos tumores, na proliferação tumoral (Ki67), no índice mitótico e em outras características histológicas que indicam um tumor menos invasivo/proliferativo e uma menor marcação para o

fator de crescimento endotelial vascular (VEGF). Indicando a possível participação do ATP extracelular e das ectonucleotidases no desenvolvimento deste tipo de tumor cerebral [89].

Em 2008, Bavaresco e colaboradores mostraram que o aumento da confluência e tempo de cultura levou a um aumento do mRNA, da proteína e da atividade da enzima CD73, que degrada AMP a adenosina, em células de glioma C6 e U138. O tratamento com AMP (1 e 3 mM) diminuiu a proliferação de células de glioma U138. Estes resultados sugerem a participação da CD73 na proliferação de células de glioma e que este processo é dependente da produção de adenosina, um fator proliferativo, e remoção de AMP, uma molécula tóxica para gliomas [90].

Em 2009, Braganhol e colaboradores analisaram a linhagem de glioma de rato C6 em cultura e as mesmas células após a sua implantação no cérebro de rato (denominado como o modelo *ex vivo*). A cultura de células C6 quando comparada com a cultura de células C6 *ex vivo* revelou morfologias celulares distintas, embora a diferenciação de células e a expressão dos marcadores angiogênicos foram semelhantes. Ambos os modelos de glioma co-expressaram múltiplos subtipos de receptores P2X e P2Y com algumas diferenças. Além disso, os dois tipos de cultura de glioma exibiram um comportamento semelhante quanto ao metabolismo de ATP extracelular e proliferação celular quando expostas a concentrações citotóxicas de ATP (0.1, 0.5 e 5 mM) por 24 horas. Assim, a ruptura da sinalização purinérgica é uma característica representada não só por linhagens de células de glioma *in vitro*, mas também nas culturas primárias de glioma [91].

Em 2010, Tamakusuku e colaboradores caracterizaram uma linhagem de glioma de camundongo GL261 como sensível ao ATP, diferentemente das linhagens de glioma estudadas até então. Tanto o ATP (5 mM) quanto o BzATP (100 μM) induziram a morte celular por ativar o receptor P2X7 em camundongos. Essas células mais sensíveis apresentaram uma maior expressão do receptor P2X7 e utilizando RNAi para o receptor P2X7 se reduziu drasticamente a morte celular induzida pelo ATP, o que sugere que este receptor é necessário para este efeito [76].

Em 2011, Ryu e colaboradores demonstraram dados similares aos de Morrone e colaboradores (2005) de que a linhagem de glioma C6 apresenta resistência à morte induzida pela via ATP-P2X7 e mostraram que a administração intravenosa do antagonista do receptor P2X7 BBG (*Brilliant Blue G*) reduziu o tamanho dos gliomas oriundos da injeção de células C6

(3×10^5 células) em ratos. O BBG reduziu a expressão do receptor P2X7 e a quimiotaxia induzida pelo BzATP *in vitro*. O resultado da imunohistoquímica indicou que o tratamento com BBG não alterou microgliose, astrogliose, ou a vascultatura dos animais injetados com C6, sugerindo que a eficácia do BBG na inibição do crescimento do tumor é primariamente mediada por ação direta do composto no receptor P2X7 em células de glioma [92].

Em 2013, Fang e colaboradores mostraram dados diferentes aos do trabalho anterior também usando a linhagem de glioma C6 (1×10^6 células) injetadas em ratos. A supressão do receptor P2X7 exerceu um efeito pró-tumoral através da indução de proliferação e angiogênese, o que foi associado com a sinalização receptor do fator de crescimento epidérmico (EGFR). Assim como no trabalho de Ryu et al., o receptor P2X7 estava acentuadamente menos expresso em células expostas ao antagonista de P2X7 BBG, porém nestas a proliferação celular e a expressão do EGFR estava aumentada, dado que foi evidenciado também através da construção de células C6 com a expressão reduzida do receptor P2X7 usando shRNA. Ressonâncias magnéticas e tomografias computadorizadas de perfusão demonstraram que tanto o BBG, quanto as células P2X7R-shRNA apresentavam um crescimento tumoral de cerca de 40% e 50%, respectivamente, e um aumento significativo da angiogênese. Mais importante ainda, ambos BBG e P2X7R-shRNA aumentaram a expressão de EGFR, HIF-1α e VEGF. Em conclusão, a supressão do receptor P2X7 induziu o crescimento de gliomas, que provavelmente está relacionado com o aumento da expressão de EGFR, fator induzido por hipoxia-1α (HIF-1α) e fator de crescimento endotelial vascular (VEGF) [93].

Em 2012, Gehring e colaboradores caracterizaram outra linhagem de glioma M059J, dessa vez humana, como sensível ao ATP [94]; assim como a linhagem GL261 de glioma de camundondo descrita anteriormente [76]. Neste estudo, se observou que essa linhagem sensível ao ATP apresentava uma captação maior de brometo de etídio após a estimulação com ATP (5 mM), quando comparado com as linhagens U138 e U251, resistentes a morte induzida pela via ATP-P2X7; mostrando que essa linhagem apresenta uma capacidade maior de abertura do poro transmembrana, que leva a morte celular [94].

Tanto nos gliomas quanto em outros tipos de tumores, se a via ATP-P2X7 exerce um efeito supressor ou promotor ainda é um assunto controverso e seu mecanismo subjacente permanece desconhecido [93]. Uma possível explicação para a resistência à morte induzida pela

via ATP-P2X7, apresentada por algumas células tumorais, pode ser a mutação/truncamento na região C-terminal do receptor (receptor P2X7B) (Figura 2), que tem um papel crucial para a formação do poro (mas não na função do canal iônico), e indução de apoptose, consequentemente, causando a falta da indução de morte celular [75, 95-96]. Esta isoforma truncada é altamente expressa em muitos tecidos especialmente nos sistemas nervoso e imune, e a um nível muito mais elevado do que o receptor P2X7 inteiro (P2X7A) e mantém a capacidade de responder ao ATP com a movimentação de cátions, porém é deficiente na formação de poros, e está correlacionada com a proliferação celular [95-96]. Outra explicação seria que, nos gliomas resistentes à citotoxicidade induzida pelo ATP-P2X7, a expressão de proteínas acessórias que são necessárias para mediar os efeitos tóxicos do ATP, como a panexina, estão faltando [97].

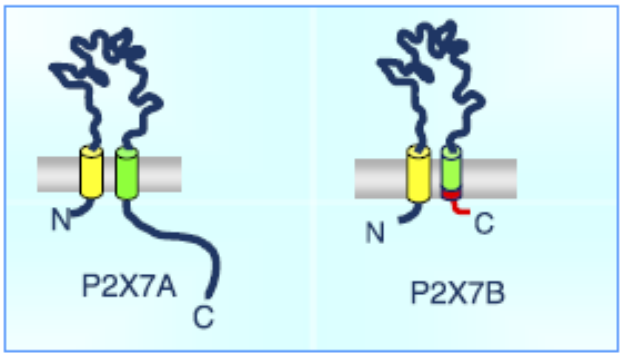

Figura 2. Topologia do receptor P2X7A e P2X7B (truncado) na membrana celular. Amarelo, TM1; verde, TM2; vermelho 18 aminoácidos extras presentes no C-terminal do receptor P2X7B. TM: transmembrana. Adaptado de Adinolfi et al., 2010 [96].

1.4.2 CD39/NTPDase1 na Imunossupressão de Gliomas

A CD39, ou nucleosídio trifosfato difosfohidrolase 1 (ENTPD1), é expressa predominantemente no endotelio vascular e em células imunes. A fonte mais freqüentemente relatada de CD39 em tecidos tumorais é as células T reguladoras (Tregs). O número de Tregs $CD39^{+}$ esta aumentado em tumores, e estas células participam da imunossupressão tumoral através da produção de adenosina. Porém, a CD39 também tem sua expressão surpreendentemente aumentada em um grande número de tumores sólidos [98]. A atividade catabólica das enzimas CD39 e CD73 representa a principal fonte de adenosina. A CD39 hidrolisa ATP e ADP extracelular em AMP, que por sua vez é hidrolisado à anti-inflamatória

adenosina, essencialmente pela ecto-5'-nucleotidase/CD73. O eixo do receptor de adenosina A2A é um mecanismo imunossupressor crítico e não redundante que atenua a inflamação e protege os tecidos normais de danos inflamatórios. Esta via imunossupressora está aberrantemente ativada em tecidos tumorais, em particular, em resposta a hipóxia, e proporciona uma proteção para as células tumorais contra a imunidade anti-tumoral [99].

Em setembro de 2013, Xu e colaboradores observaram que biópsias de glioma humano apresentavam um fenótipo $CD39^{-}CD73^{+}$ e linfócitos T infiltrantes um fenótipo $CD4^{+}$ $CD39^{alto}$ $CD73^{baixo}$, dois fenótipos distintos, mas complementares. Os dados indicaramm que a enzima CD73 expressa no glioma contribui para imunossupressão local mediada pela adenosina em sinergia com a enzima CD39 expressa nos linfócitos T infiltrados $CD4^{+}$ $CD39^{+}$. Em novembro de 2013, outro grupo mostrou este mesmo perfil fenótipico onde a grande proporção de biópsias de glioma humano foram positivas para CD73 em comparação com células apresentadoras de antígenos (APCs) e células T. Em contraste, a expressão de CD39, foi mais alta nas APCs, seguido das células T e baixa nas células tumorais (84.61% ±2.74; 15.29% ±4.05 e 6.48% ±1.98, respectivamente) [100]. Etes dados são semelhante aos publicados anteriormente por Wink et al. em 2003, em que às linhagens de glioma (U87, U138, U251, U373 e C6) apresentaram uma baixa hidrólise de ATP e em contraste uma alta atividade da enzima CD73 [87].

Após a ligação a receptores A2A de células T, a adenosina inibe a proliferação de linfócitos T efetores e a secreção de citocinas inflamatórias. A adenosina também bloqueia a atividade citotóxica e a produção de citocinas das células *natural killer* ativadas (células NK) (17). Portanto, a modulação desta via imunossupressora é uma estratégia atrativa para a terapia tumoral. Embora elas atuem em sinergia, as enzimas CD39 e CD73, os receptores de adenosina e a adenosina em si não são iguais e cada um representa um alvo único, com perfil singular. Por exemplo, o bloqueio de CD39 irá impedir a geração de adenosina, bem como a hidrólise de ATP extracelular (um potente imunoativador). O bloqueio da atividade da CD73 não irá impedir a diminuição dos níveis de ATP extracelulares, mas pode diminuir a concentração de adenosina extracelular. Alternativamente, os fármacos de adenosina desaminase podem ser utilizados para degradar a adenosina peritumoral, enquanto que antagonistas seletivos dos receptores de adenosina podem permitir uma regulação mais afinada. Portanto, uma investigação completa da expressão e o papel de cada molécula é necessária para melhor projetar futuras estratégias

terapêuticas a sinalização purinérgica [98]. O bloqueio da atividade da enzima CD39 através de inibidores farmacológicos ou anticorpos monoclonais aumenta a concentração de ATP extracelular que pode se ligar ao receptor P2X7 e causar a morte de células Tregs, células endoteliais e células tumorais. O aumento do ATP também pode levar a ativação de células Th1/Th17 e consequentemente, ativação das células NK que podem assim exercerem sua atividade antitumoral. O ATP também pode se ligar ao receptor P2X7 expresso em células dentríticas (DC) e causar a ativação do inflamossomo e consequente liberação de IL-1β, que ativa células T $CD8^+$ que induzem morte celular imunogênica (ICD). A diminuição dos níveis de adenosina, causada pelo tratamento com inibidores da CD39, leva a uma menor estimulação dos receptores de adenosina A2A que estimulam a angiogênese. Além disso, a inibição da CD39 pode auxiliar as terapias padrões no tratamento de gliomas, como a quimio e a radioterapia (Figura 3). Este tema é discutido mais aprofundadamente no Anexo I desta tese.

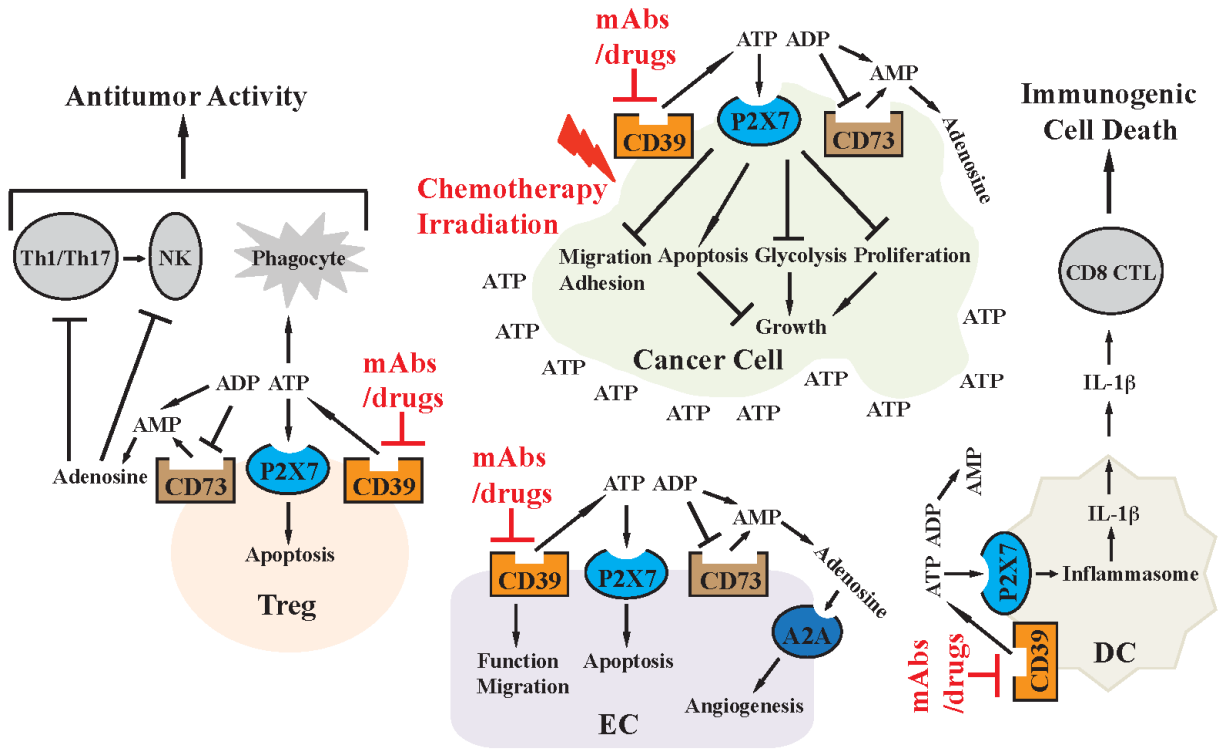

Figura 3. Impactos propostos do bloqueio da enzima CD39 em combinação com quimioterapia e/ou radioterapia. A quimioterapia e/ou radioterapia irá induzir a liberação de ATP pelas células tumorais. O bloqueio simultâneo da CD39 (via anticorpos monoclonais (mAbs) ou inibidores farmacológicos) irá alterar o microambiente do tumor, aumentando os níveis de ATP e impossibilitando a subsequente hidrólise à adenosina. Teoricamente, esta abordagem terapêutica

de combinação aborda vários aspectos distintos da biologia do tumor, como por exemplo, limita a angiogênese, aumenta as respostas imunes antitumorais, e induz diretamente a morte de células tumorais, endoteliais e imunossupressoras. Adaptado de *Frontiers in Anticancer Drug Discovery*, 2014 – Anexo I.

1.4 PAPEL DA SINALIZAÇÃO PURINÉRGICA NA RESPOSTA À RADIOTERAPIA DE GLIOMAS

Durante muito tempo, os efeitos antineoplásicos da radioterapia foram inteiramente atribuídos à sua capacidade de transferir grandes quantidades de energia para os tecidos irradiados, resultando em dano celular direto, bem como na superprodução de fatores citotóxicos, como ROS. Assim, células expostas à radiação ionizante podem tanto sofrer uma parada permanente no ciclo celular, conhecida como senescência celular ou sucumbir à ativação da resposta a danos ao DNA que na maioria das vezes (embora não exclusivamente), desencadeia a via intrínseca da apoptose [29]. Porém, descobriu-se que as respostas biológicas dos tumores à radiação se estendem além da citotoxicidade direta causada nas células tumorais. A radioterapia também influencia o microambiente tumoral estimulando a apresentação de antígenos tumorais que consequentemente causa aumento do tráfico de células imunes para o local do tumor e ativação destas [101-102].

Recentemente, descreveu-se que a radioterapia promove um tipo peculiar de apoptose chamada de "morte celular imunogênica" (ICD) [29, 103]. Assim, contrariamente às células que sofrem apoptose, as células tumorais expostas à irradiação ionizante emitem uma combinação específica de sinais enquanto morrem que estimulam as APCs a induzir respostas imunes adaptativas específicas [29, 84]. A ICD parece intensificar a fagocitose de células tumorais, o processamento de antígenos derivados do tumor e a secreção de IL-1β por células dentríticas (DC), resultando na maturação de DC e *cross-priming* com linfócitos T citotóxicos $CD8^+$ [103].

Os mecanismos intrínsecos e extrínsecos das células tumorais que fundamentam a emissão destes sinais da ICD começaram a surgir recentemente, e a autofagia parece desempenhar um papel central neste cenário [29, 84]. Entre os processos associados à ICD esta a autofagia dependente da secreção de ATP [29] e recentemente, mostrou-se que a irradiação induz à liberação de ATP via receptor P2X7 [104-106], levantado à hipótese de uma possível participação do receptor P2X7 na morte celular induzida pela irradiação. Ademais, foi

demonstrado que o ATP tem uma ação sinérgica com a radioterapia [70], e que a inibição farmacológica de ectonucleotidases que, consequentemente, causam o aumento da concentração extracelular de ATP; restaurou a sensibilidade à radiação de tumores, facilitando a infiltração de linfócitos no tumor [29], e assim revelando as NTPDases como um potencial alvo para ativar a resposta imune induzida pela radioterapia. Portanto, nossa hipótese é de que a sinalização purinérgica participe da resposta à radioterapia em gliomas.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Considerando que a sinalização purinérgica tem sido reconhecida como crítica dentro de tumores e, portanto, um alvo terapêutico promissor na oncologia e a radioterapia, que é amplamente utilizada para o tratamento de pacientes com gliomas [32], induz à liberação de ATP; e tanto a radioterapia quanto o ATP estão correlacionados atualmente com a ativação do sistema imune este estudo teve como objetivo geral investigar o papel da sinalização puriérgica na resposta à radioterapia em gliomas.

2.2 OBJETIVOS ESPECÍFICOS

Este trabalho será apresentado na forma de capítulos, constituído por artigos científicos publicados, submetidos ou em andamento, que visam cumprir os seguintes objetivos:

CAPITULO I

- Artigo científico publicado que visou investigar a relevância da via ATP-P2X7R na resposta a radioterapia usando modelo de glioma *in vitro*, *in vivo* e *ex vivo*.

CAPITULO II

- Estudo científico em andamento que visa avaliar a capacidade da ectonucleotidase CD39 em melhorar a resposta imune induzida pela radioterapia *in vivo*

CAPÍTULO III

- *Short Communication* submetido para a publicação com o objetivo de investigar a ação da IL-17 em células de glioma e outros tipos tumorais.

ANEXOS

- Outras produções dentro do período de doutorado.

3 RESULTADOS

3.1 CAPÍTULO I

P2X7 RECEPTOR AS PREDICTOR GENE FOR GLIOMA RADIOSENSITIVITY AND MEDIAN SURVIVAL

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P2X7 receptor as predictor gene for glioma radiosensitivity and median survival

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ABSTRACT

Glioblastoma multiforme (GBM) is considered the most lethal intracranial tumor and the median survival time is approximately 14 months. Although some glioma cells present radioresistance, radiotherapy has been the mainstay of therapy for patients with malignant glioma. The activation of P2X7 receptor (P2X7R) is responsible for ATP-induced death in various cell types. In this study, we analyzed the importance of ATP-P2X7R pathway in the radiotherapy response P2X7R silenced cell lines, *in vivo* and human tumor samples. Both glioma cell lines used in this study present a functional P2X7R and the P2X7R silencing reduced P2X7R pore activity by ethidium bromide uptake. Gamma radiation (2 Gy) treatment reduced cell number in a P2X7R-dependent way, since both P2X7R antagonist and P2X7R silencing blocked the cell cytotoxicity caused by irradiation after 24 h. The activation of P2X7R is time-dependent, as EtBr uptake significantly increased after 24 h of irradiation. The radiotherapy plus ATP incubation significantly increased annexin V incorporation, compared with radiotherapy alone, suggesting that ATP acts synergistically with radiotherapy. Of note, GL261 P2X7R silenced-bearing mice failed in respond to radiotherapy (8 Gy) and GL261 WT-bearing mice, that constitutively express P2X7R, presented a significant reduction in tumor volume after radiotherapy, showing *in vivo* that functional P2X7R expression is essential for an efficient radiotherapy response in gliomas. We also showed that a high P2X7R expression is a good prognostic factor for glioma radiosensitivity and survival probability in humans. Our data revealed the relevance of P2X7R expression in glioma cells to a successful radiotherapy response, and shed new light on this receptor as a useful predictor of the sensitivity of cancer patients to radiotherapy and median survival.

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1. Introduction

Glioblastoma multiforme (GBM) is a malignant astrocytic glioma (grade IV), considered the most common and lethal intracranial tumor (Furnari et al., 2007). It accounts for ~50% of cases of primary brain tumor (Wang et al., 2012). Among the defining characteristics of GBM are the high levels of proliferative and invasive cells, as well as, a wide resistance to treatment (Chandana et al., 2008; Cote et al., 2012). Patients with this tumor have a poor prognosis (Fisher et al., 2007), with a median survival time of approximately 14 months (Yang et al., 2010). The treatment of malignant gliomas remained virtually unchanged during the last years (Mathieu and Fortin, 2006; Nieder et al., 2009). Although some glioma cells present radioresistance, radiotherapy has been the mainstay of therapy for patients with malignant gliomas (Ohshima et al., 2010; Van Meir et al., 2010; Yang et al., 2010). Thus, the identification of mechanisms underlying the radioresistance of gliomas is essential for the development of new therapies. Our group has shown previous *in vitro* evidence on the possible involvement of P2X7 purinergic receptor on the radiosensitivity of glioma cells (Gehring et al., 2012).

The purinergic system has been implicated in the pathogenesis of several cancers (Burnstock and Di Virgilio, 2013; Morrone et al., 2003; White et al., 2005). The purinergic signaling consists of nucleotides and nucleosides, as adenosine triphosphate (ATP), purinergic receptors and ectoenzymes, that hydrolyze nucleotides (*e.g.* ATP to adenosine) (Burnstock and Di Virgilio, 2013). Within the tumor, ATP can be released to the extracellular space from areas of necrosis, hemorrhage, excitotoxic, immunogenic cell death and/or from injury caused by the surgical tumor resection (Ma et al., 2013b; Morrone et al., 2006). The P2X7R is a cation channel and when activated by ATP in high concentration or for a long period to ATP, can be converted to a large nonselective transmembrane pore, which ultimately causes cell death in the manner of apoptosis (White and Burnstock, 2006).

There is an apparent contradiction regarding the P2X7R oncogenic or anti-tumor role. Some studies show that P2X7R exhibits significant growth-promoting effects *in vivo* inducing a more developed vascular network and elevated amounts of VEGF (Adinolfi et al., 2012) and promoting cell invasion and migration (Qiu et al., 2014; Xia et al., 2015 {Qiu, 2014 #7180}). However, the exact molecular basis of P2X7-mediated growth-promoting activity is poorly known, but mitochondria appear to play a central role (Di Virgilio et al., 2009). On the other hand, P2X7R suppression induced glioma growth (Fang et al., 2013) and functional P2X7R activation by ATP resulted in rapid cytotoxic effects that affects cell growth/survival, leading to inhibition of tumor growth *in vitro* and *in vivo* (Bian et al., 2013; White et al., 2005).

The tumor cell response to P2X7R activation might depend on the concentration of extracellular ATP and the P2X7R isoform expressed by the tumor cells. It was shown that there are two P2X7R isoforms that respond in different ways. One is the full-length P2X7R, called P2X7A, which high extracellular ATP levels triggers sustained pore activity and consequently induces cell death. The second isoform is a naturally occurring truncated isoform, called P2X7B, that does not have the carboxyl-terminal tail, thus lacking cytotoxic activity even with high ATP concentrations because they fail to cause pore formation (Adinolfi et al., 2010). We have previously shown that ATP can increase radiotherapy-induced cell death *in vitro*, depending on the P2X7R expression and functionality, since glioma cells that do not respond to ATP-P2X7R-cytotoxicity present a less functional and a lower expression of P2X7R, when compared to ATP-P2X7 sensitive glioma cells (Gehring et al., 2012).

In this study, we demonstrate the importance of ATP-P2X7R in the radiotherapy response *in vitro, in vivo* and *ex vivo* using human tumor samples. Our data show that P2X7R could be a useful tool for evaluation of patients' response to radiotherapy.

2. Material and methods

2.1. Cell culture and treatments

The human M059J glioma cells line was purchased from American Type Culture Collection (ATCC – Rockville, MD, USA) and the mouse GL261 glioma cells line was obtained from National Cancer Institute (NCI – Frederick, MD, USA). The M059J and GL261 cell lines are considered radiosensitive as described previously (Daido et al., 2005; Hoppe et al., 2000; Szatmari et al., 2006; Virsik-Kopp et al., 2003). Cells were cultured in DMEM supplemented with 10% of fetal bovine serum (FBS) at a temperature of 37 °C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air. The cells were treated for 24 h with ATP (5 mM). Glioma cells were gamma irradiated at a 2 Gy-dose, using Cobalt Theratron Phoenix equipment (Theratronics Ltd, Ontario, Canada), at a source-to-target distance of 70 cm (Hoppe et al., 2000). Selective P2X7R antagonist A740003 (10 μM) (Riteau et al., 2010; Ulmann et al., 2010) was incubated 20 min before treatment with ATP (5 mM).

2.2. Stable RNA interference

Human P2X7R (GeneID: 18439) was silenced by transduction of cells with lentivirus produced with the plasmid clone ID NM_002562.4-801s1c1 from the Mission RNAi library from Sigma–Aldrich. Mouse P2X7R (GeneID: 18439) (Rassendren et al., 1997; Surprenant et al., 1996) was silenced by transduction of cells with lentivirus produced with the plasmid clone ID NM_011027.1-1368s1c1 from the Mission RNAi library from Sigma–Aldrich. Non-target (SHC001) sequence was used as a control. Lentiviruses were produced by co-transfecting the Mission RNAi plasmid with the helper plasmids pRSVREV, pVSV-G and pMDLgRRE in sub-confluent Hek293T cells with Superfect Reagent (Qiagen), according to the manufacturer's protocol. Three days after transfection, supernatants were collected twice every day during 1 week, filtered through a 0.22 mm membrane, and used immediately or stored at −80 °C. Virus containing medium was added to human and mouse target cells, at sub-confluent stage in 24-well plates, together with 4 μg/ml of polybrene overnight. Cells were allowed 48 h to express the selection marker and were then selected with puromycin 5.5 μM for mouse and 1.8 μM for human cell lines at least 10 days. P2X7R silencing was confirmed by western blot using 1:500 Alomone antibody against P2X7R (Alamone Cat APR004) and 1:2000 Cell Signaling secondary antibody as described before (Tamajusuku et al., 2010). The integrated optical density of western blot bands were quantified using the ImageJ software.

2.3. Cell viability

For measuring cell viability, we performed MTT assay and cell counting. The cells were seeded at 5×10^3 cells per well in 96-well plates for MTT assay or 20×10^3 cells per well in 24-well plates for cell counting and grown for 24 h. Subsequently, the cells were for 24 h. For MTT assay, 100 μl of MTT solution (90% DMEM/10% MTT 5 mg/ml in PBS) were added to the cells and incubated for 3 h. The formazan crystals, formed by tretrazolium cleavage, were dissolved with 100 μl of dimethyl sulfoxide (DMSO) for 10 min and quantified in a Spectra Max M2e (Molecular Devices) apparatus at 595 nm. For cell counting, the medium was collected and 200 μl of trypsin/EDTA solution was added to detach the cells, which were counted on hemocytometer. The cell number of the control group

(not treated cells) was considered as 100%. The experiments were carried out three times in triplicate.

2.4. Ethidium bromide (EtBr) uptake assay

Glioma cells were seeded at 8×10^3 cells per well in 96-well plates and grown for 24 h. After, the cells were incubated with 10 μM ethidium bromide in the presence or absence of ATP (5 mM) or BzATP (100 μM) for 3 min and rinsed twice with PBS. In some wells, cells were pre-incubated with the P2X7R selective antagonist A740003 (10 μM) for 15–20 min. To determine when the P2X7R receptor activation occurs after irradiation, cells were irradiated (2 Gy) and after 1, 3 and 24 h cells were incubated only with 10 μM ethidium bromide for 3 min and rinsed. The dye uptake was determined by fluorescence microscopy using an Olympus IX71 microscope. EtBr uptake was quantified by Image J software (NIH) (http://www.uhnresearch.ca/facilities/wcif/imagej/). Corrected total cell fluorescence was determined as described in http://sciencetechblog.com/2011/05/24/measuring-cell-fluorescence-using-imagej/.

2.5. Annexin V/PI flow cytometry staining technique

Human M059J mock and M059J P2X7R silenced glioma cells were seeded at 8×10^4 cells per well in 12-well plates and treated on the second day for 24 h as described previously. After this period, dead cells were quantified by annexin V-FITC–propidium iodide (PI) double staining, using Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences) 24 h after treatment, according to the manufacturer's instructions. Experiments were performed on FACS Canto II Flow Cytometer (BD Biosciences), and the results were analyzed using FlowJo Software (Tree Star). The results are representative of two independent experiments.

2.6. In vivo *glioma model*

In vivo GL261 mouse glioma model was previously validated in our laboratory at PUCRS, Brazil. GL261 WT, mock and P2X7R silenced glioma cells were cultured to approximately 70% confluence and resuspended at 3×10^5 cells/2 μl DMEM. The cells were injected using a 10-μl Hamilton microsyringe coupled to an infusion pump (1 μl/min × 2 min) at a depth of 3.0 mm into the right striatum (coordinates with regard to bregma: 2.0 mm lateral) of adult male mouse C57/BL6 (nearly 8 weeks old, 25–30 g) previously anesthetized by an intraperitoneal (i.p.) administration of ketamine/xylazine. After 20 days, mice were euthanized and the entire brain was removed, sectioned and fixed with 10% paraformaldehyde. Consecutive 4-μm-thick longitudinal sections were stained with hematoxylin-eosin (H&E). All the procedures were approved by the PUCRS' Animal Ethics Committee under protocol number 09/00123.

2.7. Radiotherapy protocol

Gamma irradiation procedure was adapted from Newcomb et al. (2010) and Vinchon-Petit et al. (2010), for mice (Newcomb et al., 2010; Vinchon-Petit et al., 2010). Briefly, mice were irradiated under mild anesthesia by i.p. administration of ketamine/xylazine 10 and 80 mg/kg, respectively; to ensure immobilization to allow radiation to be delivered directly to the tumor-bearing hemisphere. Radiation was delivered on days 11 and 15 after implantation to the head of four mice each time that were placed in a prone position as described by Vinchon-Petit et al., 2010. The total radiation dose administered was 8 Gy given on 2 days – doses of 4 Gy each (Fig. 4A). Radiation was delivered using Cobalt Theratron Phoenix equipment (Theratronics Ltd, Ontario, Canada) at Hospital São Lucas, PUCRS, Porto Alegre, Brazil.

2.8. Pathological analysis and tumor volume quantification

For this protocol, the results are representative of five animals per group. At least three H&E sections of each tumor were analyzed by an experienced pathologist, blinded for the experimental groups. For tumor size quantification, images were captured using a digital camera (G9, Canon) connected to the microscope (Stemi DV4, Zeiss) and analyzed using AxioVision 4.7 image tool software. The total volume (mm^3) of the tumor was computed by summing the segmented areas and by the multiplication of the slice resolution.

2.9. Human sample immunohistochemistry

Eighteen histological samples of human gliomas were collected between January 2004 and June 2008 from patients who underwent glioma surgical resection at the Pathology Department from the University Hospital of the Federal University of Rio Grande do Sul (HCPA-UFRGS; Porto Alegre, Brazil) and the diagnostic was made by a pathologist. All patients signed an informed consent allowing the use of part of the removed tissue for scientific purposes. Samples were obtained in accordance with approved ethical standards of the institutional research ethics committee (GPPG document number 08-259, HCPA-UFRGS). The biopsies were processed as previously described (Braganhol et al., 2009). Anti-P2X7 antibody (Santa Cruz Biotechnology) was used at the dilution of 1:100 followed by anti-IGG peroxidase and diaminobenzidine staining. The samples were lightly counterstained with H&E in order to confirm the tumor localization. P2X7R expression was quantified by WCFI Image J software (NIH) (http://www.uhnresearch.ca/facilities/wcif/imagej/) as it follows: the immunohistochemistry sample images, captured at the same light intensity, were submitted to color deconvolution with "Plugins/Color Functions" and "H&E DAB". The DAB image generated was quantified by using "Analyze/Measure" as previously described (Braganhol et al., 2009). Patients were divided according to P2X7R expression above and below median expression and the survival of the two independent groups was evaluated in a Kaplan–Meier survival plot.

2.10. mRNA expression and correlation with patient's survival

The results presented here are in whole or part based upon data generated by REpository for Molecular BRAin Neoplasia DaTa (REMBRANDT), established by NCI and NIH (National Institute of Health). Information about REMBRANDT and TCGA and the investigators and institutions that constitute the REMBRANDT research network can be found at https://caintegrator.nci.nih.gov/rembrandt/home.do. The Kaplan–Meier survival plot for Gene Expression Data was generated with the following entries: 'P2RX7', as gene symbol in all the plots, 'all gliomas' as sample group. National Cancer Institute. 2005. REMBRANDT home page http://rembrandt.nci.nih.gov (accessed 03.10.14).

2.11. Statistical analysis

Data were analyzed by test-t student or one-way analysis of variance (one-way ANOVA), followed by Tukey–Kramer *post hoc* test or, using GraphPad Software 5.0 (San Diego, CA, U.S.A.). p values <0.05 were taken to indicate statistical significance.

3. Results

3.1. P2X7R functionality and silencing on M059J and GL261 glioma cells

The cell lines used in this work are sensitive to ATP-P2X7R-induced cell cytotoxicity. The ATP dose response curve for GL261 mouse glioma cell line is shown in Fig. 1A. In order to demonstrate the participation of P2X7R in radiation-induced cell death, M059J human and GL261 mouse glioma cells were stably transduced with P2X7R RNAi vector (silenced) or with scramble-RNAi vector as a negative control (mock cell line). Relative protein quantification levels of western blot bands showed a P2X7R silencing efficiency of 60%, relative to mock cells protein levels (Fig. 1B).

To analyze the P2X7R pore functionality on GL261 and M059J cells it was performed EtBr uptake assay (Fig. 1C and D). Both ATP or BzATP treatments induced EtBr uptake on the WT and mock cells which was significantly reduced either by the selective P2X7R antagonist A740003 (10 μM) or by the P2X7R RNAi vector (P2X7R silenced cells), as shown in the corrected total cell fluorescence (Fig. 1E and F). Lastly, we observed that P2X7R silencing or P2X7R antagonist (A740003) were able to reduce the ATP-induced cytotoxicity on GL261 and M059J cells (Fig. 1G). Taking together, these data showed that GL261 and M059J cells present a functional P2X7R and the P2X7R silencing reduced ATP cytotoxicity and P2X7R pore activity.

3.2. Radiotherapy induces P2X7R activation and P2X7R silencing reduces radiotherapy-induced cell cytotoxicity in vitro

GL261 and M059J are both radiosensitive cell lines (Daido et al., 2005; Hoppe et al., 2000; Szatmari et al., 2006; Virsik-Kopp et al., 2003). Here, we show that gamma radiation (2 Gy) reduced cell number to a similar extent as seen for ATP, in a P2X7R-dependent way, since both P2X7R antagonist and P2X7R silencing blocked the cell cytotoxicity caused by gamma-radiation treatment after 24 h (Fig. 2A).

To analyze when P2X7R is activated after radiotherapy we measured the EtBr uptake 1, 3 and 24 h after radiotherapy (2 Gy) (Fig. 2B). Our data showed that P2X7R activation is time-dependent, as the highest EtBr uptake was observed 24 h after radiotherapy and both P2X7R antagonist treated cells and P2X7R silenced cells presented less EtBr uptake, indicating a P2X7R-dependent mechanism. One-hour after irradiation, EtBr uptake was not significantly different from the untreated cells, *i.e.* plate transported to the radiotherapy facility, but not irradiated (*data not shown*). However, 3-h after radiotherapy, EtBr was found incorporated when compared to control cells.

3.3. Radiotherapy-induced cell death is less efficient on P2X7R silenced glioma cell

The treatment with ATP (5 mM) or radiotherapy (2 Gy) alone induced annexin V incorporation rates of 75% and 72.1%, respectively, in M059J human glioma cells after 24 h. The combination of radiotherapy with ATP incubation induced a significant increase of annexin V incorporation (96.6%), when compared to these treatments alone, showing that ATP acts synergistically with radiotherapy. Additionally, when the cells were treated before irradiation with P2X7R antagonist A740003, it was possible to observe a decrease in annexin V incorporation (20.0%) and even with an ATP stimulus (5 mM) after A740003 + irradiation annexin V incorporation was still significantly reduced when compared to the co-treatment ATP and irradiation (25.9%) (Fig. 3A–C). Furthermore, the M059J P2X7R silenced cells also presented resistance to cell death induced by ATP, radiotherapy or both treatments together (Fig. 3B and C) – 11.5%, 12.3% and 16.2% of annexin V incorporation, respectively; suggesting that ATP-P2X7R is an important pathway to the radiotherapy response.

3.4. P2X7R expression is important for radiotherapy success in vivo

GL261 WT-bearing mice treated with radiotherapy 8 Gy (4 Gy on days 11 and 15 after implantation, Fig. 4A) showed a significant reduction in tumor volume when compared to untreated mice, in agreement with published data using the same model (Szatmari et al., 2006). Interestingly, GL261 P2X7R silenced-bearing mice developed tumors with similar size as WT, but these tumors were completely resistant to radiotherapy, showing that a P2X7R is essential for radiotherapy response in an *in vivo* glioma model (Fig. 4B and C).

3.5. High P2X7R expression is a good prognostic factor for glioma radiosensitivity and survival probability in human

Human glioma biopsies were analyzed regarding P2X7R expression by immunohistochemistry, and patients were separated into two groups: with high and low P2X7R expression. Patients whose glioma biopsies presented high P2X7R staining presented longer survival when compared to patients with low P2X7R expression (Fig. 5A). The median survival time (MST) of patients with low P2X7R was 14 months, whereas patients with high P2X7R expression have a MST higher than 80 months. This correlation was confirmed by mRNA expression data obtained by microarray analysis from the Rembrandt database. The analysis of data from 343 patients with gliomas classified in different grades also found that patients whose glioma presented an upregulation of P2X7R mRNA expression have significantly higher MSTs, when compared to those whose tumors expressing P2X7R in the intermediary and lower level (Fig. 5B). More interesting, our samples are also analogous with Rembrandt data comparing GBM occurrence. Within the low P2X7R expression group, 78% of the biopsies were GBM against 44% of this kind of tumor in high P2RX7-expressing samples. Data from Rembrandt platform also demonstrated that the majority (68%) of gliomas with down-regulated P2X7R expression was GBM, whereas in the up-regulated P2X7R expression group, GBM were just 21%, suggesting that P2X7R might be one of the genes suppressed during glioma malignant transformation.

We also used data from Rembrandt platform to analyze the P2X7R expression and 'all glioma' patients that were describe on the platform as underwent 'radiotherapy' (all types) prior or after surgery. As shown in Fig. 5C, the analysis of data from 47 patients with different gliomas showed that patients presenting higher P2X7R expression levels seem to respond better to radiotherapy with a higher MSTs, when compared to patients whose glioma present low P2X7R expression levels. Conceivably, with a larger number of samples it is possible to clearly show the correlation between radiotherapy response and P2X7R expression level.

4. Discussion

The classical glioma treatment is surgery to provide maximal tumor resection. However, total tumor resection is almost impossible due to the infiltrative nature of gliomas. Therefore, surgery is followed by radiotherapy, which clearly improves survival rates (Bassi et al., 2008). Its known that many different genes displayed significantly altered gene expression following radiotherapy (Lindgren et al., 2012). In recent years, there was an increase in the understanding of the molecular and genetic basis by identifying genes and pathways that are involved in tumor

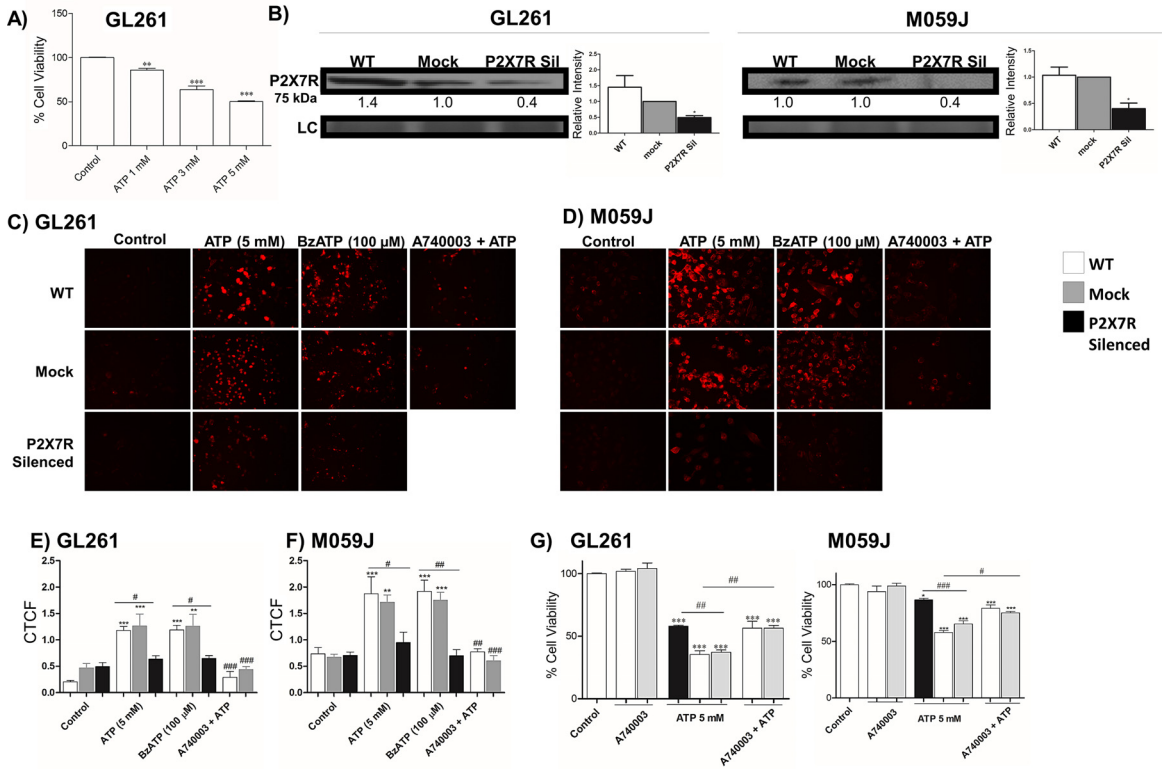

Fig. 1. P2X7R functionality and silencing on M059J and GL261 glioma cells. (A) Effect of ATP (1, 3, and 5 mM) and BzATP (100 μM) on cell viability of GL261 cell line after 24 h. (B) Quantification of western blots using scanning and ImageJ software ($n \geq 4$). Results are expressed as relative density of the bands. Mock cell line were used as control. LC: loading control. Each column represents the mean ± SEM, *$p < 0.01$. (C) GL261 and (D) M059J cells ethidium bromide uptake after stimulation with ATP (5 mM) or BzATP (100 μM). Control, treated only with ethidium bromide. (E) GL261 and (F) M059J corrected total cell fluorescence (CTCF) of ethidium bromide uptake calculated using Image J, as described in Methods. (G) Effect of ATP (5 mM) and selective P2X7R antagonist A740003 (10 μM) treatment on cell viability of GL261 and M059J cells after 24 h. All experiments were carried out at least three times. WT cells (white bars), mock cells (gray bars) and P2X7R silenced cells (black bars). Each column represents the mean ± SEM. *Comparison to the respective control and #comparison between groups. * or #$p < 0.05$; ** or ##$p < 0.01$ and *** or ###$p < 0.001$, as determined by ANOVA with Tukey's *post hoc* test.

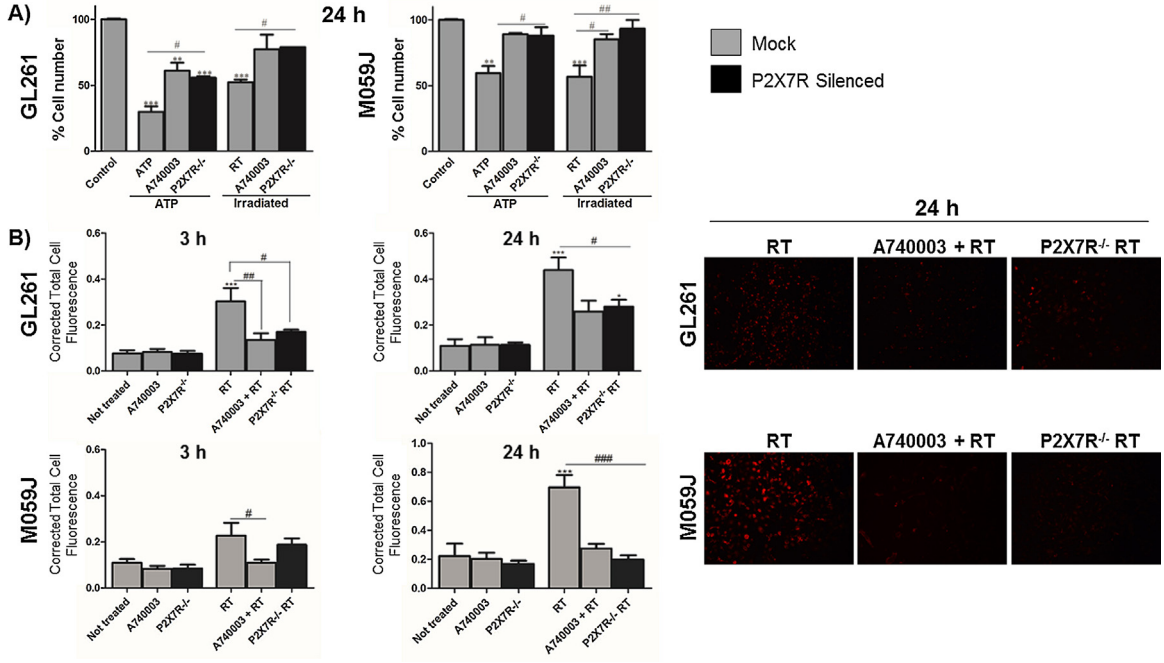

Fig. 2. Radiotherapy-induced cell death is impaired in the absence of P2X7R. (A) Effect of treatment with ATP (5 mM) or irradiation (2 Gy) on cell viability of M059J and GL261 glioma lines after 24 h. (B) Corrected total cell fluorescence (CTCF) of ethidium bromide uptake 3 and 24 h after irradiation (2 Gy) on GL261 and M059J cells, calculated using Image J, as described in Methods. The P2X7R antagonist A740003 (10 μM) was added 15–20 min prior irradiation. Mock cells (gray bars) and P2X7R silenced cells ($P2X7R^{-/-}$) (black bars). *Comparison to the respective control and #comparison between groups. Each column represents the mean ± SEM. * or #$p < 0.05$; ** or ##$p < 0.01$ and *** or ###$p < 0.001$, as determined by ANOVA with Tukey's *post hoc* test.

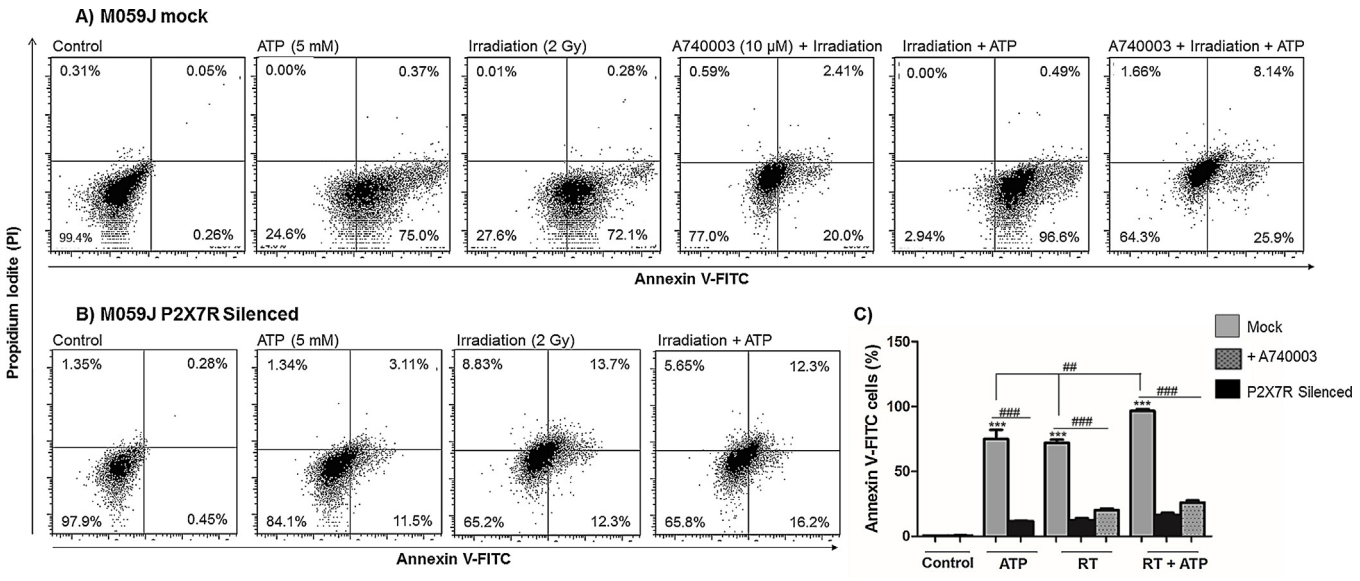

Fig. 3. Radiotherapy and ATP-P2X7R induce apoptosis and is less efficient on P2X7R silenced cells. Flow cytometry dot plot of Annexin V/PI positive cells 24 h after treatment with ATP (5 mM), radiotherapy (2 Gy) and/or pre-treatment with A740003 (10 μM) on (A) M059J mock cells, (B) M059J P2X7R silenced cells. (C) Percentage of Annexin V positive cells. Each sample has 50,000 cells. Data shown is representative of at least two independent experiments. Mock cells (gray bars), Mock cells treated prior with A740003 (dotted gray) and P2X7R silenced cells (black bars). *Comparison to the respective control and #comparison between groups. Each column represents the mean ± SEM. ##$p < 0.01$ and *** or ###$p < 0.001$, as determined by ANOVA with Tukey's *post hoc* test.

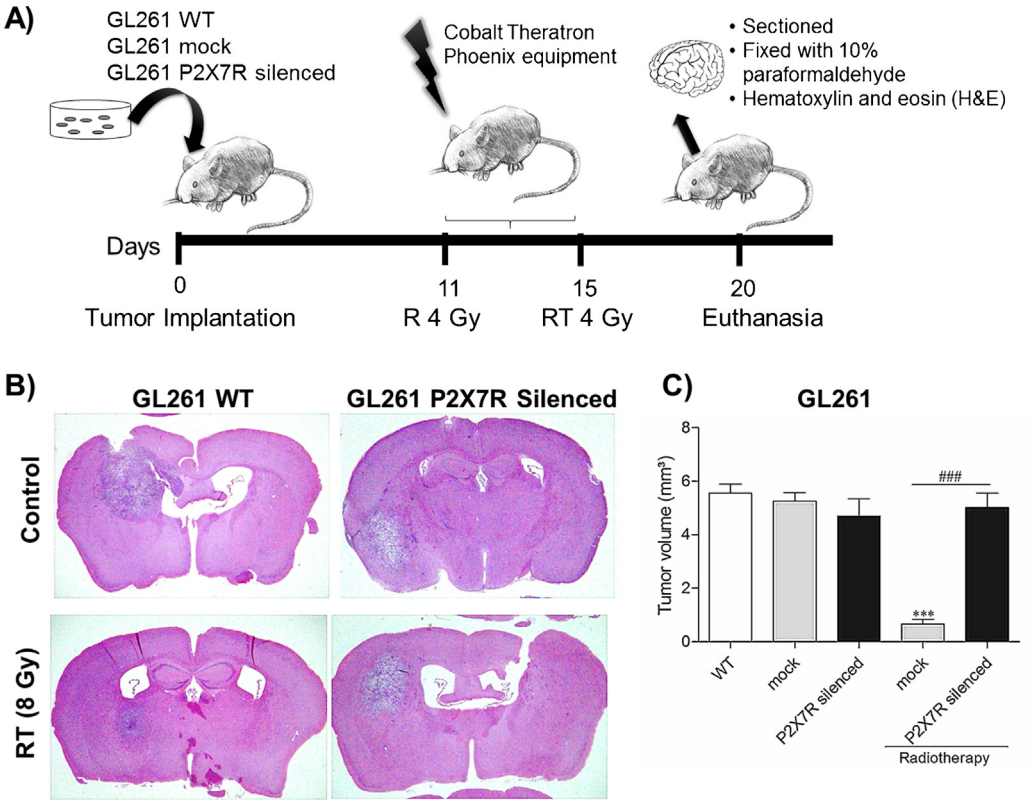

Fig. 4. P2X7R levels and sensitivity to radiotherapy-induced glioma cell death *in vivo*. (A) GL261 WT, mock and P2X7R silenced glioma cells were cultured to approximately 70% confluence and 3×10^5 cells/2 μl DMEM were injected into the right striatum of adult mouse C57/BL6. Mice were treated with radiotherapy on days 11 and 15 after implantation 4 Gy each day. The total radiation dose administered was 8 Gy. After 20 days, mice were decapitated and the entire brain was removed to further analysis. (B) Representative sections of GL261 WT and P2X7R silenced cells implanted on mice brain treated with radiotherapy (RT) or not treated (control) stained with HE. (C) Tumor size quantification of implanted gliomas. Data represent the mean ± SD of five animals per group. Each column represents the mean ± SEM *** or ###$p < 0.001$ #, as determined by ANOVA with Tukey's *post hoc* test.

radiosensitivity. DNA variants can contribute to radioresistance and the identification of these genes can be a targeted to increase the tumors sensitivity to radiation (Smirnov et al., 2012). We have shown that the P2X7R is a radiation-responsive gene since P2X7R expression and activity is significantly increased after irradiation in some glioma tumor cells (Gehring et al., 2012). Furthermore, it is already known that ATP is released from cancer cells exposed to radiation (Ohshima et al., 2010), leading to the hypothesis that

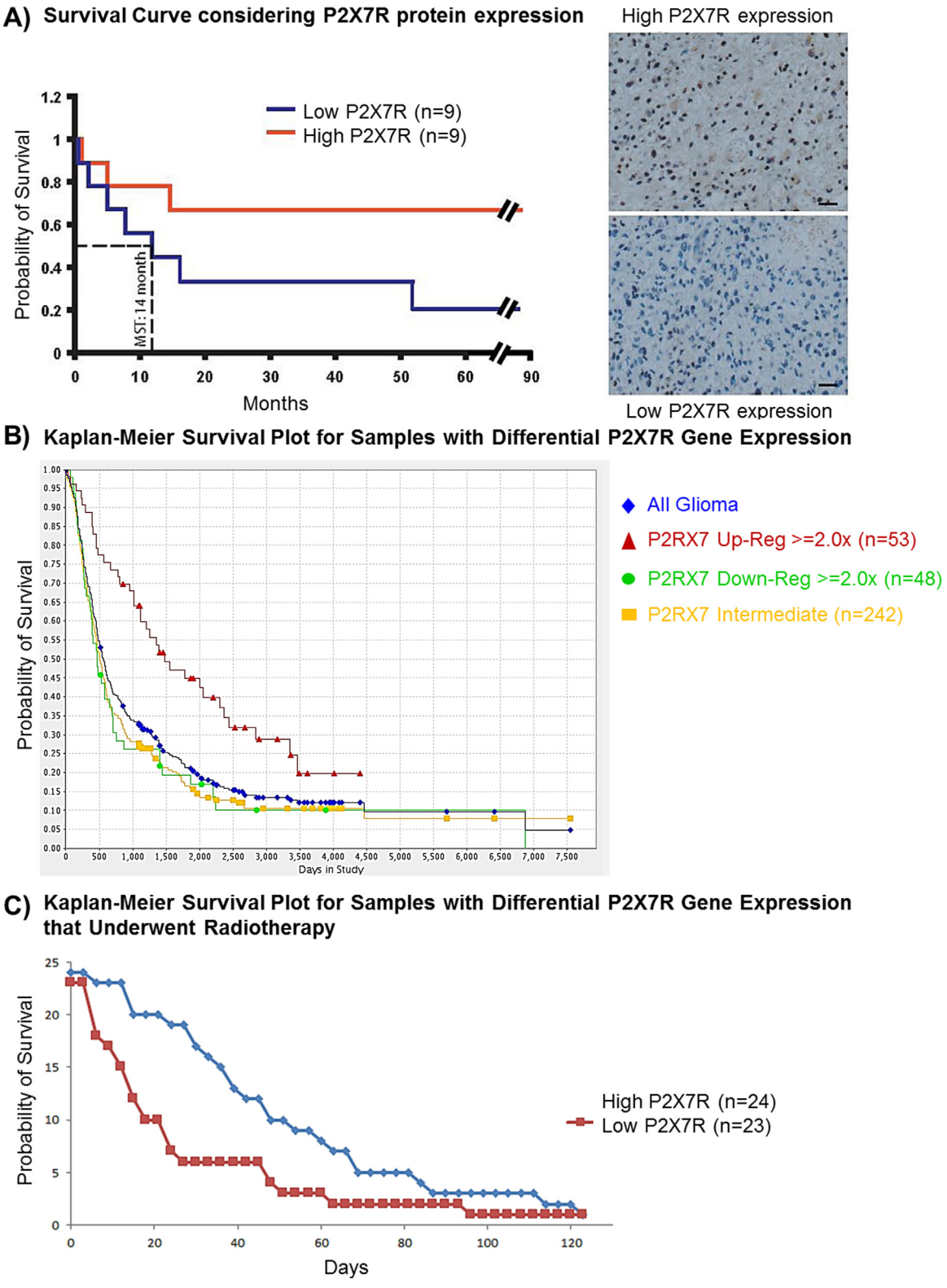

Fig. 5. High P2X7R expression in glioma positively correlates with survival probability. (A) Survival curve of 18 human glioma samples considering P2X7R protein expression above (high) and below (low) the median expression level. Representative pictures of immunohistochemistry of P2X7R in gliomas with high and low P2X7R protein expression. Bars represent 100 μm. (B) Kaplan–Meier survival plot of 343 patients with gliomas from Rembrandt database, considering P2X7R mRNA expression analyzed by microarray. (C) Kaplan–Meier survival plot of 47 patients with gliomas from Rembrandt database, considering P2X7R mRNA expression only of patients that underwent radiotherapy.

P2X7R is activated after radiotherapy, consequently inducing cell death in the manner of apoptosis. It is important to note that the tumor cells need to present a functional P2X7R to induced cell death, as we already showed that tumor cells that not respond to ATP cytotoxicity present a minor expression and functional P2X7R (Gehring et al., 2012). We believe that P2X7R radiation-responsive gene is one of the many pathways that irradiation modulate and use to induce cell death.

We have shown by EtBr uptake that GL261 and M059J cell lines present a functional P2X7R and that these cells are ATP sensitive. Either P2X7R silencing or P2X7R antagonist (A740003) were able to block the ATP-P2X7R-induced pore opening and cytotoxicity. Interestingly, M059J and GL261 cells treated with P2X7R antagonist, or P2X7R silenced cell lines failed to respond to radiotherapy-induced cell death *in vitro*, showing that there is a correlation with functional P2X7R and radiotherapy-induced cell death. P2X7R activation seems to occur at least 2 h after radiotherapy, as we observed an increase in EtBr uptake after 3 h, but not after 1 h of irradiation when compared to untreated cells, P2X7R antagonist (A740003) treated cells or P2X7R silenced cells. This result showed that the activation of P2X7R is time-dependent, as EtBr uptake significantly increases after 24 h of irradiation. These data corroborate with the idea that P2X7R pore activity is activated by high ATP concentration or, in this case, when exposure for a long period to ATP (White and Burnstock, 2006), that is released from cells undergoing immunogenic cell death (ICD) (Galluzzi et al., 2013; Kroemer et al., 2013).

We observed that either ATP or radiotherapy induced apoptosis cell death (characterized by an increased in annexin V incorporation) after 24 h. This data is in agreement with previous data describing that irradiation induces apoptotic cell death in glioma cells (Ma et al., 2013a) and ATP-P2X7R activation mediates apoptosis in different types of cancer (Fang et al., 2013). The intervention with P2X7R antagonist (A740003) or P2X7R silencing compromised the induction of apoptosis by ATP and radiotherapy, showing that there is a link between P2X7R activation and radiotherapy-induced cell death. This P2X7R relevance to radiotherapy response in glioma cells was also verified *in vivo.* Corroborating with the *in vitro* studies, our results showed that GL261 P2X7R silenced-bearing mice failed to respond to radiotherapy (8 Gy total). Otherwise, GL261 WT-bearing mice, that constitutively express a functional P2X7R, presented a satisfactory outcome following radiotherapy. In the tumor micro-environmental, the ATP released after radiotherapy (Ohshima et al., 2010) by ICD in response to radiotherapy (Galluzzi et al., 2013; Kroemer et al., 2013) activate P2X7R on dendritic cells, thereby triggering IL-1β release, which in turn enhances Ag presentation to $CD4^+$ T lymphocytes, and finally enhances antitumor immunity (Di Virgilio, 2012), resulting in a synergistic action of ATP-P2X7R and radiotherapy and an enriched radiotherapy-induced immune response.

Interpretation of our data showing an anti-tumoral effect of P2X7R is made more complex by the observation that tonic, as opposed to acute pharmacologic stimulation, may have a trophic, growth-promoting, rather than cytotoxic effect. This suggests that upon ATP stimulation of P2X7 on tumor cells can result in different outcomes: (i) as a reaction to this death-related signal, they can downregulate P2X7 to avoid apoptosis or (ii) as a cancer-promoting signal to survive and enhance invasion of new niches (Roger and Pelegrin, 2011). Some studies showed that tumor cells expressing P2X7R also expressed elevated amounts of VEGF and more developed vascular network (Adinolfi et al., 2012), increased cell invasion and migration, possibly *via* PI3K/AKT and ERK1/2 pathway, modulation of E-cadherin, MMP-13, SK3 channels- and cystein cathepsin (Jelassi et al., 2011; Qiu et al., 2014; Xia et al., 2015). However, other studies show that functional P2X7R activation induces apoptosis in melanoma, squamous cell skin cancer, lung cancer, glioma cells and cervical cancer (Tamajusuku et al., 2010; Wang et al., 2004; White et al., 2005), possibly through rapid cytotoxic effects caused by ATP exposure on tumor cells that affects cell growth/survival *in vitro* and *in vivo* (Bian et al., 2013). Another study concluded that tumor cell P2X7-dependent apoptosis is mediated by calcium influx *via* P2X7R pores, and involves the caspase-9 (mitochondrial) pathway (Fu et al., 2009; Wang et al., 2004) and the diminished pro-apoptotic effect of BzATP in cancer cells is possibly the result of low expression of the P2X7R (Fu et al., 2009). Moreover, breast cancer patients harboring a P2X7R gene with decreased activity have a significantly greater risk of progression to metastatic disease (Ghiringhelli et al., 2009). Interestingly, we also investigated the involvement of P2X7R in the glioma pathobiology using human glioma biopsies and our result is in accordance with the former result mentioned. Biopsies presenting increased P2X7R expression had less GBM tumors diagnosis, with a higher median survival. A survey of Rembrandt database revealed a similar result a positive correlation between P2X7R expression in gliomas and survival of patients. This correlation was found both at the protein level and at mRNA level. The similarity of these results with other brain tumors information points to an important influence of P2X7R on tumor progression and probability of survival. A recently study also showed a significant difference in the progression-free survival and overall survival in the non-small cell lung cancer patients with high P2X7 expression when compared with that of patients with low expression (Boldrini et al., 2015). Moreover, using Rembrandt data, even though without an expressive number of patients, we showed that patients with a higher P2X7R expression tend to respond better to radiotherapy treatment, at least in the first two months, showing that patients that dissatisfy in respond to radiotherapy express less P2RX7.

Thus, we consider that P2X7R is one of the radiation-responsive genes as part of many pathways that can be activated by irradiation to induce cell death, and that this receptor can be used to predict glioma radiosensitivity.

5. Conclusion

These data show the significance of a high and functional P2X7R expression on glioma cells to a successful radiotherapy response. Furthermore, our study sheds light on this gene as a predictor of the sensitivity of cancer patients to radiotherapy and median survival.

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References

Adinolfi, E., Cirillo, M., Woltersdorf, R., Falzoni, S., Chiozzi, P., Pellegatti, P., et al., 2010. Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor. FASEB J. 24, 3393–3404.

Adinolfi, E., Raffaghello, L., Giuliani, A.L., Cavazzini, L., Capece, M., Chiozzi, P., et al., 2012. Expression of P2X7 receptor increases in vivo tumor growth. Cancer Res. 72, 2957–2969.

Bassi, C., Mello, S.S., Cardoso, R.S., Godoy, P.D., Fachin, A.L., Junta, C.M., et al., 2008. Transcriptional changes in U343 MG-a glioblastoma cell line exposed to ionizing radiation. Hum. Exp. Toxicol. 27, 919–929.

Bian, S., Sun, X., Bai, A., Zhang, C., Li, L., Enjyoji, K., et al., 2013. P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. PLOS ONE 8, e60184.

Boldrini, L., Giordano, M., Ali, G., Melfi, F., Romano, G., Lucchi, M., et al., 2015. P2X7 mRNA expression in non-small cell lung cancer: microRNA regulation and prognostic value. Oncol. Lett. 9, 449–453.

Braganhol, E., Morrone, F.B., Bernardi, A., Huppes, D., Meurer, L., Edelweiss, M.I., et al., 2009. Selective NTPDase2 expression modulates in vivo rat glioma growth. Cancer Sci. 100, 1434–1442.

Burnstock, G., Di Virgilio, F., 2013. Purinergic signalling and cancer. Purinergic Signal.

Chandana, S.R., Movva, S., Arora, M., Singh, T., 2008. Primary brain tumors in adults. Am. Fam. Physician 77, 1423–1430.

Cote, J., Bovenzi, V., Savard, M., Dubuc, C., Fortier, A., Neugebauer, W., et al., 2012. Induction of selective blood-tumor barrier permeability and macromolecular transport by a biostable kinin B1 receptor agonist in a glioma rat model. PLoS ONE 7, e37485.

Daido, S., Yamamoto, A., Fujiwara, K., Sawaya, R., Kondo, S., Kondo, Y., 2005. Inhibition of the DNA-dependent protein kinase catalytic subunit

radiosensitizes malignant glioma cells by inducing autophagy. Cancer Res. 65, 4368–4375.
Di Virgilio, F., 2012. Purines, purinergic receptors, and cancer. Cancer Res. 72, 5441–5447.
Di Virgilio, F., Ferrari, D., Adinolfi, E., 2009. P2X(7): a growth-promoting receptor-implications for cancer. Purinergic Signal. 5, 251–256.
Fang, J., Chen, X., Zhang, L., Chen, J., Liang, Y., Li, X., et al., 2013. P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway. Int. J. Biochem. Cell Biol. 45, 1109–1120.
Fisher, J.L., Schwartzbaum, J.A., Wrensch, M., Wiemels, J.L., 2007. Epidemiology of brain tumors. Neurol. Clin. 25, 867–890, vii.
Fu, W., McCormick, T., Qi, X., Luo, L., Zhou, L., Li, X., et al., 2009. Activation of P2X(7)-mediated apoptosis Inhibits DMBA/TPA-induced formation of skin papillomas and cancer in mice. BMC Cancer 9, 114.
Furnari, F.B., Fenton, T., Bachoo, R.M., Mukasa, A., Stommel, J.M., Stegh, A., et al., 2007. Malignant astrocytic glioma: genetics, biology, and paths to treatment. Genes Dev. 21, 2683–2710.
Galluzzi, L., Kepp, O., Kroemer, G., 2013. Immunogenic cell death in radiation therapy. Oncoimmunology 2, e26536.
Gehring, M.P., Pereira, T.C., Zanin, R.F., Borges, M.C., Braga Filho, A., Battastini, A.M., et al., 2012. P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. Purinergic Signal. 8, 729–739.
Ghiringhelli, F., Apetoh, L., Tesniere, A., Aymeric, L., Ma, Y., Ortiz, C., et al., 2009. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat. Med. 15, 1170–1178.
Hoppe, B.S., Jensen, R.B., Kirchgessner, C.U., 2000. Complementation of the radiosensitive M059J cell line. Radiat. Res. 153, 125–130.
Jelassi, B., Chantome, A., Alcaraz-Perez, F., Baroja-Mazo, A., Cayuela, M.L., Pelegrin, P., et al., 2011. P2X(7) receptor activation enhances SK3 channels- and cystein cathepsin-dependent cancer cells invasiveness. Oncogene 30, 2108–2122.
Kroemer, G., Galluzzi, L., Kepp, O., Zitvogel, L., 2013. Immunogenic cell death in cancer therapy. Annu. Rev. Immunol. 31, 51–72.
Lindgren, T., Stigbrand, T., Riklund, K., Johansson, L., Eriksson, D., 2012. Gene expression profiling in MOLT-4 cells during gamma-radiation-induced apoptosis. Tumour Biol. 33, 689–700.
Ma, H., Rao, L., Wang, H.L., Mao, Z.W., Lei, R.H., Yang, Z.Y., et al., 2013a. Transcriptome analysis of glioma cells for the dynamic response to gamma-irradiation and dual regulation of apoptosis genes: a new insight into radiotherapy for glioblastomas. Cell Death Dis. 4, e895.
Ma, Y., Adjemian, S., Mattarollo, S.R., Yamazaki, T., Aymeric, L., Yang, H., et al., 2013b. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. Immunity 38, 729–741.
Mathieu, D., Fortin, D., 2006. The role of chemotherapy in the treatment of malignant astrocytomas. Can. J. Neurol. Sci. 33, 127–140.
Morrone, F.B., Jacques-Silva, M.C., Horn, A.P., Bernardi, A., Schwartsmann, G., Rodnight, R., et al., 2003. Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines. J. Neurooncol. 64, 211–218.
Morrone, F.B., Oliveira, D.L., Gamermann, P., Stella, J., Wofchuk, S., Wink, M.R., et al., 2006. In vivo glioblastoma growth is reduced by apyrase activity in a rat glioma model. BMC Cancer 6, 226.
Newcomb, E.W., Lukyanov, Y., Kawashima, N., Alonso-Basanta, M., Wang, S.C., Liu, M., et al., 2010. Radiotherapy enhances antitumor effect of anti-CD137 therapy in a mouse Glioma model. Radiat. Res. 173, 426–432.
Nieder, C., Mehta, M.P., Jalali, R., 2009. Combined radio- and chemotherapy of brain tumours in adult patients. Clin. Oncol. (R. Coll. Radiol.) 21, 515–524.
Ohshima, Y., Tsukimoto, M., Takenouchi, T., Harada, H., Suzuki, A., Sato, M., et al., 2010. gamma-Irradiation induces P2X(7) receptor-dependent ATP release from B16 melanoma cells. Biochim. Biophys. Acta 1800, 40–46.
Qiu, Y., Li, W.H., Zhang, H.Q., Liu, Y., Tian, X.X., Fang, W.G., 2014. P2X7 mediates ATP-driven invasiveness in prostate cancer cells. PLOS ONE 9, e114371.
Rassendren, F., Buell, G.N., Virginio, C., Collo, G., North, R.A., Surprenant, A., 1997. The permeabilizing ATP receptor, P2X7. Cloning and expression of a human cDNA. J. Biol. Chem. 272, 5482–5486.
Riteau, N., Gasse, P., Fauconnier, L., Gombault, A., Couegnat, M., Fick, L., et al., 2010. Extracellular ATP is a danger signal activating P2X7 receptor in lung inflammation and fibrosis. Am. J. Respir. Crit. Care Med. 182, 774–783.
Roger, S., Pelegrin, P., 2011. P2X7 receptor antagonism in the treatment of cancers. Expert Opin. Investig. Drugs 20, 875–880.
Smirnov, D.A., Brady, L., Halasa, K., Morley, M., Solomon, S., Cheung, V.G., 2012. Genetic variation in radiation-induced cell death. Genome Res. 22, 332–339.
Surprenant, A., Rassendren, F., Kawashima, E., North, R.A., Buell, G., 1996. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272, 735–738.
Szatmari, T., Lumniczky, K., Desaknai, S., Trajcevski, S., Hidvegi, E.J., Hamada, H., et al., 2006. Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. Cancer Sci. 97, 546–553.
Tamajusuku, A.S., Villodre, E.S., Paulus, R., Coutinho-Silva, R., Battasstini, A.M., Wink, M.R., et al., 2010. Characterization of ATP-induced cell death in the GL261 mouse glioma. J. Cell. Biochem. 109, 983–991.
Ulmann, L., Hirbec, H., Rassendren, F., 2010. P2X4 receptors mediate PGE2 release by tissue-resident macrophages and initiate inflammatory pain. EMBO J. 29, 2290–2300.
Van Meir, E.G., Hadjipanayis, C.G., Norden, A.D., Shu, H.K., Wen, P.Y., Olson, J.J., 2010. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J. Clin. 60, 166–193.
Vinchon-Petit, S., Jarnet, D., Jadaud, E., Feuvret, L., Garcion, E., Menei, P., 2010. External irradiation models for intracranial 9L glioma studies. J. Exp. Clin. Cancer Res. 29, 142.
Virsik-Kopp, P., Rave-Frank, M., Hofman-Huther, H., Schmidberger, H., 2003. Role of DNA-PK in the process of aberration formation as studied in irradiated human glioblastoma cell lines M059K and M059J. Int. J. Radiat. Biol. 79, 61–68.
Wang, Q., Wang, L., Feng, Y.H., Li, X., Zeng, R., Gorodeski, G.I., 2004. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. Am. J. Physiol.: Cell Physiol. 287, 1349C–1358C.
Wang, S., Chen, Y., Lal, B., Ford, E., Tryggestad, E., Armour, M., et al., 2012. Evaluation of radiation necrosis and malignant glioma in rat models using diffusion tensor MR imaging. J. Neurooncol. 107, 51–60.
White, N., Burnstock, G., 2006. P2 receptors and cancer. Trends Pharmacol. Sci. 27, 211–217.
White, N., Butler, P.E., Burnstock, G., 2005. Human melanomas express functional P2 X(7) receptors. Cell Tissue Res. 321, 411–418.
Xia, J., Yu, X., Tang, L., Li, G., He, T., 2015. P2X7 receptor stimulates breast cancer cell invasion and migration via the AKT pathway. Oncol. Rep. 34, 103–110.
Yang, I., Huh, N.G., Smith, Z.A., Han, S.J., Parsa, A.T., 2010. Distinguishing glioma recurrence from treatment effect after radiochemotherapy and immunotherapy. Neurosurg. Clin. North Am. 21, 181–186.

3.2 CAPÍTULO II

AUGMENTATION OF ANTI-TUMOR IMMUNITY AND DEPENDENCE UPON PURINERGIC RESPONSES FOLLOWING CANCER RADIOTHERAPY

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AUGMENTATION OF ANTI-TUMOR IMMUNITY AND DEPENDENCE UPON PURINERGIC RESPONSES FOLLOWING CANCER RADIOTHERAPY

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ABSTRACT

Despite over a century of effort in the treatment of cancer, there has been little. For most of this time, cancer has been viewed as a disease of hyper-proliferative, transformed cells and cancer treatments have been focused on their direct elimination. However, tumor mechanisms employed to evade immune rejection are rapidly being appreciated as a central feature of cancer. Therefore, there is a growing consensus that re-thinking therapeutic approaches to those focused on stimulating tumor rejection by the immune system. Interestingly, there has been accelerating realization that radiotherapy is a potent enhancer of antitumor immunity. Recent clinical reports showed that combining radiotherapy with immunotherapy resulted in dramatic sustained clinical responses, providing profound proof of principle for further development of similar approaches. The CD39-CD73-adenosine pathway has been recognized as a critical immunosuppressive pathway within tumors and thus a promising therapeutic target in such approaches for oncology. We hypothesized that CD39 activity may constitute an important mechanism deployed by the tumor to protect it from ATP-induced cytotoxicity and immune cytotoxic cell attack and the causes the inhibition of the immune response. Thus, we propose to test whether CD39 deletion can improve radiotherapy generating a more robust antitumor immune response. Using CD39 knockout mice bearing-GL261 glioma cell line we observed a significantly reduction of intratumoral MDSCs and a systemic decrease of Tregs on the tumor and spleen when compared to the WT irradiated mice. In addition, CD39 deletion combined to radiotherapy induced a significantly increase of CCR7 positive macrophages and dendritic cells on the spleen relative to their WT counterparts. CCR7 regulates recirculation of a variety of immune cells, guiding their chemotactic homing to lymph nodes, tumor/infected tissues and target cells. An increase in the number of cells expressing this receptor indicates a greater amount of freshly mobilized immune cells available to differentiate in immune-effector. The decrease in the MDSC and Tregs populations that suppress the activation of effector and the robust responses observed through increase of CCR7 positive cells observed in the CD39 knockout mice following irradiation of their tumors demonstrate a role for CD39 enzyme to improve radiotherapy-induced immune response.

Keywords: Glioma. CD39. Radiotherapy. Melanoma. Intratumoral immunity.

INTRODUCTION

Despite over a century of efforts to improve treatments for solid malignancies, and recent advances in immunotherapies, these diseases annually still claim millions of lives worldwide. Until of late, cancer treatments have been focused on the direct elimination of hyper-proliferative, malignant cells. However, transformed cells display multiple cell surface and secreted factors that impact elimination by the host immune system. Tumor cells undergo a form of Darwinian selection to decrease immune recognition whereby the tumor co-opts the various mechanisms that normally function to down-regulate immune responses to establish intratumoral immune privilege, the so called "The Hellström Paradox" [107-108]. It is now increasingly appreciated that commonly therapeutic approaches used in cancer treatment, counter or antagonize tumor-induced immune protective mechanisms, therefore facilitating immune rejection of the tumor cells [109] [110] [111] [112]. There is a growing consensus that re-thinking therapeutic approaches will allow boosting of immune rejection of tumors rather than attempting to achieve direct tumor cell cytotoxicity and elimination.

Radiotherapy (RT) serves a potent inducer/enhancer of antitumor immunity [101]. Supporting this hypothesis, recent clinical reports have demonstrated that combining RT with immunotherapy results in dramatically sustained clinical responses, providing proof of concept [113]. There are several proposed mechanisms for how RT stimulates anti-tumor immunity. One suggestion is that radiation-induced cell death results in the release of tumor antigens that are available for increased antigen presentation [114]. Another model postulates immune activation from the release of intracellular metabolites e.g. ATP and danger molecules e.g. high mobility group box 1 protein (HMGB1) from radiation damaged cells [29]. These mediators act in concert as potent immune stimulatory 'danger' signals that can elicit anti-tumor immune responses [104] [81]. However, CD39 and CD73 ectonucleotidases that are expressed by regulatory-immune cells within the tumor micro-environment convert extracellular ATP to immunosuppressive adenosine which blunts the production of immune effector functions that are necessary for tumor eradiation [115] [116].

Extracellular nucleotides and nucleosides play an important role in regulating inflammation and tissue homeostasis through the activation of various purinergic receptors. ATP receptors are primarily associated with pro-inflammatory responses whereas adenosine primarily acts to suppress the inflammatory effector functions [117]. The conversion of extracellular ATP/ADP to adenosine results from serial actions of cell-surface CD39 and CD73. CD39 is the dominant, rate limiting vascular endothelial and immune ectonucleotidase hydrolyzing both extracellular ATP and ADP to AMP, which is then converted to adenosine

by CD73 bioactivity [118]. This ectonucleotidase pathway constitutes a significant component of intratumoral immunosuppression and together with other CD39/NTPDase family members constitute an attractive target for cancer therapy [115].

Many tumors exhibit intrinsic expression of ATP-metabolizing enzymes, which may contribute to the pathological events observed in solid cancer given that ATP released from dying cells acts as a danger signal to drive inflammation [117]. Furthermore, ATP may work directly to induce cytotoxicity of tumor cells via P2X7R stimulation [119]. In contrast, adenosine is a critical regulator of both innate and adaptive immune responses; upon binding to A2A receptor (A2AR) (the major adenosine receptor on T cells), this nucleoside activates a negative feedback mechanism [117] [98]. Thus, CD39 ectonucleotidase is the rate-limiting enzyme of a cascade leading to the generation of immunosuppressive adenosine, which constitutes an important mechanism deployed by either tumor cells or infiltrating immune cells to protect from ATP-induced immune cytotoxicity [98].

In this manuscript, we have shown that glioma-bearing mice CD39KO exhibit increases of CCR7 (C-C chemokine receptor type 7) positive macrophages and dendritic cells in the spleen, with decreases in tumor-associated and splenic Tregs as well as intratumoral myeloid derived suppressor cells (MDSC) upon local cyberknife irradiation (8 Gy). Our data indicate mobilization of high levels of antigen presenting cells in GL261 glioma tumor-bearing mice and strongly suggest that CD39 activity abrogates therapeutic effects of radiation.

MATERIALS AND METHODS

Antibodies

FACS studies were performed using anti-mouse conjugated antibodies to CD39 PeCy, CD73 Alexa Fluor 647, CCR7 PE, CD11b PE-Cy7, CD11c PE-Cy7, CD45 Alexa 647, MHC II-APC, TCRβ APC, CD8 Alexa 700, $NKp46^{+}$ PE, CD3 APC/Cy7, or CD4 PB or CD11b PB or Gr1-PB from BioLegend. Immunohistochemistry studies were performed using anti-FOXP3 (eBioscience) and anti-CD45 (BD Pharmingen).

Mice and Cell Line

C57BL/6 wild-type FOXP3 (WT) and CD39 null FOXP3 (CD39KO) mice [120-122] were maintained at CLS Animal Facility at Beth Israel Deaconess Medical Center and used in accordance with Institutional Animal Care and Use Committee (IACUC) approved protocols. All animals were used between the ages of 8 to 14 weeks. Groups of 3 to 5 mice per group

were used for each experiment. The GL261 mouse glioma cell line was obtained from National Cancer Institute (NCI) and the B16/F10 melanoma cell line was obtained from ATCC [123]. Cells were grown in RPMI, supplemented with 10% Fetal Calf Serum (FCS) and penicillin/streptomycin at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO2 in air.

Ectonucleotidases activity of GL261 cells

The purinergic enzymes activity of GL261 cell line was analyzed by thin-layer chromatography (TLC) as previously described with slight modifications [124]. Briefly, 2 x 10^5 GL261 cells were incubated with 2 mCi/ml [C^{14}]ADP, [C^{14}]AMP, or [C^{14}]ADO (Ge Healthcare) at room temperature for 60 minutes. Aliquots of the mixture were applied onto the Silica gel TLC Plates (Sigma-Aldrich) and analyzed for the presence of hydrolysis products by TLC. The specific adenosine deaminase 1 (ADA1) inhibitor erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) and adenosine deaminase 2 (ADA2) inhibitor pentostatin were used. Three different cell culture preparations were used.

Tumor growth and Irradiation

B16/F10 (0.5 x 10^6 cells) or GL261 (1.5 x 10^6 cells) were harvested by trypsinization and resuspended in 200 µl of DMEM. The B16/F10 or GL261 glioma cells were injected (s.c.) into the flank(s) of WT or C39KO C57BL/6 mice and allowed to grow for 12 days. One day before radiotherapy (day 7), mice underwent computer tomography (CT) to allow radiation to be delivered directly to the right tumor flank. Mice were anesthetized with intraperitoneal injection of a mixture of Xylazine (10 mg/kg) and Ketamine (100 mg/kg) to ensure immobilization. Radiotherapy (8 Gy – day 8) was done at Keith C. Field CyberKnife Center Room, BIDMC, Boston. The Cyberknife (Accuracy, Sunnyvale, CA, USA) is a robotically operated linear accelerator delivering 6MV photons. The radiation beams were collimated to various diameters and delivered non-isocentrically to obtain extreme accuracy. Four days after radiotherapy, tumors were removed and cut in three pieces for: RT-qPCR (stored at -80°C), immunohistochemistry – IHC (Zinc fix for 48 h) and for flow cytometry.

qRT–PCR

Expression of inducible nitric oxide synthase (iNos), arginase 1 (Arg1), glucocorticoid-induced tumour necrosis factor receptor (GITR), interferon γ (IFN-γ), chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-C motif) ligand 3 (CCL3), chemokine (C-C motif) ligand 5

(CCL5), tumor necrosis factor α (TNF-α), interleukin (IL) IL1-β, IL-12, IL-15, IL-17A and C-C chemokine receptor type 7 (CCR7) on tumor microenvironment as well as expression of the E-NTPDases on GL261 cell line were determined by RT-qPCR. Total RNA was extracted and purified from cells using an RNeasyKit (Qiagen) according to the manufacturer's instructions, and the concentration was measured on Nanodrop ND 1000 spectrophotometer (Wilmington, DE, USA). Reverse transcription was conducted on 1 μg of total RNA using iScript cDNA Synthesis Kit (BioRad). Specific primers for quantitative real-time PCR (qRT-PCR) were obtained from Invitrogen; the sequences are shown in Table 1. The RT-qPCR analysis was performed using QuantiFast SYBR® Green PCR Kit (cat. no 204054; Quiagen) on a Stratagene Fast Real Time Machine (Mx3005P) (Agilent Technologies, Santa Clara, CA). A comparative CT (threshold cycle) was used to determine relative gene expression and analyzed against the endogenous genes of murine β-actin as an internal control.

Flow Cytometry

To assessment of tumor-infiltrating immune cells and CD39/CD73 enzymes on GL261 cell line, each tumor was chopped and the samples were put in 2 ml of RPMI 1640 medium with 2mg/ml of collagenase IV (Worthington) and 2mg/ml of DNAse I (Sigma-Aldrich) at 37°C for 30 min to tissue digestion. The reaction was stopped adding 20 μl EDTA 0.5M and the tumor samples were passed through a 70-μm cell strainer. The filtrates were centrifuged at 1300 rpm for 5 min and the pellets were resuspended in FACS Buffer to count. The spleens were also collected and grind in 10 ml HBSS using syringe pluger on 70 μm filter and 6 cm dish and after centrifuged at 1300 rpm for 5 min. Supernatants were removed and pellets were resuspended in 6 ml of 0.2% NaCl for 30 sec and then added 14 ml of 1.2% NaCl. The samples were then transfer to a second falcon tube passed through a 70-μm cell strainer. Samples were centrifuged at 1300 rpm for 5 min and the pellets were ressuspended in FACS Buffer to count. For tumor samples approximately 3 x 10^6 cells were stained and for splenocytes 1 x 10^6 cells. For tumor samples non-viable cells were excluded of the analysis using 7-amino actinomycin D (7AAD, BioLegend) staining. To analyze Tregs infiltration on tumor the samples were gated for $7AAD^-$, $CD45^+$ and $FOXP3^+$ and on the spleen Tregs were gated for $FOXP3^+$ and $CD11b^-$ cells. To analyze the myeloid derived suppressor cells population infiltrated on tumor samples were gated for 7AAD-, $CD45^+$, $CD11b^+$ and $Gr1^+$ cells. Lymphocyte infiltration on the tumor was gated for $7AAD^-$, $TCR\beta^+$, $CD4^+$ or $CD8^+$ cells. To analyze the activated antigen presenting cells the spleen samples were gated for $CD11c^+$ or $CD11b^+$ $CCR7^+$, MHC II^+ cells.

Immunohistochemistry – IHC

Tumor samples and spleens were fixed in Zinc based fixative and then paraffin-embedded. Resulting blocks were sectioned at 5 μm and then treated for antigen retrieval in 10mM pH 6 hot citrate buffer for 20 min. Sections were blocked using 7% horse serum for 30 min followed to primary antibody incubation with anti-FOXP3 (eBioscience 1:70) and anti-CD45 (BD Pharmingen 1:70) in a humid chamber overnight at 4°C. Sections were then washed in PBS and treated with H_2O_2 in PBS for 10 min followed by incubation with the appropriate biotinylated secondary IgG antibodies and developed using ABC (avidin-biotin complex) reagent (Vector Laboratories), that was formerly detected by ImmPACT 3,3'-diaminobenzidine (DAB) peroxidase substrate (Vector Laboratories), according to the manufacturer's specifications. Sections were counterstained with Gill's hematoxylin II. The IHC slides were analyzed by a pathologist in a blinded manner.

Statistical analysis

Data was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test, or by Student's t test using Graph-Pad Software (San Diego, CA). P values < 0.05 were taken to indicate statistical significance.

RESULTS

GL261 ENTPDases expression and activity

Previous work by our group showed that the B16/F10 cell line present minimal ecto-nucleoside triphosphate diphosphohydrolase family (ENTPDase) expression and no detectable ectonucleotidase activity [125]. As there are no publications regarding the ectonucleotidases expression and activity on the GL261 glioma cell line, we analyzed the transcriptional expression of known ectonucleases by RT-qPCR. These results revealed that the mRNA level of ENTPDase1 and ENTPDase4 were negligible while expressions of the following were detected in descending levels: ENTPDase3 > ENTPDase8 > ENTPDase2 > ENTPDase6 > ENTPDase7 (Figure 1A). We were also unable to detect the presence of ENTPDase1 by flow cytometry, but we detected the presence of the 5´-ectonucleotidase (CD73) (Figure 1B).

Direct analysis of ENTPDases activity by thin layer chromatography (TLC) of C^{14} labeled ATP metabolites showed that this cell line was capable of hydrolyzing ADP into AMP and subsequently generating adenosine, then hydrolyzed into inosine (Figure 1C). In order to characterize which adenosine deaminase was responsible for the conversion of

adenosine into inosine, cells were treated with either adenosine deaminase 1 (ADA1) inhibitor – EHNA or adenosine deaminase 2 (ADA2) inhibitor – pentostatin. Adenosine conversion to inosine was inhibited by EHNA, but not by pentostatin; suggesting the expression only of ADA 1 on these cells. Therefore, GL261 cells express ENTPDases 2, 3 6, 7, 8; the 5´-ectonucleotidase/CD73 and ADA1.

GL261 tumors possess more extensive immune stromal compared to B16/F10 tumors

In order to find the better model to investigate tumor immune stroma we compared the commonly used B16/F10 melanoma with the GL261 glioma tumor models. Tumor samples from both cell lines injected s.c on the flank C57BL6 mice were stained with H&E to evaluate the tumor tissue structure. Tumors derived from GL261 glioma cell line revealed dense cellularity, cellular atypia with fibrillary background and tumors derived from B16/F10 melanoma cell line presented necrotic areas, and were discohesive (tended to fall apart when handled) (Figure 2A).

Our analysis of the tumor-infiltrating immune cells on B16/F10 melanoma and GL261 glioma tumors revealed marked differences in the intratumoral content of $CD45^+$ cells by both IHC and FACS analysis, with B16/F10 melanoma tumors possessed 3.8% ± 2.4 and GL261 tumors possessed 10.9% ± 2.9 of $CD45^+$ cells (Figure 2B and 2C). Analysis of these tumors for $CD8^+$ T cell ($CD3^+CD8^+TCR\beta^+$), $CD4^+$ T cell ($CD3^+CD4^+TCR\beta^+$) and NK cells ($CD3^-NKp46^+$) showed that these populations were more highly represented in GL261 tumor compared to B16/F10, while Tregs ($CD45^+Foxp3^+$) and MDSCs ($Gr1^+CD11b^+$) were not significantly different between them (Figure 2D). These data suggested that GL261 tumors exhibited a potentially more inflammatory immune stromal than the more commonly used B16/F10 melanoma tumors. In addition, we also detected higher expression of IL-15, IL-1β, IL-17 and INF-γ in GL261 tumors when compared to the B16/F10 melanoma model (Figure 2E).

CD39KO mice exhibit increased levels of GL261 glioma tumor rejection

Consistent with our previously reported data from liver tumors [126], both B16/F10 and GL261 cell lines exhibited slower tumor growth when implanted subcutaneously in the flank of CD39KO mice (Figure 3A-C) and significantly smaller tumor size when compared to WT mice (9.46mm ± 0.85 and 14.07mm ± 1.58, respectively for B16/F10) (Figure 3B).

While this difference was modest in B16/F10 tumors, we have found that CD39 deficiency resulted in markedly slower growth rates for GL261 glioma tumors. As CD39 is

part of the extracellular ATP hydrolysis to adenosine it has been recognized to constitute a significant mechanism for the imposition of tumoral immunosuppression and thus constitutes an attractive target for cancer therapy [127]. Thus, the genetic deletion of CD39 within host prevents the ATP destruction and therefore limits the production of adenosine, leading to abrogation of adenosine A2A stimulation that may have major effects on promoting co-stimulation of immune responses against tumors. Significantly, GL261 glioma cells were able to establish tumors in WT mice but were rejected between 10 to 18 days when implanted in CD39KO mice. The tumor incidence on WT mice on day 20 was of 57% (8 of 14 tumors) while being totally rejected by this point in CD39KO mice (0 of 11 tumors) (Figure 3C). Thus, when compared relative to B16/F10 tumors, GL261 glioma tumors exhibited significantly requirement for CD39 activity in their associated stroma for their establishment and maintenance than B16/F10 cell line.

The RT-qPCR analysis of GL261 glioma tumors for IL-17A, TNF-α, INF-γ, CCL3, Arg1 and iNOS expression did not vary significantly between WT and CD39KO mice (Figure 3D). However, tumors removed from CD39KO mice exhibited higher expression of the cytokines IL-1β, IL-12 and IL15. In addition, GL261 glioma tumors from CD39KO exhibited an up-regulation of the monocytes chemokine, CCL2, and CCL5 that promotes both macrophage and lymphocyte infiltration in various types of human cancers [128]. The receptors GITR, a member of the tumor necrosis factor receptor (TNFR) super-family that is a co-stimulatory molecule that is expressed in several immune cells, including T cells, NK cells and antigen presenting cells (APCs) [129], and the chemokine C receptor 7 (CCR7) were also found upregulated on tumors from CD39KO mice. CCR7 expression is upregulated upon immune activation and drives cellular migration and proliferation [130-131]. This alteration on the expression of immunomodulatory genes suggests a link between extracellular purinergic metabolism and the immune milieu of the GL261 tumor.

As we aimed to investigate the effect of CD39 deletion in the radiotherapy-induced immune response, we chose continue our work only with the GL261 glioma cells and not with the B16/F10 melanoma cells, for the reason that our analysis suggested that the GL261 tumors were more immunogenic comparing to B16/F10 melanomas injected subcutaneous on C57BL6 WT mice. Results that are in agreement with other studies that showed that progression of B16 tumors did not spontaneously elicit concomitant immunity, these tumors also display a relative lack of infiltrating immune stroma [132], thus are considered poorly immunogenic model [133], while GL261 tumors are considered moderately immunogenic and are used for immunotherapeutic studies [134-135]. Furthermore, as melanoma is highly

radioresistant, if used, radiation works as an adjunct therapy [136-137], while for gliomas radiotherapy is part of the standard treatment [136, 138].

CyberKnife radiotherapy targeted unilateral tumor

Irradiation has been demonstrated to enhance the tumor infiltration and activation of multiple immune cell types [139]. In this study, radiation was delivered by CyberKnife that uses a linear accelerator with high accuracy. One of the advantages of the system is the ability to continuously track in real time the motion of tumors because of the patient's respiration and execute location adjustments [140-142]. Previous work established that medium-range radiation doses (7.5 Gy) gave the best tumor control and tumor immunity while maintaining low Treg number on a murine tumor model [143]. Thus, before we analyzed the specific effects of RT on the glioma tumors, we tested whether our CyberKnife radiation protocol/set up was able to deliver radiation on the specific site/tumor in mice injected in both flanks with GL261 glioma tumors. We observed by flow cytometry performed 4 days after RT (8 Gy) that irradiated tumors (right flank) had significant increases in the $CD45^+$ population when compared to the non-irradiated tumor (left flank) and when compared to tumors from non-irradiated mice (Figure 4A). These results were also confirmed by CD45 IHC staining (Figure 4B). Furthermore, we also observed significant increases in the expression IL-15, INF-ɣ, iNOS and CCL2 in the irradiated tumor compared to non-irradiated tumor (Figure 4C).

CD39 deletion decreases intratumoral immune suppressor cells in irradiated GL261 gliomas

Analysis of the GL261 tumor-infiltrating immune cells following Cyberknife irradiation (8 Gy) revealed a reduction of intratumoral Tregs ($CD45^+FOXP3^+$) in the CD39KO mice when compared to non-irradiated CD39KO mice and irradiated WT mice (0.06% ± 0.02, 0.18% ± 0.02 and 0.15% ± 0.03; respectively, Figure 5A). This result was also observed by FOXP3 IHC staining (Figure 5B). Furthermore, glioma tumor irradiation resulted in a marked decrease of splenic Tregs in GL261-bearing CD39KO mice treated when compared to WT and CD39KO mice non irradiated and to WT mice irradiated (0.44% ± 0.05, 2.13% ± 0.15, 1.75% ± 0.19 and 1.67% ± 0.37; respectively) (Figure 5C). It is known that adenosine can induce Tregs, which leads to drastically impaired antitumor immune responses [144]. Furthermore, a study showed that regulatory T cells are the major regulators of concomitant tumor immunity and demonstrates the central role played by Tregs in the blockade of tumor immunity [132]. Accordingly, our results showed that CD39 deletion

boosts RT by promoting systemic ablation of Tregs in both the tumor microenvironment as well as in the systemic lymphoid compartment, and consequently may lead to a better antitumor immune response.

We also observed differences in intratumoral, but not splenic MDSCs in response to tumor irradiation. In tumor-bearing mice and cancer patients, MDSCs accumulate in secondary lymphoid organs such as the spleen and bone marrow, as well as within the tumor [145]. Irradiation of tumors in WT mice resulted in an increase in intratumoral MDSCs ($CD45^+Gr1^+CD11b^+$) which was not observed in irradiated tumors from CD39KO mice (0.74% ± 0.12 and 0.38% ± 0.06; respectively) (Figure 5D), suggesting that either ATP somehow limits MDSC accumulation or an ATP metabolite drives their accumulation. As expected, splenic $Gr1^+CD11b^+$ cells were significantly increased on GL261 tumor-bearing mice WT and CD39KO and we were unable to detect differences in this population in upon either irradiation or CD39 deletion (*data not shown*). Interestingly, we observed that after irradiation there was a significant decrease in the number of T $CD4^+$ cells ($TCR\beta^+CD4^+$) in tumors of irradiated mice WT and CD39KO when compared to their counterparts not irradiated (0.16% ± 0.02, 0.11% ± 0.04, 0.41% ± 0.03 and 0.32% ± 0.05; respectively). However, a similar reduction was not observed in the T $CD8^+$ population ($TCR\beta^+CD8^+$) (Figure 5E). The decrease in tumoral T $CD4^+$ cells ($TCR\beta^+CD4^+$) observed on the CD39KO mice irradiated could be due to the decrease of Treg cells detected on this group, as we did not separate this $TCR\beta^+CD4^+$ population from the $FOXP3^+$.

It is of significant interest that tumor irradiation in CD39KO mice lead to systemic ablation of Tregs, suggestive of the potency of the ATP/adenosine axis in modulating intratumoral inflammation in response to irradiation while having a profound effect on $CD11b^+GR1^+$ cells. These results suggested that CD39 activity acts to blunt the therapeutic effects of radiation at several levels. Therefore we hypothesized that CD39 is an important regulator of leukocyte activation in response to irradiation.

$CCR7^+$ immune cells are increased in the spleens of CD39 deficient mice in response to radiotherapy against GL261 tumors

In order to determine if CD39 deletion results in differences in the activation states of leukocytes in response to tumor irradiation, splenocytes from GL261 bearing WT and CD39KO mice irradiated were analyzed for CCR7 expression by FACS. This revealed a significant increase of $CCR7^+$ dendritic cells ($CCR7^+$ $CD11c^+$ MHC II^+) in the spleens of CD39KO mice relative to WT non-irradiated and WT irradiated mice (0.48% ± 0.05, 0.11%

± 0.00 and 0.20% ± 0.05; respectively) (Figure 6A). Likewise, irradiation of glioma-bearing CD39KO mice induced a similar increase of $CCR7^+$ macrophage numbers ($CCR7^+$ $CD11b^+$ MHC II^+) compared to WT and CD39KO non-irradiated and WT irradiated mice (0.52% ± 0.10, 0.11% ± 0.00, 0.21% ± 0.02 and 0.22% ± 0.03; respectively) (Figure 6B). We observed a similar increase in the CCR7 expression on GL261 tumors from CD39KO mice relative to WT mice tumors (Figure 6C). Our results strongly suggest that the CD39 plays a role in regulating leukocyte trafficking and activation in response to irradiation by countering ATP-driven $CCR7^+$ upregulation.

DISCUSSION

This study highlights the importance of understanding the nature of tumor immune-privilege for the development of more successful cancer treatment regimes. To date, the ATP-CD39-CD73-adenosine pathway has been recognized as a critical immunosuppressive mechanism within tumors and thus a promising therapeutic target in oncology [115]. As ectonucleotidases are highly expressed on tumor cells and tumor-infiltrating immune cell, such as Tregs and endothelial cells, and their expression correlate with a poorer overall survival rate; these data strongly suggest an important role of this pathway in facilitating tumor development, most likely through supporting mechanisms underlying immune privilege required by the tumor for the evasion of immune elimination [115]. Thus, it is of great interest to test the effect of adenosine ablation and consequently ATP increase by inhibition of the ectonucleotidase CD39/NTPDase1 in order to promote/enhance an antitumor response [122, 146]. Therefore, we tested whether host deletion of CD39 affected responses to radiotherapy (RT) in order to generate more robust antitumor immune responses.

Looking for a good tumor model to investigate tumor-infiltrating immune cell, we compared the B16/F10 melanoma with the GL261 glioma models *in vivo*. We observed that GL261 mouse glioma cell line expresses the following ectonucleotidase activities: ENTPDases 2, 3, 6, 7 and 8, 5'-NT/CD73 and ADA1. This result demonstrates that GL261 glioma cells can hydrolyze ATP, which causes GL261 cells cytotoxicity [76], into inosine, likely using the purinergic signaling as a mechanism of protection from ATP damage. Furthermore, our data show that GL261 cells generate a more inflammatory tumor immune stromal presenting significantly more $CD45^+$, $CD4^+$ and $CD8^+$ T cells and NK cells and higher expression of IL-15, INF-γ, IL-17 and IL-1β than the more commonly used B16/F10 melanoma cell line. Thus, the B16/F10 melanoma tumor appeared to be poorly immunogenic relative to GL261 glioma tumor injected subcutaneous on C57BL6 WT mice. One of the most

likely explanations for this is that GL261 cells are more readily recognized and rejected by the host immune system of C57BL6 mice when compared to B16/F10 cells. As the former cell line was developed by 10 serial passages through hosts mice resulting in an immunoediting of the tumor cells and making them less able to evoke an immune response [147], and the opposite may occur with the GL261 cells. However, the real indication for the B16/F10 poorly immunogenic phenotype was that this cell line did not induce any rejection on the C57BL6 WT mice, while the GL261 induced rejection in some level (57% - 8 of 14 mice). When implanted subcutaneous in the flank of CD39KO mice, both B16/F10 and GL261 cell lines exhibited slower tumor growth and significantly smaller tumor size when compared to WT mice. These data is in agreement with what we have previously shown that CD39 affects tumor growth [122] [126] [118]. Interestingly, mice that lack CD39 enzyme (CD39KO) rejected GL261 glioma tumors up to 20 days showing that CD39 might be crucial to GL261 glioma tumor growth. It is already known that the GL261 cell line is sensitive to ATP-P2X7R induced cell death [76]. Thus, the higher ATP levels presented in the CD39KO mice are possibly inducing GL261 cells cytotoxicity and consequently causing tumor rejection.

Tumors extracted from GL261 glioma-bearing CD39KO mice expressed significantly more IL-1β, IL-12 and IL-15 relative to GL261 tumors from WT mice. Increases in IL-1β were expected in response to the ATP concentration rise in CD39KO, that stimulates the P2X7 receptor driving the secretion of this key pro-inflammatory cytokine [83]. Interestingly, it was shown that IL-12 expression improved immune reactions resulting in the inhibition growth of implanted glioblastoma and the increased survival time of the rats [148-149]. Another study showed that IL-15 induced antitumor immune response resulting in a prolonged survival including complete remission of tumor from GL261-tumor bearing mice [150]. The GL261 glioma-bearing CD39KO mice also expressed higher levels of GITR, CCR7, CCL2 and CCL5 than seen in GL261 glioma-bearing WT mice. CCL5 was already shown to be expressed GL261 and may be a key regulator in the crosstalk between glioblastoma and microglia/macrophages [128], and it is one of the central chemokines that regulates T cell migration towards sites of tissue injury and inflammation, as well as Th1 differentiation. CCL5 has been tested in murine models as adjuvant therapy for tumor lysate-pulsed DC vaccines and showed a significant reduction in rates of tumor growth and increased survival, which correlated with increased immune cell infiltration into the tumor [151]. Regarding CCL2 expression, there are some evidences that CCL2 expression by glioma is directly involved in accumulation of macrophages/microglia and Tregs in glioma and promotes tumor aggressiveness [152-153]. GITR is a co-stimulatory receptor expressed in

several immune cells, including T cells, NK cells and APCs. It acts to increase TCR-induced T-cell proliferation and cytokine production, which in turn abrogates Treg suppression [129].

Tregs cells have been shown to play a major role in suppression of the immune response to malignant glioma [129, 154], and T cell exposure to adenosine during activation promotes long-term T-cell anergy and the induction of Tregs, both of which lead to a drastically impair of antitumor immune responses [144]. Additionally, it was shown that RT induces upregulation of adenosine A2AR that can provide both growth and survival advantage to Tregs, suppressing the potential beneficial anti-tumor effects of RT [114]. Thus, strategies to reduce or impair Tregs activity subsequent to administration of RT would enable the endogenous anti-tumor immune cells response to emerge and be enhanced by RT [114]. In this study, we used CD39KO mice that might have reduced adenosine production and consequently less A2AR activation. Our data showed that the combination of CD39 deletion with RT caused a marked decrease of Tregs on both tumor and spleen of glioma-bearing mice showing that disrupting the purinergic signaling can enhance the RT-induced immune response. However, it is unclear from the current data whether CD39 deficiency reduces Tregs migration elicited by irradiation or if this combination causes increased Tregs cell death.

In tumor-bearing mice and cancer patients, MDSCs accumulate in secondary lymphoid organs such as the spleen and bone marrow, as well as within the tumor [145]. We observed a difference in intratumoral MDSCs but not splenic MDSCs in response to radiation. The combination of CD39 deletion and RT significantly attenuated intratumoral infiltration of MDSCs observed in the WT mice irradiated. Recruitment of myeloid-derived cells to tumors is one of the more consistent findings associated with RT. The recruited cells are primarily M2 TAMs and MDSCs and usually promote tumor growth and immune evasion [155]. The splenic MDSCs were significantly increased on GL261 tumor-bearing mice WT and CD39KO when compared to healthy mice spleens and neither RT nor CD39 deletion affected this population. However, after RT, we detected a decrease of tumoral $CD4^+$ T cells in irradiated WT and CD39KO mice when compared to non-irradiated tumors. The $CD4^+$ T cells reduction detected in the CD39KO mice irradiated can be, at least in part, because of the Tregs reduction we detected previously, as these intratumoral immune cells were stained only for $TCR\beta^+$ and $CD4^+$, but not separated from FOXP3 positives. Moreover, it is known from studies performed in mice and humans that RT induces lymphocytopenia [156]. We did not observe any difference in the intratumoral $CD8^+$ T cells content after radiotherapy.

Analysis of the spleens of GL261 glioma-bearing CD39KO mice 4 days after RT showed an increase of macrophages and dendritic cells expressing the chemokine C receptor 7 (CCR7) when compared to WT mice irradiated. The CCR7 expression levels were also significantly higher in GL261 tumors when grown in CD39KO mice compared to those grown in WT mice. The CCR7 is a receptor able to bind different ligands (CCL-19 and 21) expressed by activated DCs and other inflammatory cells and once engaged by its ligands during the immune attack, CCR7 regulates the homeostatic recirculation of a variety of activated immune cells, guiding them to secondary lymph organs [157] [131]. The CCR7 expression increase observed in this study is likely due to the decrease of adenosine in the CD39KO mice microenvironment altering the response to RT, although increased trafficking and maturation of immune cells driven by elevated ATP may play a significant role. This is consistent with observations that A2AR activation by adenosine decreased CCR7 expression and function in dendritic cells, macrophages and T cells [158] and that total body irradiation on mice results in up-regulation of the CCR7 [159]. It was also suggested that novel cancer immunotherapy protocols may include the application of antagonists of A2R and/or activation of CCR7 to prevent the apoptosis of immune cells and lead to more efficient elimination of tumors [131]. Therefore, the increased number of dendritic cells and macrophages expressing these receptors in the spleen of CD39KO mice irradiated resulted from the decrease of adenosine and/or increase of ATP indicate a greater amount of freshly mobilized antigen presenting cells (APCs) available to differentiate in immune-effector cells that sustain a more prolonged antigen-specific T-cell–mediated immune response [157].

In summary, we showed that CD39 deletion increases tumor immune inflammatory cytokines (IL-1B, IL-12 and IL-15), chemokines (CCL2 and CCL5) and that whose expression are induced following leukocyte activation (GITR and CCR7) that might support tumor rejection observed in GL261 CD39KO mice. Furthermore, the combination of CD39 deletion and RT induced a drastic decrease in tumoral and splenic Tregs, attenuated MDSC infiltration observed in WT mice after RT and increased splenic $CCR7^{+}$ dendritic cells and macrophages. The data show that CD39 deletion combined to RT might enhance the traffic and survival of immune cells through CCR7 upregulation and decrease of intratumoral immune suppressor cells demonstrating a role of CD39 and purinergic signaling in improving RT response.

The ability to counter the immune suppressive mechanisms within the tumor promises to improve both standard cancer treatments (chemo-radiotherapy) and emerging immunotherapies that are largely being administered while the intratumoral immune privilege

is unperturbed. Our data reinforces the notion that the native immunesuppressive tumor state reduces the potential strength of antitumor immune responses required to tumor elimination. The described experiments in CD39KO mice are likely to help illuminate the role of ATP/adenosine pathway on the understanding of tumor immune-privilege and help to develop more effective cancer treatment.

CONCLUSION

Accordingly, we are proposing a strategy with the use of purinergic modulators simultaneously with chemotherapy/radiotherapy that would reduce adenosine production and increase ATP stability within the tumor. This treatment could be a new avenue to improve the efficacy of the classical treatment (chemo-radiotherapy) by enhancing extracellular ATP levels, which would sustain the antitumor immune response.

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REFERENCES

1. Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor, *Immunosuppressive strategies that are mediated by tumor cells.* Annu Rev Immunol, 2007. **25**: p. 267-96.
2. Hellstrom, I., et al., *Cellular and humoral immunity to different types of human neoplasms.* Nature, 1968. **220**(5174): p. 1352-4.
3. Heylmann, D., et al., *Human CD4+CD25+ regulatory T cells are sensitive to low dose cyclophosphamide: implications for the immune response.* PLoS One, 2013. **8**(12): p. e83384.
4. Zhu, Y., et al., *CD4+Foxp3+ regulatory T-cell impairment by paclitaxel is independent of toll-like receptor 4.* Scand J Immunol, 2011. **73**(4): p. 301-8.
5. Litterman, A.J., et al., *Profound impairment of adaptive immune responses by alkylating chemotherapy.* J Immunol, 2013. **190**(12): p. 6259-68.
6. Sevko, A., et al., *Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model.* J Immunol, 2013. **190**(5): p. 2464-71.
7. Demaria, S., et al., *Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer.* Clin Cancer Res, 2005. **11**(2 Pt 1): p. 728-34.
8. Zeng, J., et al., *Immune modulation and stereotactic radiation: improving local and abscopal responses.* Biomed Res Int, 2013. **2013**: p. 658126.
9. Park, B., C. Yee, and K.M. Lee, *The effect of radiation on the immune response to cancers.* Int J Mol Sci, 2014. **15**(1): p. 927-43.
10. Galluzzi, L., O. Kepp, and G. Kroemer, *Immunogenic cell death in radiation therapy.* Oncoimmunology, 2013. **2**(10): p. e26536.
11. Ohshima, Y., et al., *gamma-Irradiation induces P2X(7) receptor-dependent ATP release from B16 melanoma cells.* Biochim Biophys Acta, 2010. **1800**(1): p. 40-6.
12. Ma, Y., et al., *Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells.* Immunity, 2013. **38**(4): p. 729-41.
13. Xu, S., et al., *Synergy between the ectoenzymes CD39 and CD73 contributes to adenosinergic immunosuppression in human malignant gliomas.* Neuro Oncol, 2013.
14. Eltzschig, H.K., M.V. Sitkovsky, and S.C. Robson, *Purinergic signaling during inflammation.* N Engl J Med, 2012. **367**(24): p. 2322-33.
15. Bergamin, L.S., et al., *Ectonucleotidases in tumor cells and tumor-associated immune cells: an overview.* J Biomed Biotechnol, 2012. **2012**: p. 959848.
16. Kunzli, B.M., et al., *Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer.* Purinergic Signal, 2011. **7**(2): p. 231-41.
17. Gehring, M.P., et al., *P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line.* Purinergic Signal, 2012. **8**(4): p. 729-39.
18. Bastid, J., et al., *ENTPD1/CD39 is a promising therapeutic target in oncology.* Oncogene, 2013. **32**(14): p. 1743-51.
19. Enjyoji, K., et al., *Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation.* Nat Med, 1999. **5**(9): p. 1010-7.
20. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.
21. Sun, X., et al., *CD39/ENTPD1 expression by CD4+Foxp3+ regulatory T cells promotes hepatic metastatic tumor growth in mice.* Gastroenterology, 2010. **139**(3): p. 1030-40.
22. Sato, A., et al., *Antitumor activity of IFN-lambda in murine tumor models.* J Immunol, 2006. **176**(12): p. 7686-94.

23. Beldi, G., et al., *Natural killer T cell dysfunction in CD39-null mice protects against concanavalin A-induced hepatitis.* Hepatology, 2008. **48**(3): p. 841-52.
24. Feng, L., et al., *Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate.* Neoplasia, 2011. **13**(3): p. 206-16.
25. Jackson, S.W., et al., *Disordered purinergic signaling inhibits pathological angiogenesis in cd39/Entpd1-null mice.* Am J Pathol, 2007. **171**(4): p. 1395-404.
26. Xu, S., et al., *Synergy between the ectoenzymes CD39 and CD73 contributes to adenosinergic immunosuppression in human malignant gliomas.* Neuro Oncol, 2013. **15**(9): p. 1160-72.
27. Pham, K., et al., *CCL5, CCR1 and CCR5 in murine glioblastoma: immune cell infiltration and survival rates are not dependent on individual expression of either CCR1 or CCR5.* J Neuroimmunol, 2012. **246**(1-2): p. 10-7.
28. Vega, E.A., M.W. Graner, and J.H. Sampson, *Combating immunosuppression in glioma.* Future Oncol, 2008. **4**(3): p. 433-42.
29. Legler, D.F., E. Uetz-von Allmen, and M.A. Hauser, *CCR7: roles in cancer cell dissemination, migration and metastasis formation.* Int J Biochem Cell Biol, 2014. **54**: p. 78-82.
30. Sun, J., et al., *Hypoxia induces T-cell apoptosis by inhibiting chemokine C receptor 7 expression: the role of adenosine receptor A(2).* Cell Mol Immunol, 2010. **7**(1): p. 77-82.
31. Turk, M.J., et al., *Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells.* J Exp Med, 2004. **200**(6): p. 771-82.
32. Wang, J., et al., *Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines.* J Immunol, 1998. **161**(10): p. 5516-24.
33. Szatmari, T., et al., *Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy.* Cancer Sci, 2006. **97**(6): p. 546-53.
34. Maes, W., et al., *DC vaccination with anti-CD25 treatment leads to long-term immunity against experimental glioma.* Neuro Oncol, 2009. **11**(5): p. 529-42.
35. Khan, M.K., et al., *Future of radiation therapy for malignant melanoma in an era of newer, more effective biological agents.* Onco Targets Ther, 2011. **4**: p. 137-48.
36. Bhatia, S., S.S. Tykodi, and J.A. Thompson, *Treatment of metastatic melanoma: an overview.* Oncology (Williston Park), 2009. **23**(6): p. 488-96.
37. Dhermain, F., *Radiotherapy of high-grade gliomas: current standards and new concepts, innovations in imaging and radiotherapy, and new therapeutic approaches.* Chin J Cancer, 2014. **33**(1): p. 16-24.
38. Shiao, S.L. and L.M. Coussens, *The tumor-immune microenvironment and response to radiation therapy.* J Mammary Gland Biol Neoplasia, 2010. **15**(4): p. 411-21.
39. Desai, A., et al., *A Retrospective Review of CyberKnife Stereotactic Body Radiotherapy for Adrenal Tumors (Primary and Metastatic): Winthrop University Hospital Experience.* Front Oncol, 2015. **5**: p. 185.
40. Jung, I.H., et al., *Clinical outcome of fiducial-less CyberKnife radiosurgery for stage I non-small cell lung cancer.* Radiat Oncol J, 2015. **33**(2): p. 89-97.
41. Song, Y., et al., *Analysis of clinical efficacy of CyberKnife((R)) treatment for locally advanced pancreatic cancer.* Onco Targets Ther, 2015. **8**: p. 1427-31.
42. Schaue, D., et al., *Maximizing tumor immunity with fractionated radiation.* Int J Radiat Oncol Biol Phys, 2012. **83**(4): p. 1306-10.
43. Novitskiy, S.V., et al., *Adenosine receptors in regulation of dendritic cell differentiation and function.* Blood, 2008. **112**(5): p. 1822-31.

44. Ma, G., et al., *Paired immunoglobin-like receptor-B regulates the suppressive function and fate of myeloid-derived suppressor cells.* Immunity, 2011. **34**(3): p. 385-95.
45. Stagg, J., et al., *CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis.* Cancer Res, 2011. **71**(8): p. 2892-900.
46. Tamajusuku, A.S., et al., *Characterization of ATP-induced cell death in the GL261 mouse glioma.* J Cell Biochem, 2010. **109**(5): p. 983-91.
47. Fidler, I.J., *Biological behavior of malignant melanoma cells correlated to their survival in vivo.* Cancer Res, 1975. **35**(1): p. 218-24.
48. Burnstock, G. and F. Di Virgilio, *Purinergic signalling and cancer.* Purinergic Signal, 2013. **9**(4): p. 491-540.
49. Chiu, T.L., M.J. Wang, and C.C. Su, *The treatment of glioblastoma multiforme through activation of microglia and TRAIL induced by rAAV2-mediated IL-12 in a syngeneic rat model.* J Biomed Sci, 2012. **19**: p. 45.
50. Campbell, I.L., M.J. Hofer, and A. Pagenstecher, *Transgenic models for cytokine-induced neurological disease.* Biochim Biophys Acta, 2010. **1802**(10): p. 903-17.
51. Mathios, D., et al., *Therapeutic administration of IL-15 superagonist complex ALT-803 leads to long-term survival and durable antitumor immune response in a murine glioblastoma model.* Int J Cancer, 2015.
52. Bobanga, I.D., A. Petrosiute, and A.Y. Huang, *Chemokines as Cancer Vaccine Adjuvants.* Vaccines (Basel), 2013. **1**(4): p. 444-62.
53. Vasco, C., et al., *Circulating T regulatory cells migration and phenotype in glioblastoma patients: an in vitro study.* J Neurooncol, 2013. **115**(3): p. 353-63.
54. Zhu, X., et al., *Systemic delivery of neutralizing antibody targeting CCL2 for glioma therapy.* J Neurooncol, 2011. **104**(1): p. 83-92.
55. Sugihara, A.Q., C.E. Rolle, and M.S. Lesniak, *Regulatory T cells actively infiltrate metastatic brain tumors.* Int J Oncol, 2009. **34**(6): p. 1533-40.
56. Vatner, R.E. and S.C. Formenti, *Myeloid-derived cells in tumors: effects of radiation.* Semin Radiat Oncol, 2015. **25**(1): p. 18-27.
57. Wei, S., et al., *Effects of tumor irradiation on host T-regulatory cells and systemic immunity in the context of adoptive T-cell therapy in mice.* J Immunother, 2013. **36**(2): p. 124-32.
58. Correale, P., et al., *Tumor infiltration by T lymphocytes expressing chemokine receptor 7 (CCR7) is predictive of favorable outcome in patients with advanced colorectal carcinoma.* Clin Cancer Res, 2012. **18**(3): p. 850-7.
59. Williams, A.J. and B.N. Cronstein, *The effect of A(2A) adenosine receptor activation on C-C chemokine receptor 7 expression in human THP1 macrophages during inflammation.* Inflammation, 2012. **35**(2): p. 614-22.
60. Cummings, R.J., et al., *Exposure to ionizing radiation induces the migration of cutaneous dendritic cells by a CCR7-dependent mechanism.* J Immunol, 2012. **189**(9): p. 4247-57.

FIGURE LEGENDS

Figure 1. Ectonucleotidases expression and activity on GL261 glioma cell line. A) Analysis of GL261 cell line ENTPDases expression by qRT-PCR. B) Analysis of the ENTPDase1/CD39 and 5´-ectonucleotidase/CD73 on the cell membrane of GL261 cell line by flow cytometry. C) Ectonucleotidases activity was accessed by thin layer chromatography (TLC) using ADP, AMP or adenosine (ADO) as substrates, and D) EHNA (adenosine deaminase 1 inhibitor) and pentostatin (adenosine deaminase 2 inhibitor) and adenosine as substrate. Control: no cells – substrates only, C: adenosine without inhibitors. EH: EHNA, Pen: pentostatin.

Figure 2. GL261 tumors are more immunegenic than B16/F10. A) H&E staining of tumor tissues, magnification, 20X. B) CD45 IHC staining of B16/F10 melanoma and GL261 glioma tissues, magnification, 200X. C) Percentage of $CD45^+$ cells on B16/F10 and GL261 tumors by flow cytometry. D) Comparison of B16/F10 and GL261 tumor-infiltrating immune cells by flow cytometry. E) Fold increase of GL261 cytokines expression relative to B16/F10 by RT-qPCR. Each column represents the mean ± SEM. p values as determined by Student's t test.

Figure 3. CD39 deletion produces smaller tumors and higher expression of immune components. A) B16/F10 melanoma tumor growth curve. B) B16/F10 tumor size on day 20 after tumor injection. C) GL261 glioma tumor growth curve. D) Level of immune components expression on GL261 glioma tumors from WT vs. CD39KO mice by RT-qPCR. Each column represents the mean ± SEM. p values as determined by Student's t test.

Figure 4. CyberKnife delivery radiotherapy to a specific mouse tumor. A) Percentage of $CD45^+$ cells infiltrated in non-irradiated (untreated mice and mice contralateral side) and irradiated GL261 glioma tumors. Mice were engrafted with tumor in both left and right flanks and irradiated using Cyberknife in only the right hand flank. B) CD45 IHC staining in irradiated and contralateral side of WT GL261 glioma-bearing mice, magnification, 200X. C) Level of immune components expression on GL261 glioma tumors from non-irradiated (Not-treated) *vs.* irradiated (Treated) WT mice by RT-qPCR. Each column represents the mean ± SEM. p values as determined by Student's t test.

Figure 5. Decrease of immune suppressor cells on GL261 glioma-bearing mice CD39KO irradiated. Percentages in dot plots are of A) 7AAD- $CD45^+$ $FOXP3^+$ Tregs on tumor. B) FOXP3 IHC staining of GL261 glioma tissue, magnification, 20X. C) $FOXP3^+$ $CD11b^-$ Tregs on spleen. D) 7AAD- $CD45^+$ $CD11b^+$ $Gr1^+$ MDSC on tumor. E) 7AAD- $TCR\beta^+$ $CD4^+$ cells and 7AAD- $TCR\beta^+$ $CD8^+$ cells on tumor. Each column represents the mean ± SEM, statistical analyses were performed using a one-way ANOVA/Tukey–Kramer test.

Figure 6. CD39 deletion combined with radiotherapy increases $CCR7^+$ immune cells in the spleen of GL261 glioma-bearing mice. CD39KO animals possessed increased splenic CCR7 expression in A) dendritic cells ($CD11c^+$ MHC II^{high} $CCR7^+$) and B) macrophages ($CD11b^+$ MHC II^{high} $CCR7^+$) 4 days after irradiation. C) CCR7 levels in GL261 tumors from WT vs. CD39KO mice. Each column represents the mean ± SEM, statistical analyses were performed using a one-way ANOVA/Tukey–Kramer test or Student's t test

Table 1

Mouse primer sequences

Gene		Primers (5' –3')	GENE ID
β-actin	F	CTCTTCCAGCCTTCCTTCCT	NM_007393
	R	AGCACTGTGTTGGCGTACAG	
Arg1	F	CAGAAGAATGGAAGAGTC AG	NM_007482
	R	CAGATATGCAGGGAGTCACC	
iNos	F	CTCTGAGGGCTGACACAAGG	NM_001313921
	R	CCAAGCCCTCACCTACTTCC	
GITR	F	GAGCAATACGGCCATTTGACT	NM_009400
	R	GAGCTGGACTGTGGTTAGGAA	
IFN-γ	F	TGGCTCTGCAGGATTTTCATG	NM_008337
	R	TCAAGTGGCATAGATGTGGAAGAA	
CCL2	F	TTAAAAACCTGGATCGGAACCAA	NM_011333
	R	GCATTAGCTTCAGAT TTACGGGT	
CCL3	F	TTCTCTGTACCATGACACTCTGC	NM_011337
	R	CGTGGAATCTTCCGGCTGTAG	
CCL5	F	GCTGCTTTGCCTACCTCTCC	NM_013653
	R	TCGGACAAACACGACTGC	
TNF-α	F	TGTAGCCCACGTCGTAGCAAA	NM_001278601
	R	CTGGCACCACTAGTTGGTTGT	
IL-12	F	ATGGCTGCTGCGTTGAGAA	NM_001159424
	R	AGCACTCATAGTCTGTCTTGGA	
IL-1β	F	GCAACTGTTCCTGAACTCAACT	NM_008361
	R	ATCTTTTGGGGTCCGTCAACT	
IL-15	F	AATCAGATACCGCAATGACCAC	NM_001254747
	R	CAGAAGTTGTTTGGGATGGTGT	
IL-17A	F	TCAGCGTGTCCAAACACTGAG	NM_010552
	R	CGCCAAGGGAGTTAAAGACTT	
CCR7	F	TGTACGAGTCGGTGTGCTTC	NM_001301713
	R	GGTAGGTATCCGTCATGGTCTTG	

F – Forward, R – Reverse

FIGURES

Figure 1

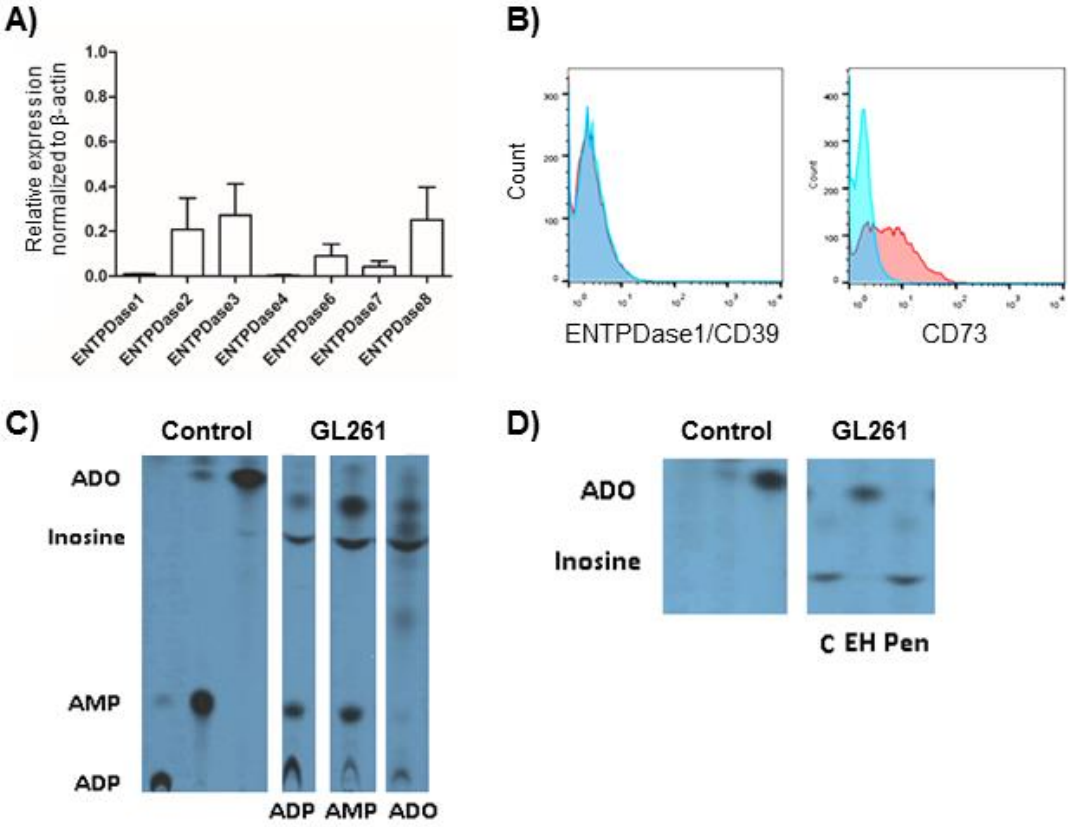


Figure 2

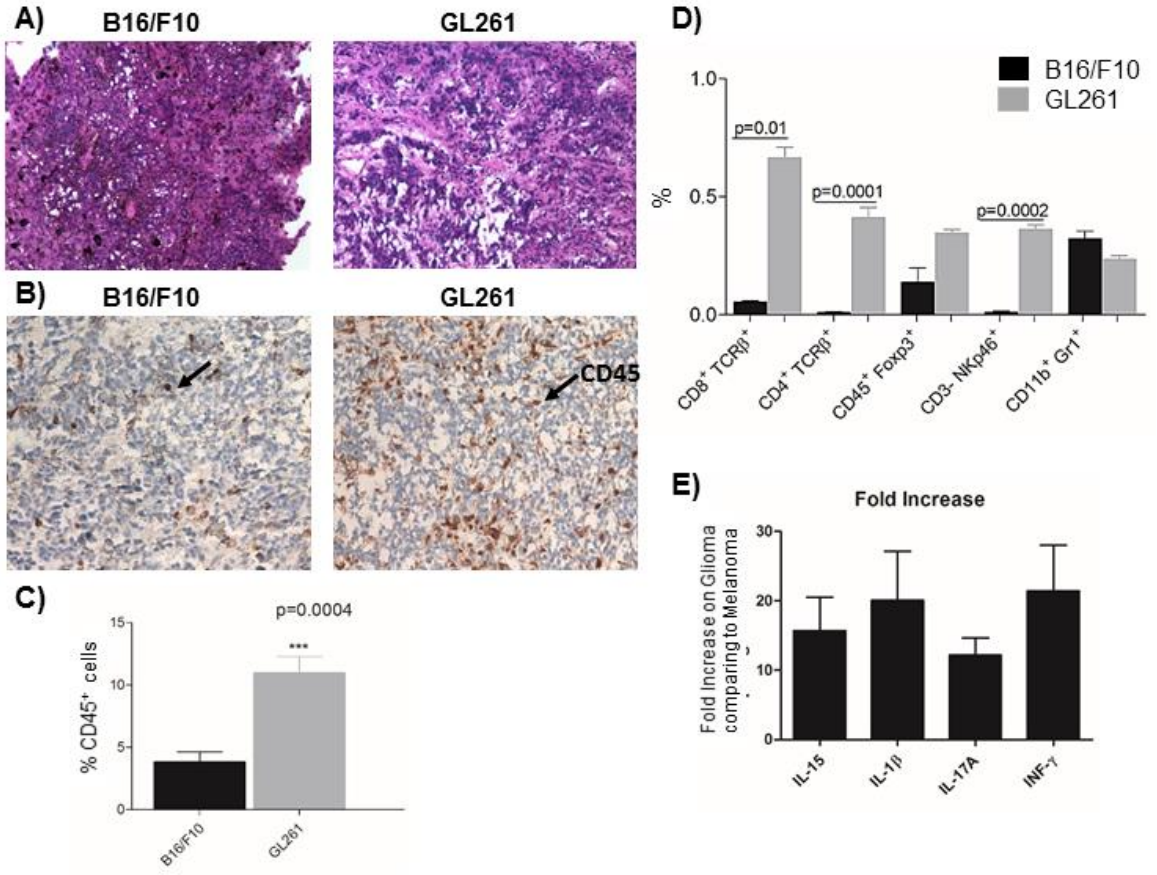

A)
B16/F10
GL261
B)
B16/F10
GL261
CD45
C)
p=0.0004
***
% CD45+ cells
B16/F10
GL261
D)
B16/F10
GL261
%
p=0.01
p=0.0001
p=0.0002
CD8+ TCRβ+
CD4+ TCRβ+
CD45+ Foxp3+
CD3- NKp46+
CD11b+ Gr1+
E)
Fold Increase
Fold Increase on Glioma comparing to Melanoma
IL-15
IL-1β
IL-17A
INF-γ

Figure 3

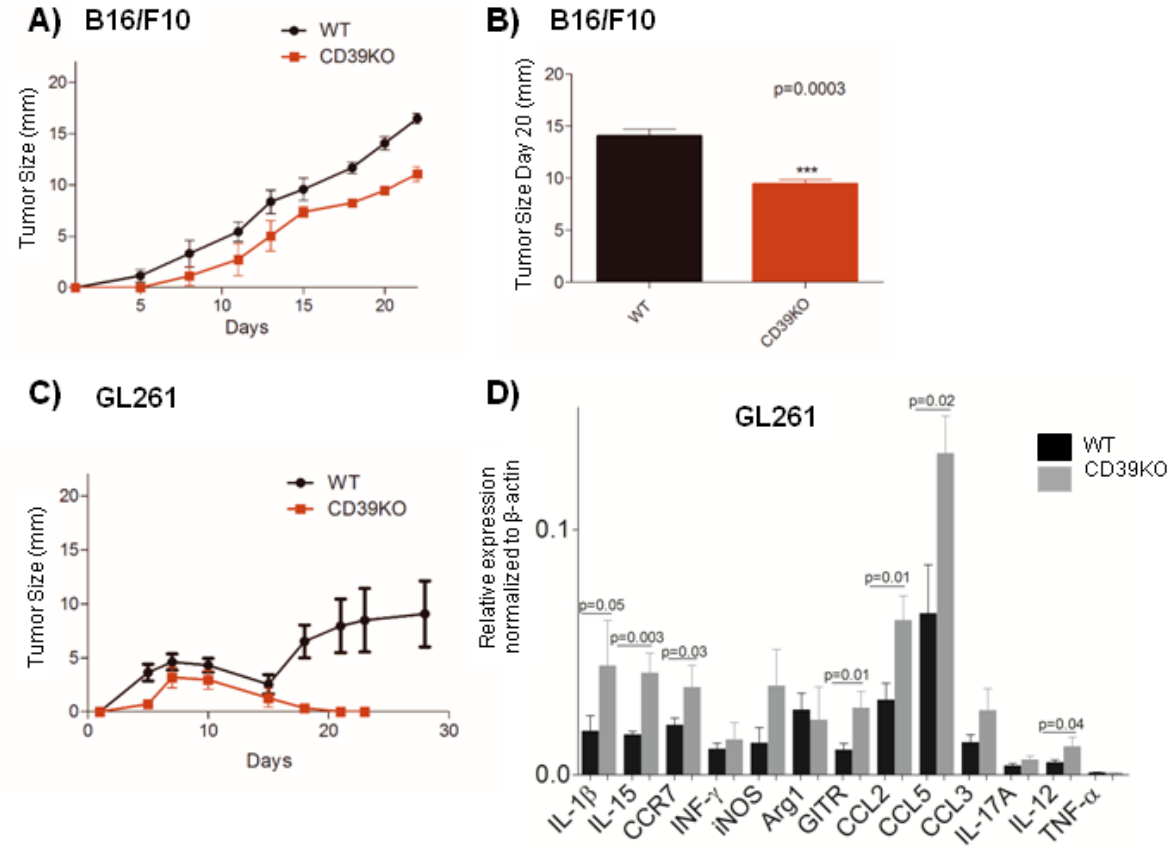

A) B16/F10
WT
CD39KO
Tumor Size (mm)
Days
B) B16/F10
Tumor Size Day 20 (mm)
p=0.0003
***
WT
CD39KO
C) GL261
WT
CD39KO
Tumor Size (mm)
Days
D)
GL261
WT
CD39KO
Relative expression normalized to β-actin
p=0.05
p=0.003
p=0.03
p=0.01
p=0.01
p=0.02
p=0.04
IL-1β
IL-15
CCR7
INF-γ
iNOS
Arg1
GITR
CCL2
CCL5
CCL3
IL-17A
IL-12
TNF-α

Figure 4

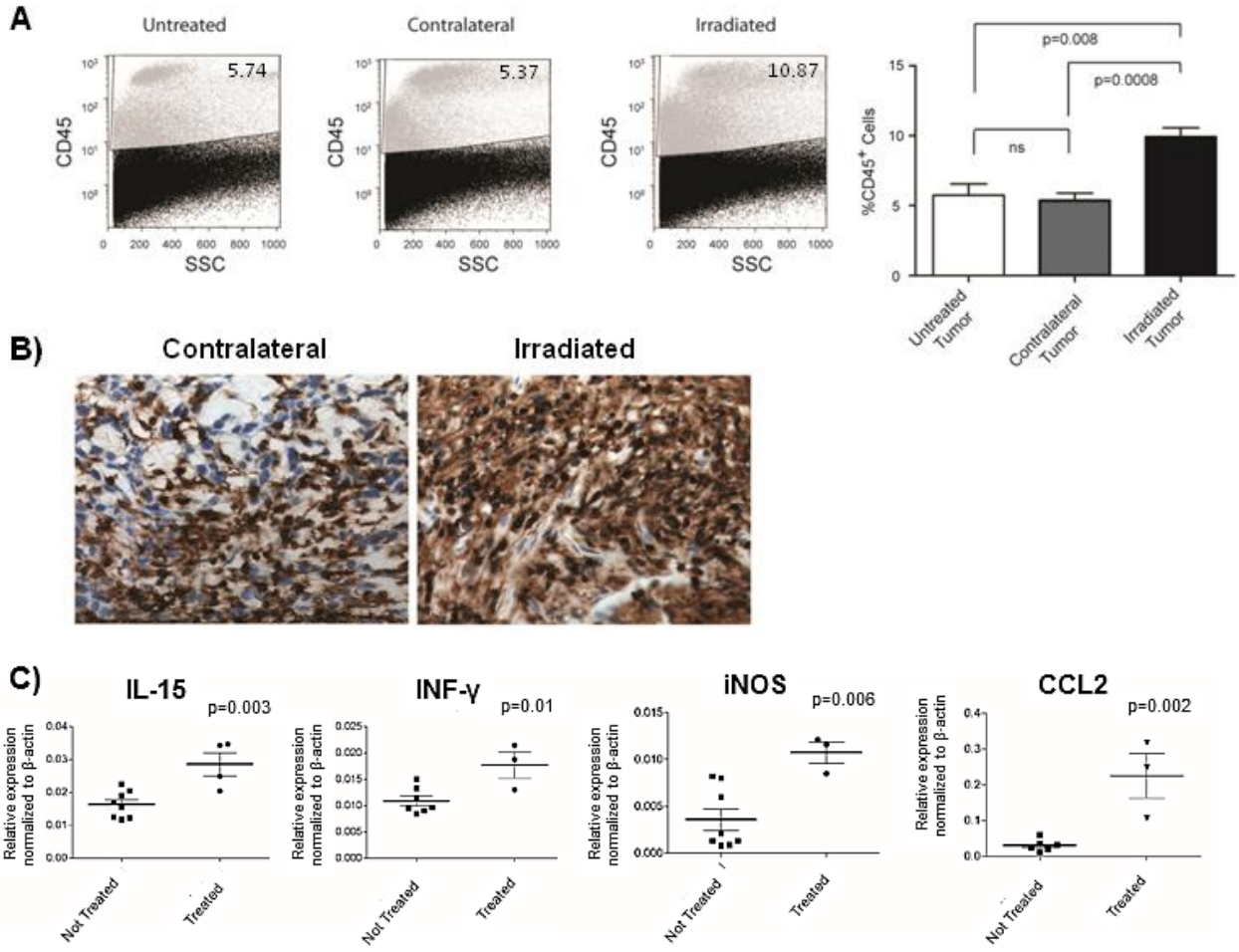
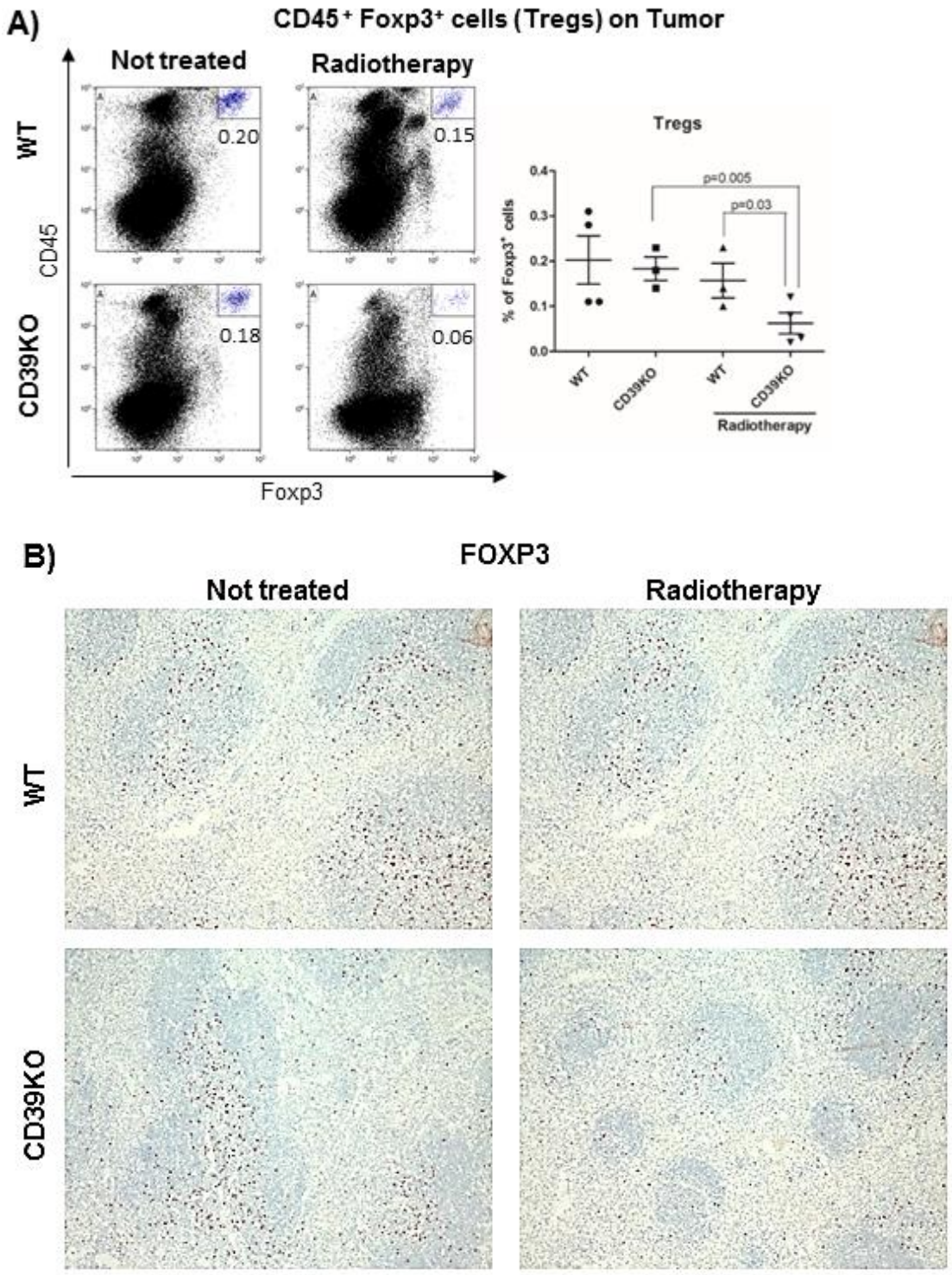
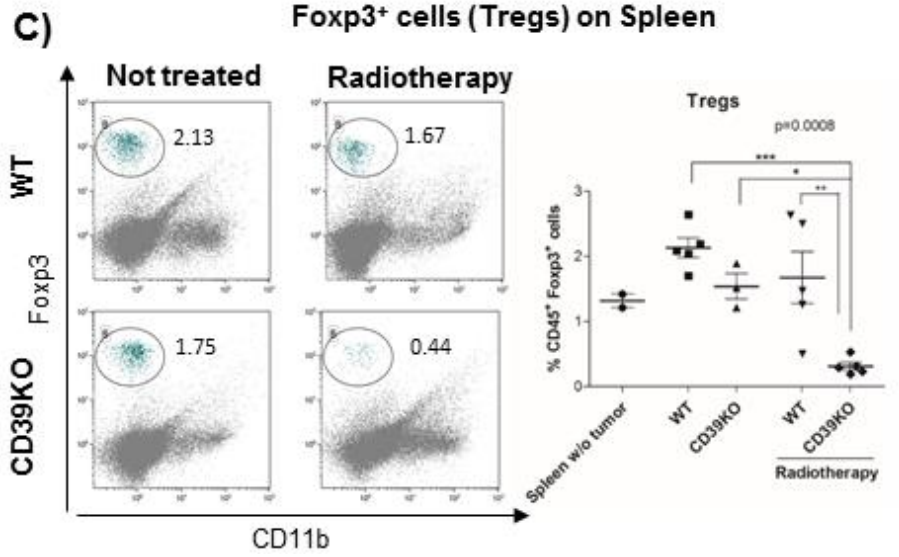
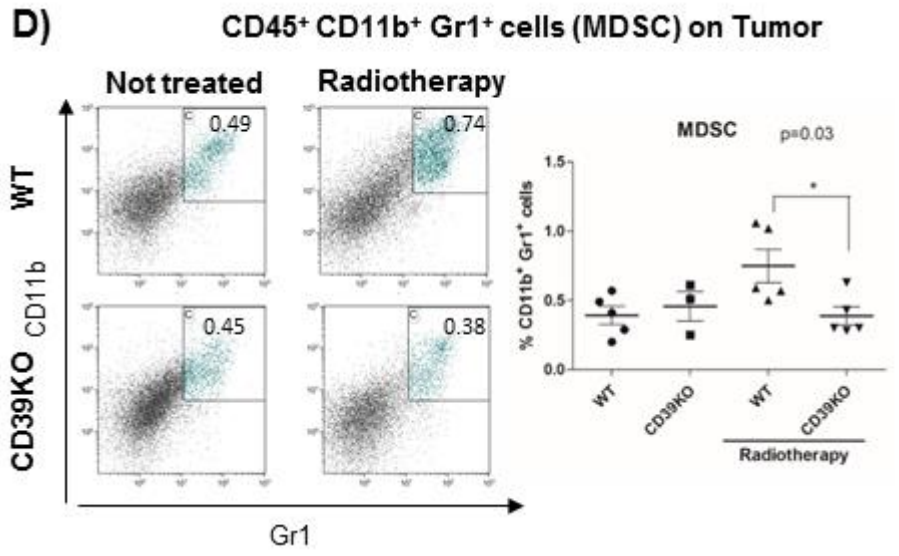

A
Untreated
Contralateral
Irradiated
5.74
5.37
10.87
CD45
SSC
p=0.008
p=0.0008
ns
%CD45+ Cells
Untreated Tumor
Contralateral Tumor
Irradiated Tumor
B)
Contralateral
Irradiated
C)
IL-15
p=0.003
INF-γ
p=0.01
iNOS
p=0.006
CCL2
p=0.002
Relative expression normalized to β-actin
Not Treated
Treated

Figure 5


A)
CD45+ Foxp3+ cells (Tregs) on Tumor
Not treated
Radiotherapy
WT
CD39KO
CD45
Foxp3
0.20
0.15
0.18
0.06
Tregs
% of Foxp3+ cells
p=0.005
p=0.03
WT
CD39KO
WT
CD39KO
Radiotherapy
B)
FOXP3
Not treated
Radiotherapy
WT
CD39KO


C)
Foxp3+ cells (Tregs) on Spleen
Not treated
Radiotherapy
WT
CD39KO
Foxp3
CD11b
2.13
1.67
1.75
0.44
Tregs
p=0.0008
% CD45+ Foxp3+ cells
Spleen w/o tumor
WT
CD39KO
WT
CD39KO
Radiotherapy


D)
CD45+ CD11b+ Gr1+ cells (MDSC) on Tumor
Not treated
Radiotherapy
WT
CD39KO
CD11b
Gr1
0.49
0.74
0.45
0.38
MDSC
p=0.03
% CD11b+ Gr1+ cells
WT
CD39KO
WT
CD39KO
Radiotherapy

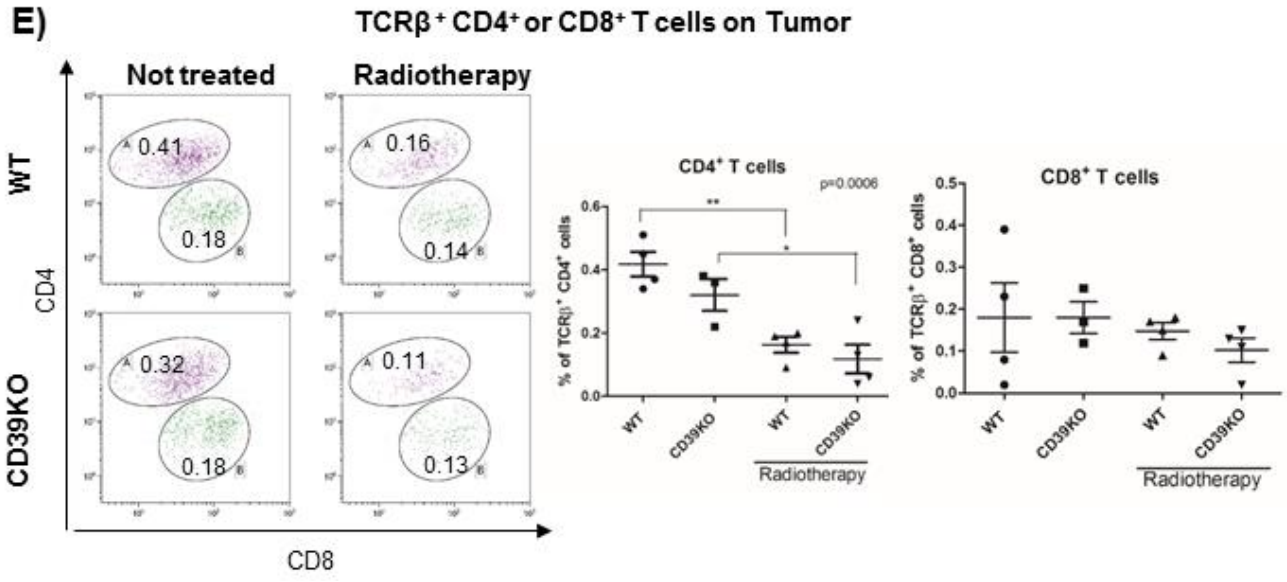
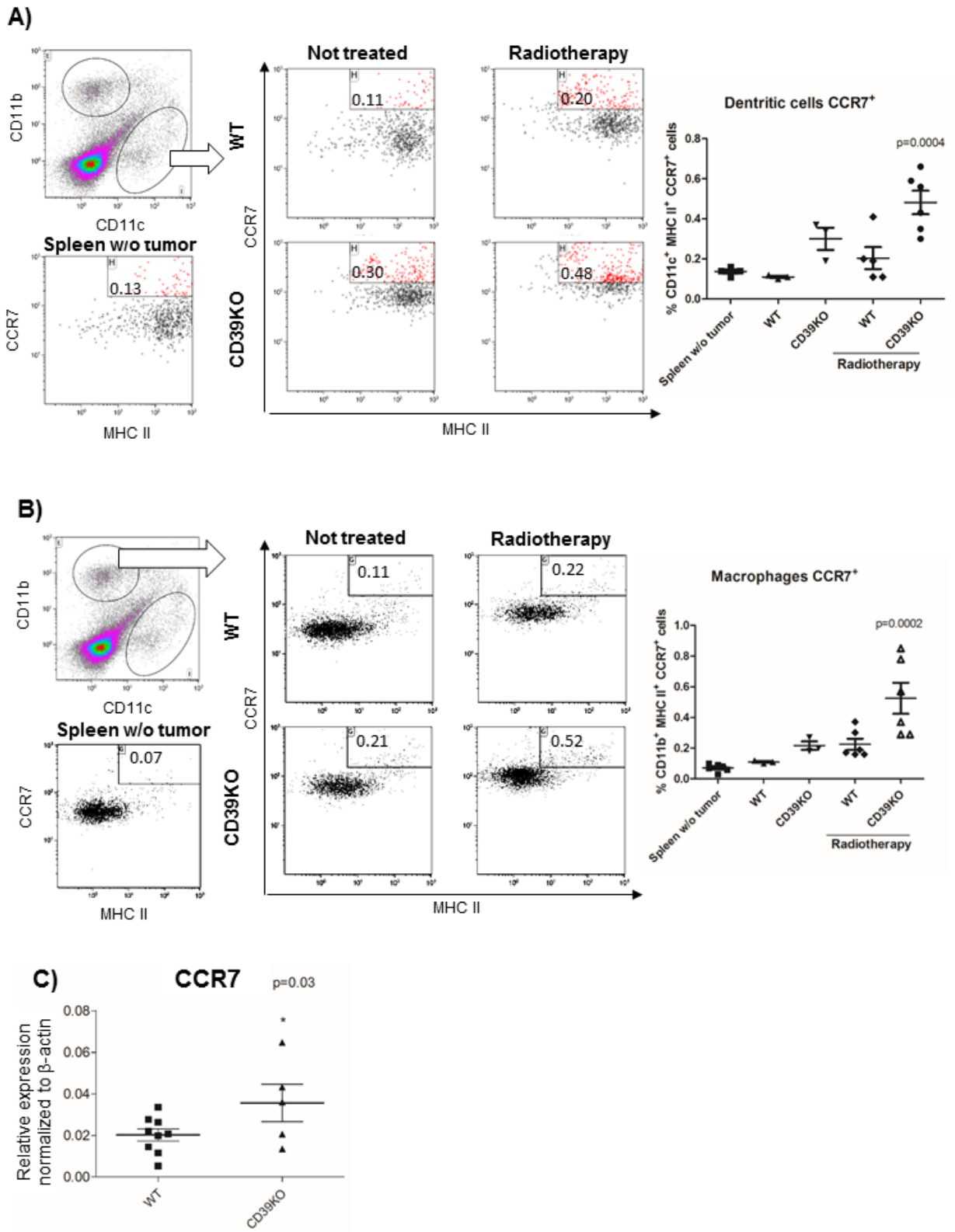

E)
TCRβ+ CD4+ or CD8+ T cells on Tumor
Not treated
Radiotherapy
WT
CD39KO
CD4
CD8
0.41
0.18
0.16
0.14
0.32
0.18
0.11
0.13
CD4+ T cells
p=0.0006
% of TCRβ+ CD4+ cells
CD8+ T cells
% of TCRβ+ CD8+ cells
WT
CD39KO
WT
CD39KO
Radiotherapy

Figure 6


A)
CD11b
CD11c
Spleen w/o tumor
0.13
CCR7
MHC II
Not treated
Radiotherapy
WT
CD39KO
0.11
0.20
0.30
0.48
CCR7
MHC II
Dentritic cells CCR7+
p=0.0004
% CD11c+ MHC II+ CCR7+ cells
Spleen w/o tumor
WT
CD39KO
WT
CD39KO
Radiotherapy
B)
CD11b
CD11c
Spleen w/o tumor
0.07
CCR7
MHC II
Not treated
Radiotherapy
WT
CD39KO
0.11
0.22
0.21
0.52
CCR7
MHC II
Macrophages CCR7+
p=0.0002
% CD11b+ MHC II+ CCR7+ cells
Spleen w/o tumor
WT
CD39KO
WT
CD39KO
Radiotherapy
C)
CCR7
p=0.03
Relative expression normalized to β-actin
WT
CD39KO

3.3 CAPÍTULO III

IL-17 PROMPTS HUMAN TUMOR CELLS MIGRATION AND AKT, ERK, P38 PHOSPHORILATION *IN VITRO*

Short Communication submetido ao periódico *Cytokine*

IL-17A PROMPTS HUMAN TUMOR CELLS MIGRATION AND AKT, ERK, P38 PHOSPHORYLATION *IN VITRO*

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ABSTRACT

Changes of interleukin levels have been associated with advanced stages of several cancer types. Increased levels of cytokines induce tumorigenesis, being a poor prognostic marker for malignant disease. Chronic inflammation is a pathological feature of cancer and interleukin-17 (IL-17A) is an inflammatory cytokine with diverse functions in host defense. This study aimed to clarify the direct role of IL-17 on tumor cells. For this study, we used human tumor cells from glioma, bladder and esophagus, which were treated with different IL-17 concentrations, ranging from 10 pg/mL to 100 ng/mL. We analyzed IL-17 receptor (IL-17RA) expression on tumor cells through qRT-PCR, and investigated IL-17 ability to modify tumor cell proliferation and viability through cell counting and MTT assay. Cell migration capacity and intracellular signaling pathways activated by IL-17 were assessed by wound healing assay and flow cytometry, respectively. IL-17RA mRNA expression was detected on the cell lines tested, but the incubation of IL-17 did not alter tumor cell viability or proliferation. However, the incubation of IL-17 was able to promote tumor cell migration and AKT, ERK and P38 phosphorylation *in vitro*. Our data show, for the first time, the ability of IL-17RA/IL-17 to induce migration of human tumor cell lines *in vitro*, via activation of MAP kinases and AKT.

Keywords: IL-17A; IL-17RA; Glioma; Bladder tumor; Esophageal tumor; Cell migration.

INTRODUCTION

Interleukin-17 (IL-17A) is classified as a pro-inflammatory cytokine and it is shown to be elevated in several types of cancer [160]. Data regarding the role of IL-17 on tumor development are not consensual. It is important to highlight that exogenously delivered IL-17 might display different effects in comparison to endogenous IL-17 [39]. Recently, researches on this field point out the idea that IL-17 promotes tumor growth, especially through the activation of IL-6-STAT3 cascade, an oncogenic transcription factor that upregulates pro-survival and pro-angiogenic genes [39, 52, 56, 160]. In contrast, other studies suggest that IL-17 may protect against tumors, by promoting immune system-mediated tumor rejection [160-162].

After binding to its receptor (IL-17/R), intracellular IL-17 signaling includes transcription factor NF-κB [44, 48], which induces the coordinated expression of several inflammatory genes causing the perpetuation of the inflammatory response [48]; PI3K, MAPKs, JNK, ERK and p38 are clearly involved in IL-17-induced responses [44, 48].

Studies about the role of IL-17 in cancer are still very few regarding to different types of cancer such as bladder, brain and esophageal cancer. In relation to gliomas, Hu et al. 2011, described that IL-17 expression may play an important role in tumorigenesis and progression, since they observed high mRNA-positive ratios of IL-17 in glioma tissues, but not in tissues submitted to trauma [56]. For bladder cancer, the tumor was reduced in IL-17$^{-/-}$ mice, indicating a role of this cytokine in promoting tumor growth through IL-6/STAT3 pathway [52]. Another study suggests that IL-17 may play an important role in the recruitment and infiltration of antitumor immune cells in early stages of bladder cancer [161]. In esophageal squamous cell carcinoma (ESCC), IL-17 led to the production of inflammatory chemokines (CXCL9, CXCL10 and CCL2, CCL20), which are associated with the migration of T cells, NK cells, and DCs, respectively. In addition, IL-17 enhanced the cytotoxic effects of NK cells against tumor cells [162]. The present study aimed to clarify the direct action of IL-17 in a series of solid tumor human cells, from glioma, esophageal carcinoma and bladder cancer.

MATERIALS AND METHODS

Cell lines and cell culture

GL261 mouse glioma cells, M059J and U138 human glioma cells and T24 human bladder transitional cell carcinoma cells were obtained from ATCC (Rockville, Maryland, USA). OE-21 human esophageal squamous cell carcinoma was a gift from INCA (Rio de Janeiro, Brazil). M059J and U138 were cultured in DMEM/10% FBS and OE-21 and T24 were

cultured in RPMI/10% FBS at a temperature of 37°C, a minimum relative humidity of 95%, and an atmosphere of 5% CO_2 in air.

Analysis of IL-17RA mRNA expression

Cells (2 x 10^5) were collected and processed as previously described [163]. Briefly, total RNA was isolated using Trizol LS reagent (Invitrogen) and cDNA species synthesized with ImProm-II™ Reverse Transcription System (Promega). Quantitative PCR, using SYBR Green I as intercalating dye, was performed for *IL-17RA* (F: 5'-GCCCTGGACAGGTTCCGGGACTG-3'; R: 5'-CCCCTCCTCTGCGGCGAGCAC-3') and for *18S* and *β-actin* as reference genes. Relative mRNA levels were determined using $2^{-\Delta\Delta CT}$ method including individual efficiency calculated per sample using LinReg 11.0 Software (http:// LinRegPCR.nl).

Cell viability and proliferation

For measure cell viability and proliferation, we performed MTT assay and cell counting, respectively, as previously described [163]. Cells were treated with IL-17A (10, 20, 50, 100 pg/mL and 10, 20, 100 ng/mL) for 24 h.

Wound-healing migration assay

Cells were seeded in medium containing 10% FBS at 3 x 10^5 cells per well in 24-well plates. In order to minimize cell proliferation, cell cultures were grown to 80-90% confluence and deprived of serum medium for 18 h. The objective was to obtain a reproducible measurement of the migration of the wound edge towards the wound space with minimum cell proliferation, but without loss of cell viability over the experimental period. Wounds were made by sterile pipette tips and remaining cells were washed twice with CMF to eliminate detached cells and it was added DMEM/RPMI deprived serum medium. Cells were treated with IL-17A (10 ng/ml) and then incubated at 37°C. After 48 h, migrating cells at the wound front were photographed by Olympus inverted microscope IX71 (Tokyo, Japan) with a magnification × 100 and compared. The cell-free area was correlated with tumor cell ability to migrate into the scratch. TScratch software was used to calculate the cell-free area. Three independent experiments were performed.

Intracellular signaling pathways activated by IL-17

In order to analyze IL-17 intracellular signaling activation, tumor cells were treated with IL-17A (10 ng/ml) for 1, 15 and 30 min and then processed according to the manufacturer's instructions using anti-AKT, anti-p38 MAPK and anti-ERK 1/2 antibodies (BD Phosflow, BD Biosciences). Cells were analyzed on FACSCanto II Flow Cytometer (BD Biosciences) and the results were analyzed using FlowJo Software (Tree Star).

Statistical analysis

Data were analyzed by one-way analysis of variance (one-way ANOVA), followed by Tukey post-hoc test, using GraphPad Software version 5.0 (San Diego, CA, U.S.A.). p values <0.05 were taken as statistically significant.

RESULTS AND DISCUSSION

IL-17 receptor (IL-17RA) is expressed on human glioma, esophageal and bladder tumor cells

In this study, we demonstrated, for the first time, that M059J and U138 human glioma cells, 0E-21 human esophageal cancer cells and T24 human bladder cancer cells express IL-17A receptor (IL-17RA) (Figure 1A). A previous study has already shown the IL-17RA expression on GL261 cells [57]. When we tested the effects of different concentrations of IL-17 on these tumor cell lines, the exogenous IL-17 had no direct effect on the tumor cells proliferation or viability *in vitro* (Figure 1B and C, respectively). These results are in agreement with studies performed with GL261 mouse glioma cell line [57] and human hepatocarcinoma cell lines [164]. Another study showed that recombinant IL-17 protein or retroviral transduction of IL-17 gene into tumors did not affect *in vitro* proliferation, but *in vivo*-IL-17-transfected cells grew more rapidly when compared with controls [50].

IL-17 prompts tumor cell migration in vitro

Although IL-17 is primarily associated with the induction of tissue inflammation, the other biological functions of IL-17, as wound-healing functions, still remains to be thoroughly explored [165]. We have shown that tumor cells stimulated with IL-17 (10 ng/ml) showed an increase in the wound closure rates after 48 h when compared to non treated tumor cells (Figure 2A), suggesting that IL-17 is capable of promoting tumor cell migration *in vitro*. A study has showed that IL-17 increases the invasive capability of the JEG-3 human choriocarcinoma cell line, but the mechanisms of action involved in this effect remain unclear

[166]. It is known that matrix metalloproteinases (MMPs) degrade ECM and facilitate cell migration. Regarding this mechanism, a study showed that IL-17 is able to induce invasion through MMPs (MMP-2, MMP-9) transcription and expression [164] leading to cell migration [165]. Furthermore, the IL-17 capacity to stimulate IL-8 production can, in turn, promote granulocyte recruitment and stimulates MMPs expression, which causes the extracellular matrix remodeling and promotes cancer cell invasion [166].

Intracellular signaling activated by IL-17 on tumor cells

As shown in Figure 2B, IL-17 (10 ng/ml) induces ERK1/2 phosphorylation in all human tumor cells tested, occurring continuously (from the first minute until 30 min after IL-17 stimulation) on the U138 and OE-21 cell lines and only after 15 min or 30 min on T24 and on M059J cell lines, respectively. IL-17-induced p38 phosphorylation was continuous on 0E-21 and T24 cells and occurred only after 15 or 30 min of stimulation on M059J and U138 cells, respectively. IL-17-induced AKT phosphorylation occurred faster (only on the first minute of stimulation) on U138 and T24 cells, decreasing this activation in the following min. On the OE-21 cell line, IL-17-induced AKT phosphorylation in a continuously way and on the M059J cells, IL-17 failed to induce AKT phosphorylation. It was shown that IL-17-induced MMPs expression occurs via p38 and MAPK activation, suggesting that IL-17 enhances cell migration by increasing MMP-1 expression through these signal transduction pathways [165]. AKT signaling may also play an important role in inducing pro-invasive factors and hence tumor progression, as the expression of IL-6, IL-8, MMP2, and VEGF increased in parallel with AKT activation in cells treated with IL-17 [164].

CONCLUSION

In conclusion, our results suggest that IL-17 directly promotes the migration of different human tumor cultured cells, possible through the activation of ERK, AKT and p38. This study provides additional understanding on the IL-17 direct action on tumors.

Acknowledgments

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Conflicts of Interest Notification

Potential conflicts of interest do not exist.

REFERENCES

1. Zeng, Y., et al., *Prognostic significance of interleukin-17 in solid tumors: a meta-analysis.* Int J Clin Exp Med, 2015. **8**(7): p. 10515-36.
2. Zou, W. and N.P. Restifo, *T(H)17 cells in tumour immunity and immunotherapy.* Nat Rev Immunol, 2010. **10**(4): p. 248-56.
3. Hu, J., et al., *The profile of Th17 subset in glioma.* Int Immunopharmacol, 2011. **11**(9): p. 1173-9.
4. Wang, L., et al., *IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway.* J Exp Med, 2009. **206**(7): p. 1457-64.
5. Baharlou, R., et al., *Increased interleukin-17 transcripts in peripheral blood mononuclear cells, a link between T-helper 17 and proinflammatory responses in bladder cancer.* Iran Red Crescent Med J, 2015. **17**(2): p. e9244.
6. Lu, L., et al., *IL-17A promotes immune cell recruitment in human esophageal cancers and the infiltrating dendritic cells represent a positive prognostic marker for patient survival.* J Immunother, 2013. **36**(8): p. 451-8.
7. Ivanov, S. and A. Linden, *Interleukin-17 as a drug target in human disease.* Trends Pharmacol Sci, 2009. **30**(2): p. 95-103.
8. Kehlen, A., et al., *Interleukin-17 stimulates the expression of IkappaB alpha mRNA and the secretion of IL-6 and IL-8 in glioblastoma cell lines.* J Neuroimmunol, 1999. **101**(1): p. 1-6.
9. Gehring, M.P., et al., *P2X7 receptor as predictor gene for glioma radiosensitivity and median survival.* Int J Biochem Cell Biol, 2015. **68**: p. 92-100.
10. Cantini, G., et al., *A critical role for regulatory T cells in driving cytokine profiles of Th17 cells and their modulation of glioma microenvironment.* Cancer Immunol Immunother, 2011. **60**(12): p. 1739-50.
11. Gu, F.M., et al., *IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma.* Mol Cancer, 2011. **10**: p. 150.
12. Numasaki, M., et al., *Interleukin-17 promotes angiogenesis and tumor growth.* Blood, 2003. **101**(7): p. 2620-7.
13. Wu, Y., et al., *Interleukin-17A stimulates migration of periodontal ligament fibroblasts via p38 MAPK/NF-kappaB -dependent MMP-1 expression.* J Cell Physiol, 2014. **229**(3): p. 292-9.
14. Zhu, X., et al., *IL-17 expression by breast-cancer-associated macrophages: IL-17 promotes invasiveness of breast cancer cell lines.* Breast Cancer Res, 2008. **10**(6): p. R95.

FIGURES

Figure 1. A) Relative gene expression profile of IL-17 on human tumor cells. Overall results from N = 4 independent experiments using *18S* and *β-actin* as reference genes. **B)** Effect of IL-17 (100 pg/mL and 10, 100 ng/mL) on human tumor cells proliferation after 24 h. Control: non treated cells were considered 100% and used to compare cell viability between the same cell line treated with IL-17. The experiments were carried out at least three times in triplicate. **C)** Effect of IL-17 (10, 20, 50, 100 pg/mL and 10, 20, 100 ng/mL) on tumor cells viability after 24 h. The experiments were carried out at least three times in triplicate. Each column represents the mean ± SEM, as determined by ANOVA/Tukey test.

Figure 2. A) Effect of IL-17 (10 ng/ml) on cell migration after stimulation for 48 h (100× magnification). The experiment was carried out at least three times. Percentage of open area were calculated using TScratch software [26]. Each column represents the mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, as determined by Student's t-test. **B)** Effect of IL-17 (10 ng/ml) on phosphorylation of ERK, P38 and AKT after 1, 15 and 30 minutes of IL-17 (10 ng/ml) stimulation. The experiments were carried out at least two times. Each column represents the mean ± SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$, as determined by ANOVA/Tukey test. NC: negative control. PC: positive control

FIGURES

Figure 1

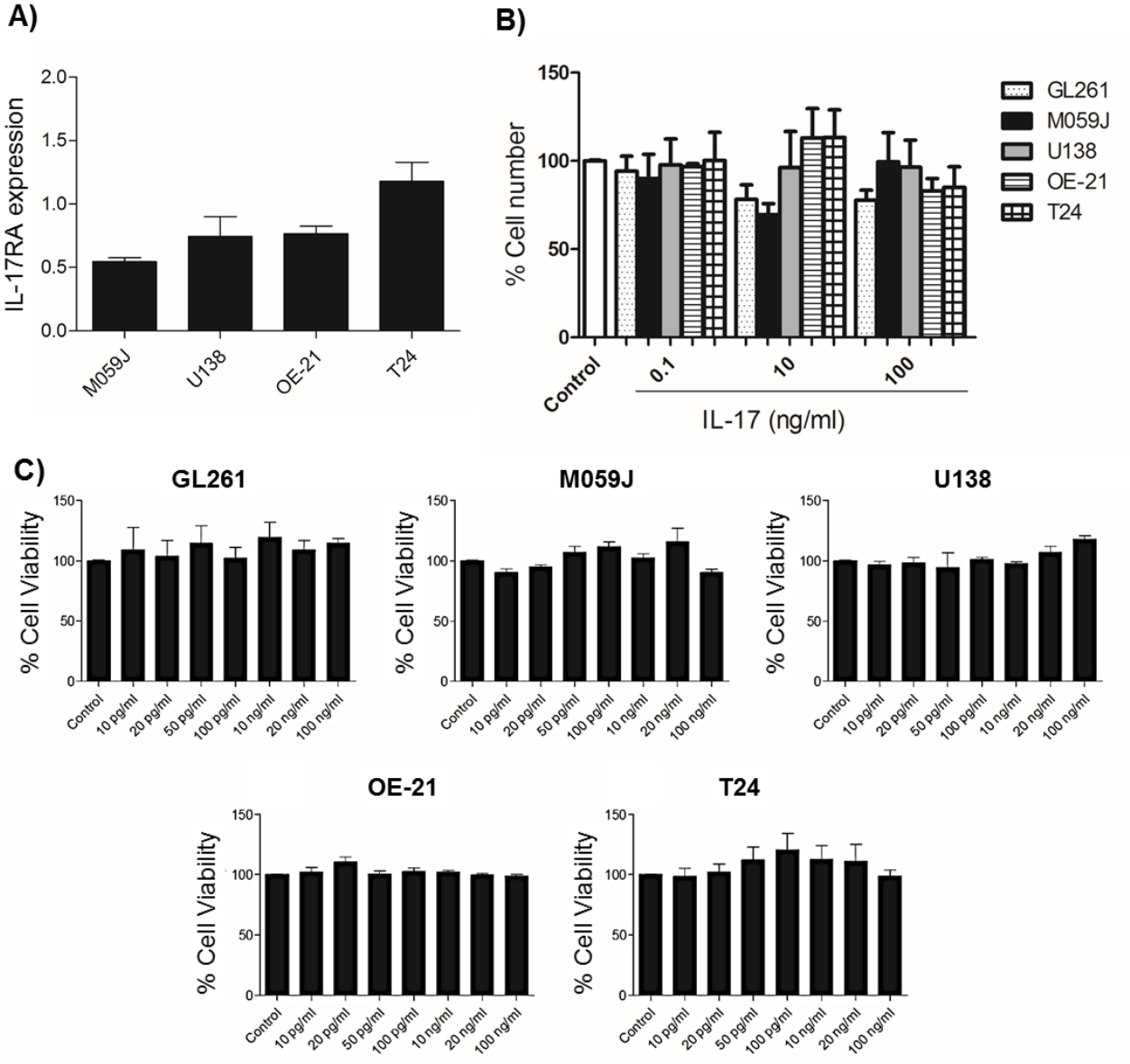
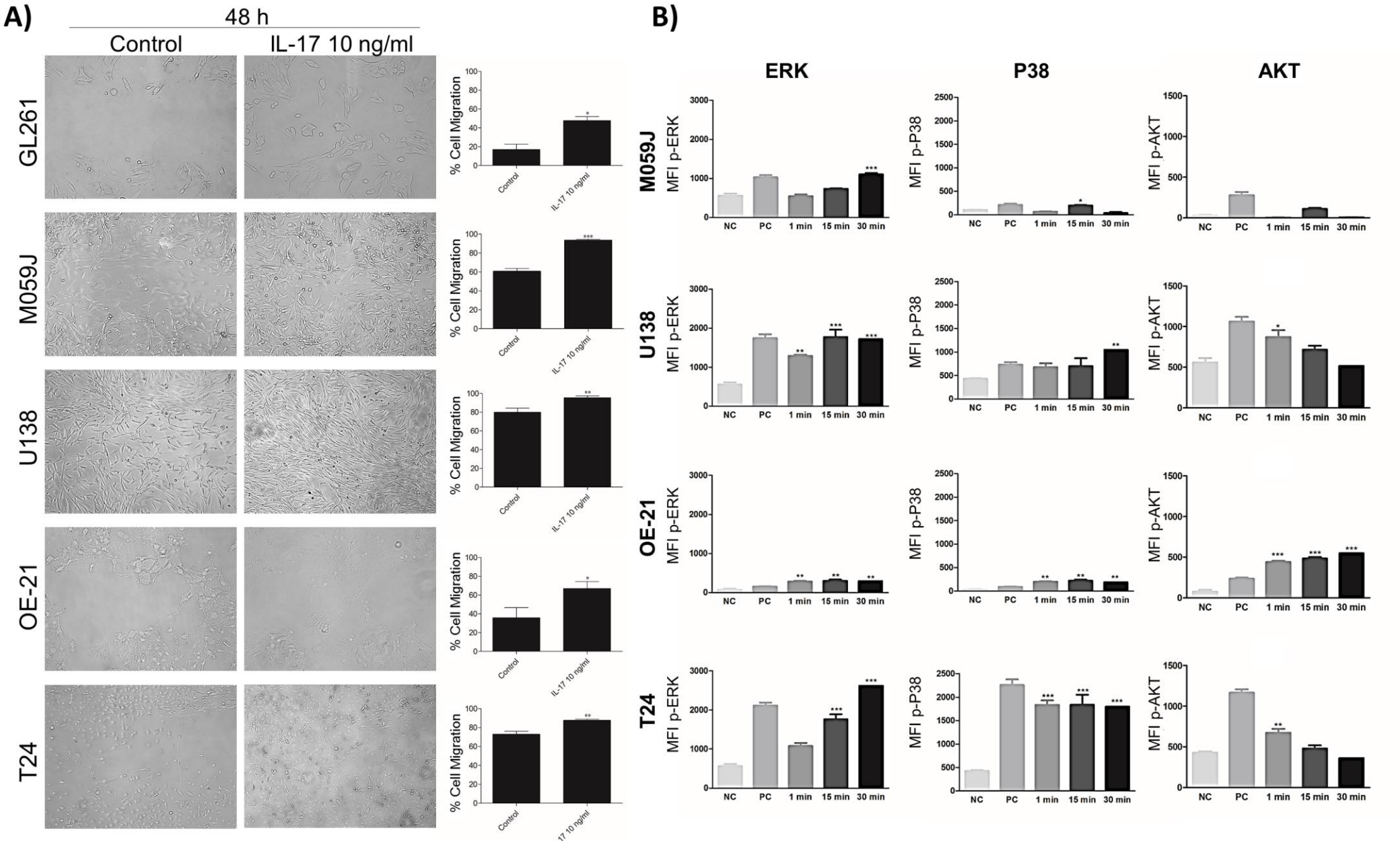


Figure 2


A)
48 h
Control
IL-17 10 ng/ml
GL261
M059J
U138
OE-21
T24
% Cell Migration
Control
IL-17 10 ng/ml
17 10 ng/ml
B)
ERK
P38
AKT
M059J
U138
OE-21
T24
MFI p-ERK
MFI p-P38
MFI p-AKT
NC
PC
1 min
15 min
30 min

4 CONSIDERAÇÕES FINAIS

O câncer é uma doença sistêmica, e não uma formação solitária, geralmente iniciada como resultado da acumulação gradual de alterações genéticas e epigenéticas no compartimento epitelial; no entanto, cada vez mais evidências indicam que o microambiente tumoral pode ditar a função do tecido e desempenhar um papel crítico no desenvolvimento de neoplasias mais avançadas e refratárias [167]. Por muito tempo, o câncer foi visto como uma doença de células hiper-proliferativas e os tratamentos para o câncer têm sido focados na sua eliminação direta. No entanto, a capacidade de células tumorais em evadir respostas imunes específicas, que evoluiriam para o reconhecimento e eliminação das células malignas, é agora reconhecida como uma das características centrais do câncer [168]. Portanto, compreender os mecanismos utilizados pelos tumores para induzir esta imuno-modulação e resistência a tratamentos utilizados atualmente como quimio e radioterapia a fim de evitar sua eliminação é de grande importância para se pensar em terapias baseadas na reativação do sistema imune para a eliminação das células tumorais. Ao iniciar e amplificar respostas imunes antitumorais específicas, as estratégias de imunoterapia trazem uma abordagem promissora na luta contra o câncer. Atualmente sabe-se que muitas terapias para tratar o câncer, consideradas eficazes na clínica, como o paclitaxel, ciclofosfamida e a radioterapia, atuam também através da interrupção da supressão imunológica induzida pelo tumor [109-110, 112]. Evidências sugerem que a radioterapia tem o potencial de induzir a morte celular imunogênica, que dentre as características está a liberação de ATP. Portanto, em alguns casos, a morte celular desencadeada pela quimio ou radioterapia permite o reconhecimento de células tumorais mortas pelas células apresentadoras de antígeno (APC), provocando uma resposta imune específica para a resolução do tumor [101].

Neste contexto, o ATP extracelular juntamente com os receptores e as enzimas do sistema purinérgico são considerados atualmente como novos e importantes moduladores da inflamação e da imunidade e, como tal, importantes peças na interação tumor-hospedeiro. O ATP e a adenosina se acumulam dentro do microambiente tumoral, dado o papel estabelecido do ATP como um sinal de perigo e um mediador pró-inflamatório. Se o acúmulo de ATP extracelular vai ser benéfico ou prejudicial para o hospedeiro dependerá da sua concentração, taxa de degradação pelas econucleotidases, e o painel de receptores P2 expressos pelas células do tumor e pelas

células inflamatórias infiltradas. Portanto, prever o resultado final sobre a interferência da sinalização purinérgica na progressão do tumor é muito difícil [169]. As ectonucleotidases que são altamente expressas em algumas células tumorais, células imunossupressoras e células endoteliais e correlacionadas com uma baixa taxa de sobrevida global [170], sugerem fortemente um importante papel das ectonucleotidases no sentido de facilitar o desenvolvimento tumoral, provavelmente através do auxílio do privilégio imunológico que as células malignas exigem para a evasão da eliminação pelo sistema imune. A ectonucleotidase CD39 parecem axiliar nessa imunossupressão através da hidrolize ATP extracelular, um ativador imune, à adenosina, que tem um efeito oposto. O ATP extracelular, se não degradado pela CD39, ativa o receptor P2X7 em células dendríticas (DC), induzindo a liberação de IL-1β, que por sua vez aumenta a apresentação de antígenos para os linfócitos T $CD4^+$, e, finalmente, potencia a imunidade antitumoral [169]. Portanto, a presença de ATP extracelular pode melhorar a eficácia da quimio e radioterapia, pois a redução da liberação de ATP *in vivo*, está correlacionada com um recrutamento deficiente de monócitos, macrófagos, e células dentríticas (DCs), ausência de resposta imune antitumoral e falha terapêutica [84]. Além disso, um estudo mostrou que a injeção local do inibidor de ectonucleotidase ARL67156 (NTPDase1 e NTPDase3) [171], suficiente para aumentar os níveis de ATP extracelular em tumores deficientes para autofagia, restabeleceu o recrutamento de linfócitos T e DCs no microambiente tumoral, restaurado a resposta à quimioterapicos, mas apenas em hospedeiros imunocompetentes. Mostrando a ação do aumento das concentrações de ATP extracelular na melhora da eficácia de tratamentos antineoplásicos através da ativação do sistema imune [172]. Neste contexto, neste estudo utilizamos camundongos *knockout* para a enzima CD39/NTPDase1 e observamos que a deleção desta enzima e consequente aumento da concentração de ATP/diminuição da concentração de adenosina, levou a uma rejeição até o dia 20 dos tumores GL261 injetados subcutâneo no flanco destes camundongos, mostrando a necessidade da atividade da enzima CD39 no estroma deste tumor para seu estabelecimento e manutenção. Provavelmente esta rejeição tumoral ocorreu, pois a linhagem GL261 nas nossas análises se mostrou bastante imunogênica, levando a uma maior infiltração de células imunes ativadas no tumor e consequentemente uma melhor resposta imune anti-tumoral, quando comparado com tumores injetados em camundongos do tipo selvagem (WT); combinado ao fato de que esta linhagem celular é sensível ao ATP, que está aumentado

nos camundongos CD39KO. Estes dados são diferentes do observado por Morrone e colaboradores que fizeram o oposto – analisaram o efeito da depleção do ATP no crescimento de outra linhagem de glioma (C6) através da co-injeção destas células com apirase, uma tipo de NTPDase solúvel, no cérebro de ratos. O grupo observou uma redução significativa no tamanho dos tumores com a menor concentração de ATP, indicando a possível participação deste nucleotídeo extracelular no desenvolvimento deste tipo de tumor cerebral [89]. Porém, é importante notar que a linhagem de glioma utilizada no trabalho de Morrone e colaboradores apresenta resistência a citotoxicidade induzida pelo ATP diferentemente da linhagem GL261 que utilizamos neste estudo, e os modelos utilizados foram diferentes, mostrando a importância de se investigar a fundo a expressão e atividade dos compontes purinérgicos nos tumores. Neste trabalho ainda observamos que a linhagem de glioma GL261 expressa as NTPDases 2, 3 e 8, CD73 e adenosina deaminase 1, sendo capaz de hidrolisar ADP até inosina. Um perfil de atividade das ectonucleotidases diferente do observado por Wink e colaboradores que observaram uma baixa atividade da enzima CD39 e alta atividade de CD73 em outras linhagens de glioma, sendo essas resistentes a citotoxicidade induzida pelo ATP [87]. Nossos dados sugerem que a GL261, sensível ao ATP, utiliza as enzimas purinérgicas como um mecanismo contra os danos causados pelo ATP.

Além disso, observamos que o ATP liberado pela radioterapia é capaz de ativar o receptor P2X7 em células de glioma de forma tempo-dependente e reduz o número de células positivas para anexina V, sugerindo morte por apoptose, de um modo dependente do receptor P2X7 após 24 h, mostrando a participação deste receptor na morte induzida pela irradiação (Figure 4A). O co-tratamento com ATP e radioterapia aumentou significativamente a incorporação de anexina V, em comparação com radioterapia isolada, indicando que o ATP pode atuar sinergicamente com a radiação, aumentando o efeito deste tratamento. *In vivo*, camundongos injetados com GL261 P2X7R$^{-/-}$ falharam em responder à radioterapia enquanto que, camundongos injetados com GL261 WT, que expressa o receptor P2X7 constitutivamente, apresentaram uma significativa redução do volume tumor após aradiação, mostrando que a expressão do receptor P2X7 funcional é essencial para uma resposta eficiente à radioterapia em gliomas. Nós também demonstramos que pacientes com glioma que expressaram mais o receptor P2X7, apresentaram uma melhor resposta à radioterapia, mostrando que o receptor P2X7 é um

bom marcador para sensibilidade a radioterapia [163]. Também observamos que a combinação do aumento de ATP e/ou diminuição de adenosina e radioterapia causou uma redução significativa das células Tregs tumorais e esplênicas, mostrando uma sinergia eficiente deste co-tratamento em reduzir a immunossupressão presente nestes tumores. Este redução das células Tregs nos camunsongos CD39KO tratados com radioterapia provavelmente ocorrou pois a ativação do receptor P2X7 pelo ATP inibe o potencial de supressão e a estabilidade desta linhagem [173] e a radioterapia em doses em torno de 8Gy que utilizamos leva é capaz de manter um baixo número de células T regulatórias [143]. Este tratamento também atenuou a infiltração de células supressoras mielóides (MDSC), causada pela radiação, e aumentou a expressão de CCR7 em DCs e macrófagos localizados no baço indicando a presença APCs recém-mobilizadas e disponíveis para se diferenciarem em células imunes efetoras que sustentam uma resposta imune mais prolongada mediada por células T antígeno específicas (Figura 4B). O aumento da expressão de APC $CCR7^{+}$ provavelmente foi devido a diminuição dos níveis de adenosina nos camundongos CD39KO, que através da estimulação dos receptores A2A diminui a expressão do receptor CCR7 [158], em combinação com o efeito da radioterapia que também é capaz de modular a expressão de CCR7, aumentando sua expressão principalmente quatro dias após a radiação [159], dia em que fizemos nossas análises.

Visto que a inflamação pode afetar cada aspecto do desenvolvimento e da progressão tumoral, assim como a resposta a terapias e sabendo-se que somente tratando cada aspecto da biologia tumoral nós poderemos ter verdadeiros ganhos na luta contra essa doença incurável nós buscamos entender qual o efeito direto da citocina IL-17 em células de glioma e outros tipos tumorais. Nossos dados mostraram que esta citocina parece ter um efeito sobre a migração das células tumorais e é capaz de induz a fosforilação das vias da p38, AKT e ERK. Estudos mostram que a migração célular causada pela IL-17 parece ser induzida pela expressão de metaloproteinases (MMP-2, MMP-9) [164], através da ativação de P38 e MAPK [165]. Portanto, quanto mais aprofundarmos nosso estudo em todo o contexto do microambiente tumoral para entende-lô melhor, mais perto estaremos de atender a nossa maior responsabilidade; trazer a promessa de uma cura para pacientes com câncer.

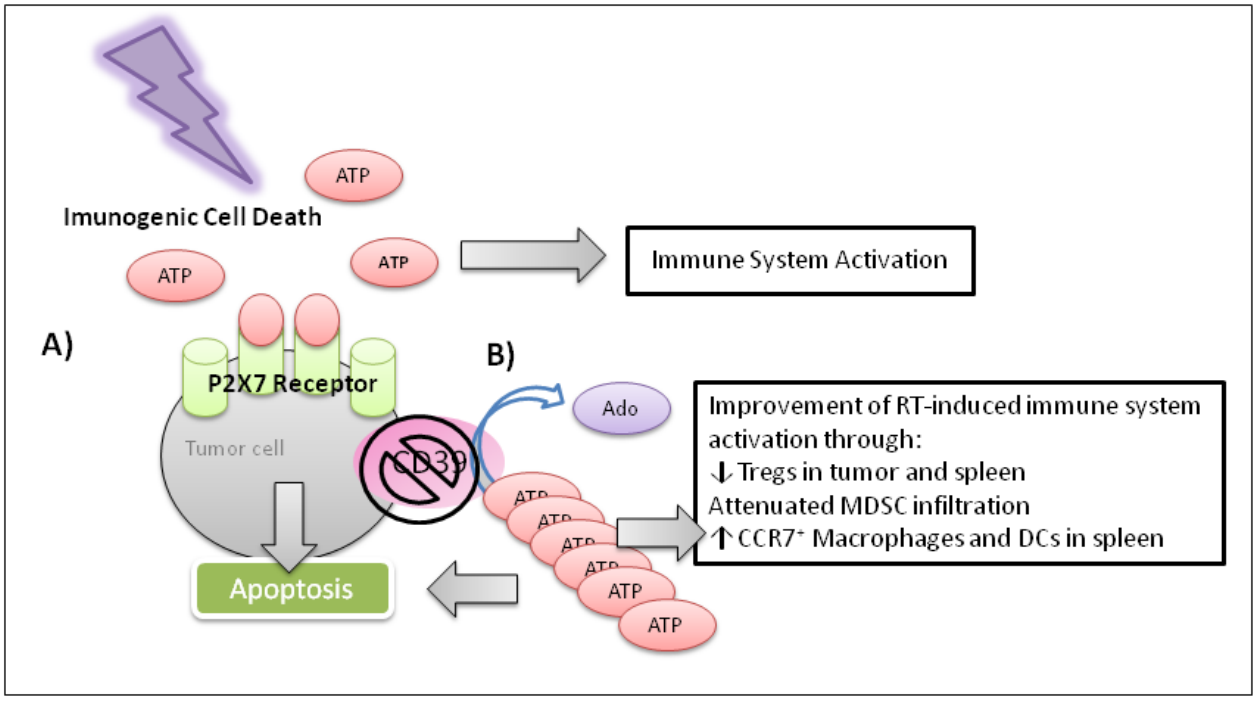


Figura 4. Participação da Sinalização Purinérgica na resposta à radioterapia em gliomas. A RT é capaz de ativar o sistema imune através da indução da morte celular imunogênica que dentre os sinais está a liberação de ATP, uma molécula imunoativadora. **A)** Este ATP liberado pela RT ativa o receptor P2X7, que quando funcional e altamente expresso em células tumorais, auxilia na radiosensibilidade destas células e indução de apoptose pela RT. **B)** Uma das formas do tumor evitar sua destruição pelas células do sistema imune, que é um dos Hallmarks do Câncer, é através da expressão da enzima NTPDase1/CD39 ou recrutamente de células que expressem essa enzima como as células Tregs, que leva a uma imunossupressão via produção de adenosina. A deleção da NTPDase1/CD39 no hospedeiro, que causa o aumento da concentração de ATP e consequente diminuição dos níveis de adenosina, foi capaz de melhorar a resposta imune induzida pela RT em gliomas através da diminuição das células imunosupressoras Treg e aumento de macrófagos e células dentríticas (DC) $CCR7^{+}$.

A investigação no tratamento do GBM está em rápida e vasta evolução. No entanto, não importa quantos alvos diferentes são descobertos e quantas moléculas são contruídas para atingir estes alvos, o resultado final é que tem se obtido pouco progresso na melhoria da sobrevida global destes pacientes. Porém, a cada passo, novas lições nos conduzem adiante e pequenos detalhes nos ajudam a contornar os obstáculos apresentados. Novos alvos, novos antagonistas, uma melhor penetração das substâncias através da barreira hematoencefálica e novos métodos de manipulação da resposta imune são todos enigmas a serem resolvidos no futuro próximo [11].

Nossos dados lançam a possibilidade de se melhorar a resposta imune induzida pela radiorerapia através da modulação da sinalização purinérgica. Em conjunto, podemos observar que a capacidade de se controlar mecanismos imunossupressores gerados pelo tumor promete melhorar tanto o tratamento padrão com radioterapia, bem como imunoterapias emergentes, como vacinas de células dendríticas e inibidores de ponto de verificação (immuno *check-point*), como os anticorpos anti-PD1 e anti-CTLA4. Já que atualmente, estas terapias são em grande parte testadas na presença do imuno privilégio presente no microambiente tumoral, o que dificulta sua resposta para efetuar a eliminação do tumor.

Em suma, nossos resultados revelaram que a radioterapia pode ativar o receptor P2X7 em células de glioma e a expressão funcional deste receptor pelas células de glioma é essencial para uma resposta bem-sucedida à radioterapia, mostrando, portanto, este receptor como um possível marcador para prever a sensibilidade à radioterapia. Além disso, a utilização de radioterapia juntamente com a deleção da enzima CD39/NTPDase1 no hospedeiro, reduz significativamente o número das células imunossupressoras Tregs, o que pode melhorar a resposta imune induzida pela radioterapia. Com isso, nossos dados lançam luz sobre a sinalização purinérgica como uma ferramenta para modular e prever a resposta à radiorerapia em gliomas.

5 PERSPECTIVAS

I. Investigar qual a isoforma do receptor P2X7 (A ou B) é expressa pelas diferentes linhagens de glioma utilizadas neste estudo por RT-qPCR.

II. Analisar a ativação do receptor P2X7 após a radioterapia através da medida de cálcio intracelular nas células de glioma.

III. Confirmar a participação do receptor P2X7 na resposta à radioterapia por outra técnica, irradiando as células de glioma e tratando-as com apirase.

IV. Investigar a expressão dos outros receptores purinérgicos após a radioterapia em células de glioma pela técnica de RT-qPCR.

V. Medir a atividade enzimática das ectonucleotidases na linhagem GL261 também através da medida de liberação de fosfato inorgânico.

VI. Avaliar o crescimento da linhagem de glioma GL261 com o receptor P2X7 silenciado em camundongos CD39KO.

VII. Investigar se a redução de células Tregs observada em camundongos CD39 knockout tratados com radioterapia é devido à morte celular ou a migração.

VIII. Analisar se a redução de células T $CD4^+$ observada após a radioterapia são células T $CD4^+$ $FOXP3^+$.

IX. Explorar o efeito da intervenção terapêutica com o inibidor farmacológico de CD39 (POM1), na resposta imune induzida pela radioterapia.

X. Investigar se a modulação da sinalização purinérgica, através da inibição da atividade da enzima CD39, pode intensificar a resposta imune antitumoral induzida pelo anticorpo anti-PD-1 em glioma.

REFERÊNCIAS BIBLIOGRÁFICAS

1. Xu, W., et al., *Association between EGF promoter polymorphisms and cancer risk: a meta-analysis.* Med Oncol, 2010. **27**(4): p. 1389-97.
2. Ma, Y., et al., *Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy.* J Exp Med, 2011. **208**(3): p. 491-503.
3. Ganau, L., et al., *Management of Gliomas: Overview of the Latest Technological Advancements and Related Behavioral Drawbacks.* Behav Neurol, 2015. **2015**: p. 862634.
4. Meijer, D.H., et al., *Controlling brain tumor growth by intraventricular administration of an AAV vector encoding IFN-beta.* Cancer Gene Ther, 2009. **16**(8): p. 664-71.
5. Robins, H.I., A.B. Lassman, and D. Khuntia, *Therapeutic advances in malignant glioma: current status and future prospects.* Neuroimaging Clin N Am, 2009. **19**(4): p. 647-56.
6. Van Meir, E.G., et al., *Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma.* CA Cancer J Clin, 2010. **60**(3): p. 166-93.
7. Wei, J., et al., *Glioma-associated cancer-initiating cells induce immunosuppression.* Clin Cancer Res, 2010. **16**(2): p. 461-73.
8. Noch, E. and K. Khalili, *Molecular mechanisms of necrosis in glioblastoma: the role of glutamate excitotoxicity.* Cancer Biol Ther, 2009. **8**(19): p. 1791-7.
9. Mendonca, R., et al., *[Primary connus medullaris glioblastoma: case report]*. Arq Neuropsiquiatr, 2005. **63**(2B): p. 539-42.
10. Dai, C. and E.C. Holland, *Glioma models.* Biochim Biophys Acta, 2001. **1551**(1): p. M19-27.
11. Alifieris, C. and D.T. Trafalis, *Glioblastoma multiforme: Pathogenesis and treatment.* Pharmacol Ther, 2015. **152**: p. 63-82.
12. Yamanaka, R. and H. Saya, *Molecularly targeted therapies for glioma.* Ann Neurol, 2009. **66**(6): p. 717-29.
13. Schwartzbaum, J.A., et al., *Epidemiology and molecular pathology of glioma.* Nat Clin Pract Neurol, 2006. **2**(9): p. 494-503; quiz 1 p following 516.
14. Chi, J., et al., *Human herpesvirus 6 latent infection in patients with glioma.* J Infect Dis, 2012. **206**(9): p. 1394-8.
15. Crawford, J.R., et al., *Detection of human herpesvirus-6 variants in pediatric brain tumors: association of viral antigen in low grade gliomas.* J Clin Virol, 2009. **46**(1): p. 37-42.
16. Martin-Villalba, A., A.F. Okuducu, and A. von Deimling, *The evolution of our understanding on glioma.* Brain Pathol, 2008. **18**(3): p. 455-63.
17. Weller, M., *Novel diagnostic and therapeutic approaches to malignant glioma.* Swiss Med Wkly, 2011. **141**: p. w13210.
18. Lassman, A.B., *Molecular biology of gliomas.* Curr Neurol Neurosci Rep, 2004. **4**(3): p. 228-33.
19. Laws, E.R., Jr. and M.E. Shaffrey, *The inherent invasiveness of cerebral gliomas: implications for clinical management.* Int J Dev Neurosci, 1999. **17**(5-6): p. 413-20.
20. Oliver, L., et al., *Hypoxia and the malignant glioma microenvironment: regulation and implications for therapy.* Curr Mol Pharmacol, 2009. **2**(3): p. 263-84.

21. Wippold, F.J., 2nd, et al., *Neuropathology for the neuroradiologist: palisades and pseudopalisades.* AJNR Am J Neuroradiol, 2006. **27**(10): p. 2037-41.
22. Tyurikova, O., et al., *Perspectives in Intraoperative Diagnostics of Human Gliomas.* Comput Math Methods Med, 2015. **2015**: p. 479014.
23. Minniti, G., et al., *Chemotherapy for glioblastoma: current treatment and future perspectives for cytotoxic and targeted agents.* Anticancer Res, 2009. **29**(12): p. 5171-84.
24. Milojkovic Kerklaan, B., et al., *Strategies to target drugs to gliomas and CNS metastases of solid tumors.* J Neurol, 2015.
25. Yang, I., et al., *Distinguishing glioma recurrence from treatment effect after radiochemotherapy and immunotherapy.* Neurosurg Clin N Am, 2010. **21**(1): p. 181-6.
26. Ho, I.A., K.M. Hui, and P.Y. Lam, *Isolation of peptide ligands that interact specifically with human glioma cells.* Peptides, 2010. **31**(4): p. 644-50.
27. Deng, L., et al., *Irradiation and anti-PD-L1 treatment synergistically promote antitumor immunity in mice.* J Clin Invest, 2014. **124**(2): p. 687-95.
28. Begg, A.C., F.A. Stewart, and C. Vens, *Strategies to improve radiotherapy with targeted drugs.* Nat Rev Cancer, 2011. **11**(4): p. 239-53.
29. Galluzzi, L., O. Kepp, and G. Kroemer, *Immunogenic cell death in radiation therapy.* Oncoimmunology, 2013. **2**(10): p. e26536.
30. Bedford, J.S. and W.C. Dewey, *Radiation Research Society. 1952-2002. Historical and current highlights in radiation biology: has anything important been learned by irradiating cells?* Radiat Res, 2002. **158**(3): p. 251-91.
31. Schmidt-Ullrich, R.K., *Molecular targets in radiation oncology.* Oncogene, 2003. **22**(37): p. 5730-3.
32. Bassi, C., et al., *Transcriptional changes in U343 MG-a glioblastoma cell line exposed to ionizing radiation.* Hum Exp Toxicol, 2008. **27**(12): p. 919-29.
33. Li, H.F., J.S. Kim, and T. Waldman, *Radiation-induced Akt activation modulates radioresistance in human glioblastoma cells.* Radiat Oncol, 2009. **4**: p. 43.
34. McLaughlin, N., et al., *The Survivin-mediated radioresistant phenotype of glioblastomas is regulated by RhoA and inhibited by the green tea polyphenol (-)-epigallocatechin-3-gallate.* Brain Res, 2006. **1071**(1): p. 1-9.
35. Dutreix, M., J.M. Cosset, and J.S. Sun, *Molecular therapy in support to radiotherapy.* Mutat Res, 2010. **704**(1-3): p. 182-9.
36. Grivennikov, S.I., F.R. Greten, and M. Karin, *Immunity, inflammation, and cancer.* Cell, 2010. **140**(6): p. 883-99.
37. Iwami, K., A. Natsume, and T. Wakabayashi, *Cytokine networks in glioma.* Neurosurg Rev, 2011. **34**(3): p. 253-63; discussion 263-4.
38. Coussens, L.M. and Z. Werb, *Inflammation and cancer.* Nature, 2002. **420**(6917): p. 860-7.
39. Zou, W. and N.P. Restifo, *T(H)17 cells in tumour immunity and immunotherapy.* Nat Rev Immunol, 2010. **10**(4): p. 248-56.
40. Patel, M.A., et al., *The future of glioblastoma therapy: synergism of standard of care and immunotherapy.* Cancers (Basel), 2014. **6**(4): p. 1953-85.
41. Mantovani, A., et al., *Cancer-related inflammation.* Nature, 2008. **454**(7203): p. 436-44.
42. Maniati, E., R. Soper, and T. Hagemann, *Up for Mischief? IL-17/Th17 in the tumour microenvironment.* Oncogene, 2010. **29**(42): p. 5653-62.

43. Aggarwal, B.B., et al., *Inflammation and cancer: how hot is the link?* Biochem Pharmacol, 2006. **72**(11): p. 1605-21.
44. Ivanov, S. and A. Linden, *Interleukin-17 as a drug target in human disease.* Trends Pharmacol Sci, 2009. **30**(2): p. 95-103.
45. Moseley, T.A., et al., *Interleukin-17 family and IL-17 receptors.* Cytokine Growth Factor Rev, 2003. **14**(2): p. 155-74.
46. Kawaguchi, M., et al., *IL-17 cytokine family.* J Allergy Clin Immunol, 2004. **114**(6): p. 1265-73; quiz 1274.
47. Gaffen, S.L., *Structure and signalling in the IL-17 receptor family.* Nat Rev Immunol, 2009. **9**(8): p. 556-67.
48. Kehlen, A., et al., *Interleukin-17 stimulates the expression of IkappaB alpha mRNA and the secretion of IL-6 and IL-8 in glioblastoma cell lines.* J Neuroimmunol, 1999. **101**(1): p. 1-6.
49. Chen, Y., A. Kijlstra, and P. Yang, *IL-17A stimulates the production of inflammatory mediators via Erk1/2, p38 MAPK, PI3K/Akt, and NF-kappaB pathways in ARPE-19 cells.* Mol Vis, 2011. **17**: p. 3072-7.
50. Numasaki, M., et al., *Interleukin-17 promotes angiogenesis and tumor growth.* Blood, 2003. **101**(7): p. 2620-7.
51. Numasaki, M., et al., *IL-17 enhances the net angiogenic activity and in vivo growth of human non-small cell lung cancer in SCID mice through promoting CXCR-2-dependent angiogenesis.* J Immunol, 2005. **175**(9): p. 6177-89.
52. Wang, L., et al., *IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway.* J Exp Med, 2009. **206**(7): p. 1457-64.
53. Benchetrit, F., et al., *Interleukin-17 inhibits tumor cell growth by means of a T-cell-dependent mechanism.* Blood, 2002. **99**(6): p. 2114-21.
54. Gnerlich, J.L., et al., *Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer.* J Immunol, 2010. **185**(7): p. 4063-71.
55. Wainwright, D.A., et al., *The presence of IL-17A and T helper 17 cells in experimental mouse brain tumors and human glioma.* PLoS One, 2010. **5**(10): p. e15390.
56. Hu, J., et al., *The profile of Th17 subset in glioma.* Int Immunopharmacol, 2011. **11**(9): p. 1173-9.
57. Cantini, G., et al., *A critical role for regulatory T cells in driving cytokine profiles of Th17 cells and their modulation of glioma microenvironment.* Cancer Immunol Immunother, 2011. **60**(12): p. 1739-50.
58. Burnstock, G. and F. Di Virgilio, *Purinergic signalling and cancer.* Purinergic Signal, 2013.
59. Burnstock, G., *Pathophysiology and therapeutic potential of purinergic signaling.* Pharmacol Rev, 2006. **58**(1): p. 58-86.
60. Wei, W., et al., *Expression and function of the P2X(7) receptor in rat C6 glioma cells.* Cancer Lett, 2008. **260**(1-2): p. 79-87.
61. Burnstock, G., *Introductory overview of purinergic signalling.* Front Biosci (Elite Ed), 2011. **3**: p. 896-900.
62. Morrone, F.B., et al., *Increased resistance of glioma cell lines to extracellular ATP cytotoxicity.* J Neurooncol, 2005. **71**(2): p. 135-40.

63. Zimmermann, H., *Ectonucleotidases in the nervous system.* Novartis Found Symp, 2006. **276**: p. 113-28; discussion 128-30, 233-7, 275-81.
64. Zimmermann, H., *Signalling via ATP in the nervous system.* Trends Neurosci, 1994. **17**(10): p. 420-6.
65. Robson, S.C., J. Sevigny, and H. Zimmermann, *The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance.* Purinergic Signal, 2006. **2**(2): p. 409-30.
66. Roszek, K. and J. Czarnecka, *Is Ecto-nucleoside Triphosphate Diphosphohydrolase (NTPDase)-based Therapy of Central Nervous System Disorders Possible?* Mini Rev Med Chem, 2015. **15**(1): p. 5-20.
67. Verkhratsky, A., O.A. Krishtal, and G. Burnstock, *Purinoceptors on neuroglia.* Mol Neurobiol, 2009. **39**(3): p. 190-208.
68. Di Virgilio, F., et al., *Purinergic signalling in inflammation of the central nervous system.* Trends Neurosci, 2009. **32**(2): p. 79-87.
69. Burnstock, G., *Geoffrey Burnstock: most highly cited scientist.* Mol Interv, 2004. **4**(4): p. 192-5.
70. White, N. and G. Burnstock, *P2 receptors and cancer.* Trends Pharmacol Sci, 2006. **27**(4): p. 211-7.
71. Burnstock, G., *Introduction: P2 receptors.* Curr Top Med Chem, 2004. **4**(8): p. 793-803.
72. Kim, M., et al., *Proteomic and functional evidence for a P2X7 receptor signalling complex.* EMBO J, 2001. **20**(22): p. 6347-58.
73. Stagg, J. and M.J. Smyth, *Extracellular adenosine triphosphate and adenosine in cancer.* Oncogene, 2010. **29**(39): p. 5346-58.
74. Ralevic, V. and G. Burnstock, *Receptors for purines and pyrimidines.* Pharmacol Rev, 1998. **50**(3): p. 413-92.
75. Bianco, F., et al., *Different properties of P2X(7) receptor in hippocampal and cortical astrocytes.* Purinergic Signal, 2009. **5**(2): p. 233-40.
76. Tamajusuku, A.S., et al., *Characterization of ATP-induced cell death in the GL261 mouse glioma.* J Cell Biochem, 2010. **109**(5): p. 983-91.
77. Zimmermann, H., M. Zebisch, and N. Strater, *Cellular function and molecular structure of ecto-nucleotidases.* Purinergic Signal, 2012. **8**(3): p. 437-502.
78. Coutinho-Silva, R., et al., *P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation.* Am J Physiol Gastrointest Liver Physiol, 2005. **288**(5): p. G1024-35.
79. Morrone, F.B., et al., *Extracellular nucleotides and nucleosides induce proliferation and increase nucleoside transport in human glioma cell lines.* J Neurooncol, 2003. **64**(3): p. 211-8.
80. White, N., P.E. Butler, and G. Burnstock, *Human melanomas express functional P2 X(7) receptors.* Cell Tissue Res, 2005. **321**(3): p. 411-8.
81. Ma, Y., et al., *Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells.* Immunity, 2013. **38**(4): p. 729-41.
82. Jantaratnotai, N., H.B. Choi, and J.G. McLarnon, *ATP stimulates chemokine production via a store-operated calcium entry pathway in C6 glioma cells.* BMC Cancer, 2009. **9**: p. 442.

83. Burnstock, G. and F. Di Virgilio, *Purinergic signalling and cancer.* Purinergic Signal, 2013. **9**(4): p. 491-540.
84. Kroemer, G., et al., *Immunogenic cell death in cancer therapy.* Annu Rev Immunol, 2013. **31**: p. 51-72.
85. Rathbone, M.P., et al., *Adenosine and its nucleotides stimulate proliferation of chick astrocytes and human astrocytoma cells.* Neurosci Res, 1992. **13**(1): p. 1-17.
86. Bradley, K.K. and M.E. Bradley, *Purine nucleoside-dependent inhibition of cellular proliferation in 1321N1 human astrocytoma cells.* J Pharmacol Exp Ther, 2001. **299**(2): p. 748-52.
87. Wink, M.R., et al., *Altered extracellular ATP, ADP and AMP catabolism in glioma cell lines.* Cancer Lett, 2003. **198**(2): p. 211-8.
88. Jacques-Silva, M.C., et al., *ERK, PKC and PI3K/Akt pathways mediate extracellular ATP and adenosine-induced proliferation of U138-MG human glioma cell line.* Oncology, 2004. **67**(5-6): p. 450-9.
89. Morrone, F.B., et al., *In vivo glioblastoma growth is reduced by apyrase activity in a rat glioma model.* BMC Cancer, 2006. **6**: p. 226.
90. Bavaresco, L., et al., *The role of ecto-5'-nucleotidase/CD73 in glioma cell line proliferation.* Mol Cell Biochem, 2008. **319**(1-2): p. 61-8.
91. Braganhol, E., et al., *A comparative study of ectonucleotidase and P2 receptor mRNA profiles in C6 cell line cultures and C6 ex vivo glioma model.* Cell Tissue Res, 2009. **335**(2): p. 331-40.
92. Ryu, J.K., et al., *Block of purinergic P2X7R inhibits tumor growth in a C6 glioma brain tumor animal model.* J Neuropathol Exp Neurol, 2011. **70**(1): p. 13-22.
93. Fang, J., et al., *P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway.* Int J Biochem Cell Biol, 2013. **45**(6): p. 1109-20.
94. Gehring, M.P., et al., *P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line.* Purinergic Signal, 2012. **8**(4): p. 729-39.
95. Cheewatrakoolpong, B., et al., *Identification and characterization of splice variants of the human P2X7 ATP channel.* Biochem Biophys Res Commun, 2005. **332**(1): p. 17-27.
96. Adinolfi, E., et al., *Trophic activity of a naturally occurring truncated isoform of the P2X7 receptor.* FASEB J, 2010. **24**(9): p. 3393-404.
97. Pelegrin, P. and A. Surprenant, *Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor.* EMBO J, 2006. **25**(21): p. 5071-82.
98. Bastid, J., et al., *ENTPD1/CD39 is a promising therapeutic target in oncology.* Oncogene, 2013. **32**(14): p. 1743-51.
99. Bastid, J., et al., *Inhibition of CD39 enzymatic function at the surface of tumor cells alleviates their immunosuppressive activity.* Cancer Immunol Res, 2015. **3**(3): p. 254-65.
100. Kmiecik, J., et al., *Elevated CD3+ and CD8+ tumor-infiltrating immune cells correlate with prolonged survival in glioblastoma patients despite integrated immunosuppressive mechanisms in the tumor microenvironment and at the systemic level.* J Neuroimmunol, 2013. **264**(1-2): p. 71-83.
101. Demaria, S., et al., *Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer.* Clin Cancer Res, 2005. **11**(2 Pt 1): p. 728-34.

102. Kwilas, A.R., et al., *In the field: exploiting the untapped potential of immunogenic modulation by radiation in combination with immunotherapy for the treatment of cancer.* Front Oncol, 2012. **2**: p. 104.
103. Golden, E.B. and L. Apetoh, *Radiotherapy and immunogenic cell death.* Semin Radiat Oncol, 2015. **25**(1): p. 11-7.
104. Ohshima, Y., et al., *gamma-Irradiation induces P2X(7) receptor-dependent ATP release from B16 melanoma cells.* Biochim Biophys Acta, 2010. **1800**(1): p. 40-6.
105. Gameiro, S.R., et al., *Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing.* Oncotarget, 2014. **5**(2): p. 403-16.
106. Golden, E.B., et al., *Radiation fosters dose-dependent and chemotherapy-induced immunogenic cell death.* Oncoimmunology, 2014. **3**: p. e28518.
107. Rabinovich, G.A., D. Gabrilovich, and E.M. Sotomayor, *Immunosuppressive strategies that are mediated by tumor cells.* Annu Rev Immunol, 2007. **25**: p. 267-96.
108. Hellstrom, I., et al., *Cellular and humoral immunity to different types of human neoplasms.* Nature, 1968. **220**(5174): p. 1352-4.
109. Heylmann, D., et al., *Human CD4+CD25+ regulatory T cells are sensitive to low dose cyclophosphamide: implications for the immune response.* PLoS One, 2013. **8**(12): p. e83384.
110. Zhu, Y., et al., *CD4+Foxp3+ regulatory T-cell impairment by paclitaxel is independent of toll-like receptor 4.* Scand J Immunol, 2011. **73**(4): p. 301-8.
111. Litterman, A.J., et al., *Profound impairment of adaptive immune responses by alkylating chemotherapy.* J Immunol, 2013. **190**(12): p. 6259-68.
112. Sevko, A., et al., *Antitumor effect of paclitaxel is mediated by inhibition of myeloid-derived suppressor cells and chronic inflammation in the spontaneous melanoma model.* J Immunol, 2013. **190**(5): p. 2464-71.
113. Zeng, J., et al., *Immune modulation and stereotactic radiation: improving local and abscopal responses.* Biomed Res Int, 2013. **2013**: p. 658126.
114. Park, B., C. Yee, and K.M. Lee, *The effect of radiation on the immune response to cancers.* Int J Mol Sci, 2014. **15**(1): p. 927-43.
115. Xu, S., et al., *Synergy between the ectoenzymes CD39 and CD73 contributes to adenosinergic immunosuppression in human malignant gliomas.* Neuro Oncol, 2013.
116. Eltzschig, H.K., M.V. Sitkovsky, and S.C. Robson, *Purinergic signaling during inflammation.* N Engl J Med, 2012. **367**(24): p. 2322-33.
117. Bergamin, L.S., et al., *Ectonucleotidases in tumor cells and tumor-associated immune cells: an overview.* J Biomed Biotechnol, 2012. **2012**: p. 959848.
118. Kunzli, B.M., et al., *Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer.* Purinergic Signal, 2011. **7**(2): p. 231-41.
119. Gehring, M.P., et al., *P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line.* Purinergic Signal, 2012. **8**(4): p. 729-39.
120. Enjyoji, K., et al., *Targeted disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis and thromboregulation.* Nat Med, 1999. **5**(9): p. 1010-7.
121. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells.* Nature, 2006. **441**(7090): p. 235-8.

122. Sun, X., et al., *CD39/ENTPD1 expression by CD4+Foxp3+ regulatory T cells promotes hepatic metastatic tumor growth in mice.* Gastroenterology, 2010. **139**(3): p. 1030-40.
123. Sato, A., et al., *Antitumor activity of IFN-lambda in murine tumor models.* J Immunol, 2006. **176**(12): p. 7686-94.
124. Beldi, G., et al., *Natural killer T cell dysfunction in CD39-null mice protects against concanavalin A-induced hepatitis.* Hepatology, 2008. **48**(3): p. 841-52.
125. Feng, L., et al., *Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate.* Neoplasia, 2011. **13**(3): p. 206-16.
126. Jackson, S.W., et al., *Disordered purinergic signaling inhibits pathological angiogenesis in cd39/Entpd1-null mice.* Am J Pathol, 2007. **171**(4): p. 1395-404.
127. Xu, S., et al., *Synergy between the ectoenzymes CD39 and CD73 contributes to adenosinergic immunosuppression in human malignant gliomas.* Neuro Oncol, 2013. **15**(9): p. 1160-72.
128. Pham, K., et al., *CCL5, CCR1 and CCR5 in murine glioblastoma: immune cell infiltration and survival rates are not dependent on individual expression of either CCR1 or CCR5.* J Neuroimmunol, 2012. **246**(1-2): p. 10-7.
129. Vega, E.A., M.W. Graner, and J.H. Sampson, *Combating immunosuppression in glioma.* Future Oncol, 2008. **4**(3): p. 433-42.
130. Legler, D.F., E. Uetz-von Allmen, and M.A. Hauser, *CCR7: roles in cancer cell dissemination, migration and metastasis formation.* Int J Biochem Cell Biol, 2014. **54**: p. 78-82.
131. Sun, J., et al., *Hypoxia induces T-cell apoptosis by inhibiting chemokine C receptor 7 expression: the role of adenosine receptor A(2).* Cell Mol Immunol, 2010. **7**(1): p. 77-82.
132. Turk, M.J., et al., *Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells.* J Exp Med, 2004. **200**(6): p. 771-82.
133. Wang, J., et al., *Eliciting T cell immunity against poorly immunogenic tumors by immunization with dendritic cell-tumor fusion vaccines.* J Immunol, 1998. **161**(10): p. 5516-24.
134. Szatmari, T., et al., *Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy.* Cancer Sci, 2006. **97**(6): p. 546-53.
135. Maes, W., et al., *DC vaccination with anti-CD25 treatment leads to long-term immunity against experimental glioma.* Neuro Oncol, 2009. **11**(5): p. 529-42.
136. Khan, M.K., et al., *Future of radiation therapy for malignant melanoma in an era of newer, more effective biological agents.* Onco Targets Ther, 2011. **4**: p. 137-48.
137. Bhatia, S., S.S. Tykodi, and J.A. Thompson, *Treatment of metastatic melanoma: an overview.* Oncology (Williston Park), 2009. **23**(6): p. 488-96.
138. Dhermain, F., *Radiotherapy of high-grade gliomas: current standards and new concepts, innovations in imaging and radiotherapy, and new therapeutic approaches.* Chin J Cancer, 2014. **33**(1): p. 16-24.
139. Shiao, S.L. and L.M. Coussens, *The tumor-immune microenvironment and response to radiation therapy.* J Mammary Gland Biol Neoplasia, 2010. **15**(4): p. 411-21.
140. Desai, A., et al., *A Retrospective Review of CyberKnife Stereotactic Body Radiotherapy for Adrenal Tumors (Primary and Metastatic): Winthrop University Hospital Experience.* Front Oncol, 2015. **5**: p. 185.

141. Jung, I.H., et al., *Clinical outcome of fiducial-less CyberKnife radiosurgery for stage I non-small cell lung cancer.* Radiat Oncol J, 2015. **33**(2): p. 89-97.
142. Song, Y., et al., *Analysis of clinical efficacy of CyberKnife((R)) treatment for locally advanced pancreatic cancer.* Onco Targets Ther, 2015. **8**: p. 1427-31.
143. Schaue, D., et al., *Maximizing tumor immunity with fractionated radiation.* Int J Radiat Oncol Biol Phys, 2012. **83**(4): p. 1306-10.
144. Novitskiy, S.V., et al., *Adenosine receptors in regulation of dendritic cell differentiation and function.* Blood, 2008. **112**(5): p. 1822-31.
145. Ma, G., et al., *Paired immunoglobin-like receptor-B regulates the suppressive function and fate of myeloid-derived suppressor cells.* Immunity, 2011. **34**(3): p. 385-95.
146. Stagg, J., et al., *CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis.* Cancer Res, 2011. **71**(8): p. 2892-900.
147. Fidler, I.J., *Biological behavior of malignant melanoma cells correlated to their survival in vivo.* Cancer Res, 1975. **35**(1): p. 218-24.
148. Chiu, T.L., M.J. Wang, and C.C. Su, *The treatment of glioblastoma multiforme through activation of microglia and TRAIL induced by rAAV2-mediated IL-12 in a syngeneic rat model.* J Biomed Sci, 2012. **19**: p. 45.
149. Campbell, I.L., M.J. Hofer, and A. Pagenstecher, *Transgenic models for cytokine-induced neurological disease.* Biochim Biophys Acta, 2010. **1802**(10): p. 903-17.
150. Mathios, D., et al., *Therapeutic administration of IL-15 superagonist complex ALT-803 leads to long-term survival and durable antitumor immune response in a murine glioblastoma model.* Int J Cancer, 2015.
151. Bobanga, I.D., A. Petrosiute, and A.Y. Huang, *Chemokines as Cancer Vaccine Adjuvants.* Vaccines (Basel), 2013. **1**(4): p. 444-62.
152. Vasco, C., et al., *Circulating T regulatory cells migration and phenotype in glioblastoma patients: an in vitro study.* J Neurooncol, 2013. **115**(3): p. 353-63.
153. Zhu, X., et al., *Systemic delivery of neutralizing antibody targeting CCL2 for glioma therapy.* J Neurooncol, 2011. **104**(1): p. 83-92.
154. Sugihara, A.Q., C.E. Rolle, and M.S. Lesniak, *Regulatory T cells actively infiltrate metastatic brain tumors.* Int J Oncol, 2009. **34**(6): p. 1533-40.
155. Vatner, R.E. and S.C. Formenti, *Myeloid-derived cells in tumors: effects of radiation.* Semin Radiat Oncol, 2015. **25**(1): p. 18-27.
156. Wei, S., et al., *Effects of tumor irradiation on host T-regulatory cells and systemic immunity in the context of adoptive T-cell therapy in mice.* J Immunother, 2013. **36**(2): p. 124-32.
157. Correale, P., et al., *Tumor infiltration by T lymphocytes expressing chemokine receptor 7 (CCR7) is predictive of favorable outcome in patients with advanced colorectal carcinoma.* Clin Cancer Res, 2012. **18**(3): p. 850-7.
158. Williams, A.J. and B.N. Cronstein, *The effect of A(2A) adenosine receptor activation on C-C chemokine receptor 7 expression in human THP1 macrophages during inflammation.* Inflammation, 2012. **35**(2): p. 614-22.
159. Cummings, R.J., et al., *Exposure to ionizing radiation induces the migration of cutaneous dendritic cells by a CCR7-dependent mechanism.* J Immunol, 2012. **189**(9): p. 4247-57.
160. Zeng, Y., et al., *Prognostic significance of interleukin-17 in solid tumors: a meta-analysis.* Int J Clin Exp Med, 2015. **8**(7): p. 10515-36.

161. Baharlou, R., et al., *Increased interleukin-17 transcripts in peripheral blood mononuclear cells, a link between T-helper 17 and proinflammatory responses in bladder cancer.* Iran Red Crescent Med J, 2015. **17**(2): p. e9244.
162. Lu, L., et al., *IL-17A promotes immune cell recruitment in human esophageal cancers and the infiltrating dendritic cells represent a positive prognostic marker for patient survival.* J Immunother, 2013. **36**(8): p. 451-8.
163. Gehring, M.P., et al., *P2X7 receptor as predictor gene for glioma radiosensitivity and median survival.* Int J Biochem Cell Biol, 2015. **68**: p. 92-100.
164. Gu, F.M., et al., *IL-17 induces AKT-dependent IL-6/JAK2/STAT3 activation and tumor progression in hepatocellular carcinoma.* Mol Cancer, 2011. **10**: p. 150.
165. Wu, Y., et al., *Interleukin-17A stimulates migration of periodontal ligament fibroblasts via p38 MAPK/NF-kappaB -dependent MMP-1 expression.* J Cell Physiol, 2014. **229**(3): p. 292-9.
166. Zhu, X., et al., *IL-17 expression by breast-cancer-associated macrophages: IL-17 promotes invasiveness of breast cancer cell lines.* Breast Cancer Res, 2008. **10**(6): p. R95.
167. Chen, F., et al., *New horizons in tumor microenvironment biology: challenges and opportunities.* BMC Med, 2015. **13**: p. 45.
168. Hanahan, D. and R.A. Weinberg, *Hallmarks of cancer: the next generation.* Cell, 2011. **144**(5): p. 646-74.
169. Di Virgilio, F., *Purines, purinergic receptors, and cancer.* Cancer Res, 2012. **72**(21): p. 5441-7.
170. Ghiringhelli, F., et al., *Production of adenosine by ectonucleotidases: a key factor in tumor immunoescape.* J Biomed Biotechnol, 2012. **2012**: p. 473712.
171. Levesque, S.A., et al., *Specificity of the ecto-ATPase inhibitor ARL 67156 on human and mouse ectonucleotidases.* Br J Pharmacol, 2007. **152**(1): p. 141-50.
172. Michaud, M., et al., *Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice.* Science, 2011. **334**(6062): p. 1573-7.
173. Schenk, U., et al., *ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors.* Sci Signal, 2011. **4**(162): p. ra12.

ANEXO I

PURINERGIC MODULATION AND CD39/ENTPD1 IN CANCER

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CHAPTER

PURINERGIC MODULATION AND CD39/ENTPD1 IN CANCER

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Abstract: Multiple and pleiotropic functional traits are acquired by transformed cells during progression to the neoplastic state. These include genomic instability with several defined mutations that are associated with uncontrolled proliferation, resistance to cell death with induction of immortality, altered cellular metabolism, loss/inactivation of tumor suppressor responses, evasion of immune surveillance, induction of angiogenesis with vascular perturbation, and activation of cell invasiveness resulting in metastasis. A better understanding of any overlapping pathogenetic mechanisms underpinning several of these properties would facilitate development of novel and more effective modalities to treat cancer. Dissecting out the molecular basis for these unique properties of malignancy has already resulted in the discovery and development of novel anticancer drugs.

Extracellular nucleotides and nucleosides have been recently identified as crucial signal mediators in the tumor microenvironment and are known to specifically interact with purinergic receptors. These cellular activation processes provoke different intracellular signaling transduction pathways, termed as "purinergic signaling". Ectonucleotidases, especially those of CD39/ENTPD family, regulate pericellular levels of pro-inflammatory adenosine 5'-triphosphate (ATP) to ultimately generate antagonistic anti-inflammatory nucleosides such as adenosine thereby tightly modulating purinergic signaling. Such regulated cascades of purinergic signaling have been shown to participate in many of the above fundamental pathophysiological processes in the context of inflammation and immune responses.

In this chapter, we review some of the purinergic mechanisms involved in cancer. We specifically highlight the discovery and development and the potential uses of drugs based on ectonucleotidases that would be applicable to cancer therapy. We also discuss recent advances using purinergic modulation in cancer therapy and consider several of the therapeutic obstacles that would need to be overcome.

Keywords: ATP, adenosine, ectonucleotidases, CD39, CD73, purine-based drugs, cancer therapy, adjunctive therapy

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At A Glance

- Purinergic mechanisms play distinct and opposing roles in the biology of transplanted and autochthonous tumors.
- ATP-mediated P2 receptor signaling is crucial in augmenting antitumor immune responses; whereas adenosine-P1 receptor signaling attenuates antitumor immunity.
- CD39/ENTPD1 is the dominant and rate-limiting ectoenzyme for extracellular ATP phosphohydrolysis to adenosine and one of the master switches or control nodes in the purinergic system.
- In the tumor microenvironment, the balance of extracellular nucleotide- and nucleoside-initiated purinergic signaling dictates biology and integrates those specific metabolic changes associated with cell growth, inflammation and immunity.
- Chronic inflammation with scarring predisposes to malignancy and is associated with the perturbation of purinergic mechanisms.
- CD39 activity and downstream purinergic signaling pathways are novel therapeutic targets with great promise as adjunctive anticancer therapies.

INTRODUCTION

ATP and Other Purinergic Mediators

Indispensable throughout evolution, nucleotides such as adenosine triphosphate (ATP) are highly conserved in all organisms. As the well-recognized universal energy currency of the cell, intracellular ATP is an absolute requirement for the maintenance of cell metabolism and intracellular signals. At steady state, ATP concentration in the cytosol is at the millimolar (mM) range (3 to 10 mM), whereas it is thought to be around 10 nanomolar (nM) in the extracellular microenvironment [1]. As such, there is a 10^6-fold gradient for ATP efflux. In response to tissue damage and cellular stress, cells actively release ATP. Even small leaks of ATP from the intracellular space elicit a dramatic elevation of extracellular ATP concentration and therefore purinergic signaling [1].

Extracellular purines are tremendously important metabolic signals. Since the first description of ATP as a specific signaling molecule, it has been well documented that extracellular nucleotides and their derivatives function as "signal transmitters" that induce intracellular signaling transduction through interaction with specific membrane purinergic receptors (purinoceptors) [2,3]. This process termed "purinergic signaling" has been shown to participate in many physiological and pathological processes, regulating cell growth, metabolism, angiogenesis, inflammation and immunity.

Purinergic Systems

Purinergic systems have three essential components: extracellular nucleotides and their sources, specific receptors for these molecular transmitters (or derived products such as adenosine diphosphate (ADP) and adenosine), and ectonucleotidases that hydrolyze nucleotides (to nucleosides) [4]. Each of these has important effects on purinergic signal transduction, leading to changes in cellular responses.

We discuss these separately.

Release of Extracellular Nucleotides and Cellular Sources

ATP release can occur virtually in all cell types. Multiple mechanisms underlying ATP release by stressful, injured, or stimulated cells, either well-regulated or uncontrolled, have been reported [5]. Synaptic vesicles co-release ATP and neurotransmitters upon fusion with the cell membrane [6]. Lysosomes mediate ATP release by way of exocytosis [7-9]. Apoptotic or inflammatory cells release ATP through various types of membrane channels/efflux pathways, including connexin and pannexin hemichannels [10,11], maxi-anion channels [12], volume-regulated anion channels [13], and the type 2 purinergic (P2) receptors P2X4 and P2X7 [14]. Finally, direct, uncontrolled ATP leakage can occur, whether at the center of rapidly proliferating solid tumors which become necrotic as they outstrip their vascular supply or from the disrupted membranes of dying tumor cells treated with radiation or chemotherapy [15-17].

Purinergic Receptors

The precise actions of extracellular nucleotides or nucleosides are determined by the receptor(s) with which these mediators interact. Differing largely by their ligand binding affinity and cell/tissue distribution, purinergic receptors are broadly distributed on the cell surface of virtually all cell types. Chiefly, almost all biological processes can be modulated by purinergic receptors, *e.g.* cell growth and metabolism, angiogenesis, platelet activation, and inflammatory and immune responses [18].

In 1978, Burnstock proposed a taxonomy for purinergic receptors that divided them into two distinct subclasses. Type 1 purinergic (P1) receptors are selective for adenosine, whereas type 2 purinergic (P2) receptors specifically bind ATP (as well as other nucleoside tri and diphosphates) [19]. To date, four P1 receptors (A1, A2A, A2B, and A3), seven ionotropic P2Xreceptors (P2X1-7), and eight metabotropic P2Y receptors (P2Y1, 2, 4, 6, 11–14) have been characterized [20,21]. Different subtypes in the P2 and P1 receptor families often mediate opposing physiological functions by initiating different downstream signaling cascades. Any given cell type expresses a broad-spectrum of purinergic receptors. As different purinergic receptors contain differential agonist affinity and specificity, the relative pericellular purine concentrations regulate distinct cellular functions in both health and disease [22-24].

P2X receptors are ATP-gated ion channels for Na^+, K^+ and Ca^{2+}. The diversity of P2X receptor function is tremendous. The infertility of P2X1 null male mice, for example, is a consequence of defective P2X1 signaling in the ductus deferens [25]. P2X2, 4, and 6 are the most abundant subunits found in neurons [26]. P2X3 expression is limited to a subgroup of afferent neurons. By forming a heteromeric channel with P2X2, P2X3 fully executes the transmission of nociception by ATP [27-29]. P2X4, distributed in various tissues, is mainly involved in modulation of epithelial and endothelial function, controlling ciliary function and calcium flux [23]. Moreover, several P2X receptors (inclusive of P2X1-4 and 7) have been shown to associate with lipid rafts [30-33]. Amongst all P2X receptors, P2X7 is the most extensively characterized subtype, owing to its critical roles in many fundamental pathophysiological processes including inflammation, immunity, and carcinogenesis [34].

P2Y receptors are seven-transmembrane-domain G protein-coupled receptors (GPCRs) [3]. They can be further divided into five Gq/11-coupled subtypes (P2Y1, 2, 4, 6 and 11) and three Gi/o-coupled subtypes (P2Y12-14). In general, elicitation of Gq/11-coupled P2Y receptors often induces Ca^{2+} release from the intracellular stores, through activation of phospholipase C (PLC) and inositol triphosphate (IP3) pathways; whereas Gi/o-coupled P2Y subtypes modulate ion channel function *via* the inhibition of adenylyl cyclase activity [35]. In addition, adenylate cyclase can be stimulated by P2Y1 by Gs protein [3,36,37]. Alterations in cytoplasmic cyclic AMP (cAMP) levels, in turn, impact protein kinase A (PKA) activity. Finally, P2Y receptors are ubiquitously expressed on hematopoietic cells. Indeed, P2Y2 is the major P2 receptor subtype responsible for recruitment and activation of neutrophils, dendritic cells (DCs), and macrophages at the inflammatory sites [38]. Beneficial effects of P2 receptor antagonists have been noted in various pathological conditions, including stroke and inflammatory diseases [39,40].

P1 receptors are also GPCRs, each with downstream signaling pathways of tremendous importance to both health and disease. A2A and A2B subtypes possess high adenosine binding affinity, whereas A1 and A3 receptors have low affinity for adenosine binding. A2B activates PLC through the Gq protein [41]. A1 activation also stimulates PLC, by suppressing adenylate cyclase, thereby decreasing intracellular cAMP levels [42]. A3 signaling differentially impacts normal *vs.* tumor cells [42,43]. In normal cells, A3 activation is linked to NF-κB signaling to further drive production of growth factors. By contrast, in tumor cells, A3 agonists result in NF-κB and Wnt signaling-mediated apoptosis and tumor growth inhibition. Finally, all P1 receptor signals have been coupled to the mitogen-activated protein kinase (MAPK) pathway (inclusive of extracellular signal-regulated kinase 1 and 2 (ERK1/2), JUN N-terminal kinase (JNK), and p38) [44]. Important functional implications of P1 receptors have been indicated in numerous essential pathophysiological processes, *e.g.* vascular integrity and function and immunomodulation [45].

Ectonucleotidases

Ectonucleotidases perform as an essential regulatory component of purinergic signaling. By hydrolyzing extracellular purines, ectonucleotidase activity maintains the physiological 10^6-fold ATP gradient between the intracellular and extracellular compartment and thereby tightly controls purinergic responses.

To date, eight different ectonucleoside triphosphate diphosphohydrolase (ENTPDase) subtypes of the CD39 family have been identified (ENTPD1-8, each with distinct cellular location and catalytic properties). ENTPD1, 2, 3, and 8 are prototypical plasma membrane-associated enzymes with an extracellular catalytic site. Properties that are shared by CD39/ENTPD1 and other ectonucleotidases include localization in membrane lipid rafts [46] and the regulation of cellular signaling pathways [47]. Wu *et al* found that CD39 could directly interact with Ran-binding protein M (RanBPM), a membrane scaffolding protein with GTPase activity [48]. Distinct phosphohydrolytic activities among ENTPDase subtypes are, in large part, as a result of substantial differences in their sequences as well as in secondary, tertiary and quaternary structures [49,50]. Beyond that, they have distinct preferences for substrates and divalent cations, hydrolyze nucleoside triphosphates at varying rates, and generate different products [51]. Micromolar levels of Ca^{2+} or Mg^{2+} ions are absolutely requisite for these four cell surface-located ectoenzymes to exert maximal activity. CD39/ENTPD1, with a preference of Mg^{2+} over Ca^{2+}, equally degrades ATP and ADP. ENTPD2, originally identified as an ecto-ATPase, favors hydrolysis of nucleoside triphosphates and also prefers Mg^{2+} over Ca^{2+} [51]. In contrast, ENTPD3 and 8 preferentially metabolize ATP over ADP and are in favor of Ca^{2+} over Mg^{2+} [52-54]. ENTPD5 and 6 are intracellularly located and, after being heterologously expressed, can be secreted. Finally, ENTPD4 and 7 are found entirely within cells [55].

While CD39 is the rate-limiting enzyme in the adenosine generation cascade, other ectoenzymes participate in purine metabolism cascades [56]. These include amongst others CD73 (ecto-5'-nucleotidase; or Nt5e) which degrades AMP to adenosine and the adenosine deaminases (ADAs) which further hydrolyze adenosine to inosine. The conversion of ATP into AMP can be reversed by the action of extracellular soluble kinases such as adenylate kinase (AK). However, AMP conversion to adenosine seems to be largely reversible within cells, catalyzed by adenosine kinase after adenosine transporter-mediated adenosine uptake [57].

These ectoenzymes are inextricably linked by their dynamic substrate concentrations and resultant feedback loops. The first evidence in this regard was reported by Camici *et al* in 1985 [58,59]. The authors described that ATP and ADP can potently inhibit CD73 enzymatic activity in a competitive manner. Furthermore, this group found that the ADP's inhibition is more effective than ATP, albeit both function at the low micromolar ranges. In 1986, Gordon and colleagues also observed that the higher the initial ATP concentration, the slower the appearance of adenosine. This suggests a "feed-forward inhibition" of CD73 by ADP [60]. This is further corroborated by the recent observation that loss of CD39 bioactivity causes the accumulation of ATP and ADP and results in local "feed-forward inhibition" of CD73 function, in turn inhibiting adenosine generation [61]. At the same time, an increased ecto-ATPDase activity shifts the adenine nucleotide composition towards increased AMP, which is a substrate for CD73 to generate adenosine [62]. The result is a compensatory upregulation of CD73, which implied significant demand for conversion of ATP/ADP into adenosine [63]. The two major cell types expressing CD39 and/or CD73 are the vascular endothelium and immune system [64-69]. CD39 is expressed by many types of activated hematopoietic cells including B cells, natural killer (NK) and NKT cells, activated T cell subsets, monocytes/macrophages, and leukocytes. CD73, on the other hand, is expressed by activated B cells but neither naive T cells nor follicular DCs [57]. Indeed, CD73 has been suggested as a phenotypic marker of

effector memory T helper type 17 (Th17) cells to monitor inflammatory response [70]. Similarly, dual expression of CD39 and CD73 distinguishes $CD4^+Foxp3^+$regulatory T (Treg) cells from other T cells [71,72]. More importantly, this dual expression, *via* production of adenosine, provides a novel immunosuppressive mechanism that is independent of conventional inhibitory cytokines [34,73,74]. $Foxp3^-CD39^+$ cells are able to mitigate TGF-β-induced conversion of $Foxp3^-CD39^-$ cells into Tregs (iTregs) [75]. Additionally, expression of CD39 is also observed on pre-existing $CD4^+CD44^+CD62L^-Foxp3^-$ memory T cells (inclusive of T helper type 1 (Th1), type 2 (Th2), and Th17 subsets), accompanied by elevated alloreactivity. NKT cells coexpress CD39 and CD73, whereas NK cells only contain CD39 [76]. Finally, expression of CD39 and/or CD73, which is regulated by hypoxia, has also been observed on many types of tumor cells [18-21,35,38,45,77-85].

Aside from the enzymatic activity-dependent actions of CD39 and CD73, these ectonucleotidases may function independently of their ectoenzymatic activities. CD39 directly associates with RanBPM and CD73 has the capacity to both transmit strong activation signals in T cells upon antibody ligation and act as a cell adhesion molecule [48,86]. More recently, it has been shown that blocking monoclonal antibodies (mAbs) against CD73 inhibit primary tumor growth and metastasis in mice, in both CD73 enzymatic activity-dependent and -independent manners. The underlying mechanism of this effect involves clustering and internalization of the CD73 protein, which does not require cross-linking of CD73 mAbs [87].

CANCER AND PURINERGIC MECHANISMS

The specific constitution of the tumor microenvironment is critical for tumorigenesis. Chronic inflammation predisposes tissues to malignancy. The putative link between inflammation and cancer has been further corroborated by countless epidemiological studies [88], since the first observation by Rudolf Virchow of predominant accumulation of cancers at the chronically inflamed tissues in the 19^{th} century. Yet, the mechanistic role of chronic inflammation in tumorigenesis has only been clearly established during the past decade [89,90]. As critical components of tumor progression, tumor-infiltrating inflammatory cells participate in malignant transformation of premalignant cells as well as proliferation, survival, and migration of cancer cells [91]. Tumor cells also release inflammatory mediators that activate and recruit leukocytes to tumor vascular endothelium, driving production of angiogenic, chemotactic, and growth factors as well as proteolytic enzymes. These processes will, in turn, promote recruitment of additional inflammatory cells thereby supporting tumor growth and metastasis [90]. In summary, tumors often develop mechanisms to evade the host antitumor immune system and instead use it to their own advantage to survive, proliferate, and spread [92].

Numerous important signal mediators, including extracellular ATP and adenosine, provide the essential local milieu for cancer development, which is further governed by reciprocal and dynamic tumor-host interactions. In recent years, there has been an increasing focus on the role of purinergic signaling and its potential as a therapeutic target in a large variety of diseases inclusive of cancer. Because of the broad-spectrum of P2 and P1 receptor expression on both tumor bulk and host tissues, purinergic signaling that integrates distinct cellular changes/responses has been explicitly ascribed to virtually all aspects of cancer biology. In general, ATP is thought to be pro-inflammatory whereas adenosine is anti-inflammatory.

In this section, we will summarize key findings delineating the purinergic mechanisms underpinning select aspects of carcinogenesis. A few concepts should be clarified at the outset. First, since ATP is the very first signal released by inflammatory or damaged tumor cells and CD73 function and adenosine generation is dependent on CD39, we will focus

on discussing fundamental molecular mechanisms underlying modulation of tumor biology by ATP-P2 receptor/CD39 signaling. Second, as the majority of research investigations thus far have utilized transplanted tumor models (either xenograft or syngenic), we will focus on how purinergic modulation impacts transplanted tumor biology (post malignant transformation), with introduction of limited, if seemingly very important, studies on autochthonous tumor development (premalignant and malignant transformation). Third, though these cancer aspects will be discussed in separate subsections for a clearer presentation, the following concepts should always be kept in mind: i) each trait is certainly not independent. Crosstalk among different traits are indispensable for their functions; ii) pericellular ATP/adenosine would impact any cells capable of sensing purine level alterations, regardless of cellular sources of purines. Therefore, ATP/adenosine effects may be autocrine or paracrine, that is determined by the differential expression pattern of CD39/CD73 on a given cell type; iii) purinergic signaling can act alone or synergistically with other oncogenic signaling pathways (*e.g.* hypoxia, growth factors, chemokines, and toxins) to modulate cellular responses.

Intrinsic Tumor Responses

Virtually all types of cancer cells possess more than one functional purinoreceptor that can efficiently sense changes in purine concentrations within the local milieu. As a consequence, these purinergic signals can activate multiple intracellular pathways that control intrinsic properties of the tumor cells, including metabolism and growth.

Tumor Metabolism

Cell metabolism is fundamental to cell growth. In normal differentiated tissues, cell metabolism is largely dependent upon the availability of nutrients. In the presence of oxygen, normal cells metabolize glucose to CO_2 *via* oxidative phosphorylation to maximize production of the energy currency ATP. When oxygen is scarce, cells switch to anaerobic glycolysis to generate lactate and produce ATP. Proliferating or cancer cells, on the other hand, regardless of the availability of oxygen, primarily rely on glycolytic metabolism that converts most glucose to lactate leading to rapid production of ATP [93]. Termed the “Warburg effect”, this metabolic phenomenon serves to provide essential building blocks supporting rapid cell division. Activation of oncogenic signaling, such as Ras and PI3K-AKT-mTOR, has been shown to drive changes in metabolism and has been implicated in the regulation of aspects of the “Warburg effect” [94,95].

Many recent innovative anticancer therapeutic avenues have been focusing on targeting the key regulators in tumor metabolism. Several groups have successfully developed clinically promising inhibitors for glutaminase 1 (GLS1), a glutaminase isoform that is highly upregulated in cancer cells. Similarly promising, modulating the activity of pyruvate kinase M2 (PKM2), a glycolytic enzyme that is frequently elevated in tumors. Furthermore, several groups have developed FASN inhibitors to target tumorigenesis. Many inhibitors for hypoxia-inducible factor (HIF) and HIF targets, are also potential anticancer drugs. Early phase clinical trial data show that blocking lactate production using dichloroacetate (DCA) also inhibits HIF-1α signaling and activates p53, ultimately suppressing tumor angiogenesis in glioblastoma patients with minor side effects. Glycolysis inhibitors are also of interest in anticancer therapy. For instance, FX11, a selective suppressor of lactate dehydrogenase A (LDH-A) activity, is among the most advanced inhibitors of tumor metabolism in clinical trials (Phase II). It is well known that metformin, commonplace in the medical therapy of insulin resistance, inhibits mitochondrial complex I, thereby decreasing ATP production. Lack of ATP subsequently stimulates LKB1-AMPK pathway and blocks gluconeogenesis, leading to lower blood glucose concentrations, improved sensitivity to insulin and diminished insulin production. Metformin has been well documented to improve cancer patient survival and also harm cancer stem cells (CSCs). It is unclear whether metformin improves cancer clinical outcomes by

lowering blood glucose levels and insulin/insulin-like growth factor production or by directly targeting cancer cells. Nevertheless, the scope of promising interventions for disrupting tumor metabolism that leverage purinergic mechanisms is broad and ranges from the investigational to readily available [96].

Our studies have linked purinergic signaling with metabolic pathways in premalignant and tumor cells. Sun and Han *et al,* for example, recently demonstrated how metabolic pathways are altered to meet the continuous demands of constitutively proliferating premalignant hepatocytes, as a result of aberrant activation of ATP-P2Y2 receptor signaling [97]. Specifically, in the CD39-free liver, elevated extracellular ATP (ranging from 10 to 100 μM) facilitates anabolic pathways by way of altering expression/activity of several critical glycolytic switch enzymes (*e.g.* PKM2 and LDH-A) as well as by abrogating autophagy, a fundamentally catabolic process. These changes are associated with disruptions in multiple genes involved in mitochondrial biogenesis and regulation (*e.g.* mitochondrial uncoupling protein (UCP) 2). The end result of these processes push cells towards glycolysis.

UCP2 is an important player in this process. UCP2 controls both reactive oxidative species (ROS) production and mitochondrial ATP biosynthesis [98]. Disruptions in these processes dictate the fate of cells through reprogramming proliferative and glycolytic pathways. Elevated UCP2 levels have been noted in some cancers [99,100], with putative roles in both impaired tumor suppression and chemoresistance, mediated largely *via* diverting metabolic signals [100,101]. Other recent findings have elucidated several molecular underpinnings of the critical contributions of UCP2 to metabolic transformation of cancer cells [98]. Owing to UCP2's indispensable role in the maintenance of mitochondrial homeostasis, the lethality of altered mitochondrial uncoupling has limited identification of the downstream components. Recently, p53 is recognized as a downstream target of UCP2's uncoupling activity in cancer cells [101]. p53 signaling can be altered by intracellular ROS levels, which are determined by UCP2 function. Whether p53 provokes anti-oxidant or pro-oxidant responses is mainly dependent upon the oxidative stress levels. UCP2-driven aberrant p53 signals are an encouraging opportunity for novel anticancer strategies [98].

Additionally, a recent study by Fang *et al* has unraveled an important role for ENTPD5 in mediating the "Warburg effect" in cancer cells. Heightened levels of ENTPD5, an UDPase located on endoplasmic reticulum (ER), have been noted in several human malignancy models, including both cell lines and tumor specimens [102]. ENTPD5 not only facilitates protein folding *via* driving N-glycosylation, but also in concert with cytidine monophosphate kinase-1 and adenylate kinase-1, orchestrates an ATP metabolism cycle hydrolyzing ATP to AMP [102]. The latter process tightly modulates the PTEN and PI3K-AKT pathways providing a novel compensatory mechanism driving aerobic glycolysis [102]. Moreover, combined suppression of ENTPD5 and PTEN markedly inhibits tumor growth (both *in vitro* and *in vivo*), associated with increased ER stress and diminished growth factor receptor expression. These novel findings support ENTPD5 as a potential therapeutic target in cancer [102].

Tumor Growth

The established roles of extracellular ATP and adenosine in tumor growth are paradoxical, either tumor-promoting or tumoricidal. These are largely determined by the specific expression/activation of P2 and P1 receptor subtypes, which impact the cellular machinery of proliferation, apoptotic/necrotic/autophagic death, and differentiation.

Pro-proliferative Activities of Purinergic Signaling

P2Y1 receptor activation is shown to be pro-proliferative in human thyroid cancer [103]. A generalized tumor-promoting activity has also been suggested for the P2Y2 receptor in melanoma, squamous cell carcinoma, breast, lung, thyroid, and liver cancer cells [104-109]. Recently, extracellular ATP is reported to inactivate Forkhead Box O (FoxO) transcription factors and drive cell cycle progression through the P2Y2-mediated PI3K/AKT pathway in MCF-7 breast cancer cells [110]. In studies exposing human lung cancer A549 cells to radiotherapy, P2Y6 and P2Y12 receptor signaling activates cellular DNA repair machinery and facilitates cell survival through an ERK1/2 pathway mechanism [109,111,112]. In 132N1 astrocytoma cells, P2Y6-provoked PKC activation abrogates tumor necrosis factor (TNF)-induced apoptosis [113], while P2Y12 signaling activates Ca^{2+} signals to regulate cell proliferation and differentiation [114].

In addition, accumulation of ATP/ADP in the local tumor microenvironment may result in activation of platelets. P2Y1 and P2Y12 activation by ADP promotes thrombosis [36]. Thrombosis, in turn, leads to further tumor ischemia/necrosis and thus a positive feedback loop of ATP release which, together with platelet-derived growth factor release, stimulates tumor growth.

A2A and A2B receptors are generally thought to promote tumor cell proliferation. Their activation has been reported to stimulate proliferation of lung, pancreatic, intestinal, and prostate cancer cells [115-117]. Adenosine also enhances chemoresistance of chronic lymphocytic leukamia cells through this mechanism [118]. Moreover, A1 receptor function has also been shown to be required for proliferation of MDA-MB-468 breast cancer cells [119] and poorly differentiated HT-29 human colon cancer cells [120].

Tumor-killing Actions of Purinergic Signaling

The anti-neoplastic activity of ATP was first described by Rapaport *et al* in 1983 [121]. The authors demonstrated that *in vitro* supplementation of exogenous ATP could block growth of pancreatic and colon cancer cells, by promoting cell-cycle arrest at S phase. Since then, tumoricidal actions of ATP have been extensively exploited in the laboratory setting using both *in vitro* experimentation systems and *in vivo* animal models. Chiefly, all types of tumor cells express one or several purinergic receptor subtypes that differentially sense alterations in extracellular ATP/adenosine levels, and thereby initiate distinct downstream intracellular signaling regulatory networks to dictate the cell fate – growth, quiescence, or death [104,122-125].

Amongst these P2 receptors, P2X7 has been labeled as the “death receptor”. It has been associated with ATP-evoked tumor cell death in many types of cancer, namely colon cancer, melanoma [65,126,127], squamous cell carcinomas [128], glioma [129], cervical cancer [130], and endometrial cancer [131]. P2X7 activation leads to membrane pore formation and cytoskeletal re-organization, often leading to tumor cell apoptosis. Indeed, low level or defective P2X7 expression have been linked to cancer development by allowing escape from the P2X7 controlled pro-apoptotic mechanism [132]. P2X7 receptor expression as a diagnostic biomarker and/or therapeutic target has been explicitly explored not only in solid tumors [131], but also in haematologic malignancies [133].

Feng *et al* showed that high levels of exogenous purines, inclusive of ATP, ADP, and adenosine, all effectively and dose-dependently inhibit cell proliferation and induce apoptotic/necrotic cell death in murine B16/F10 melanoma and MCA38 colon cancer cells [65]. Thereafter, Bian *et al* linked the concentration-dependent ATP-P2X7 triggered

autophagic cell death to two downstream signaling axes – the PI3K-AKT pathway and the AMPK-PRAS40-mTOR signaling. These two signaling pathways, synergistic but independent, are not modulated by Ca^{2+} signaling [127]. The clinical import of P2X7 signaling is becoming increasingly clear. Induction of ATP-P2X7-mediated apoptosis has also been indicated as a novel chemoprevention strategy for epithelial cancers [134]. In human breast cancer cells, upregulation of microRNA (miR)-150 is found to inhibit P2X7 expression and function, thereby driving cell growth and aggressive phenotype [132]. In glioma, P2X7 blockade also exerts a growth-promoting effect, concurrent with upregulated expression levels of epithelial growth factor receptor (EGFR), HIF-1α, and vascular endothelial growth factor (VEGF) [135]. P2X7 activation elicited by high extracellular ATP alone, or in combination with cholesterol-lowing drugs statins, decreases phosphorylated AKT levels in the nuclei, thereby inhibiting cancer cell growth. This is largely mediated through interactions of P2X7 with several protein/lipid phosphatases including phosphatase and tensin homolog (PTEN) [136-138]. These findings speak to the potential anticancer therapeutic strategies using purinergic modulation alone or in combination with other oncogenic pathway inhibitors.

Activation of multiple purinergic receptor subtypes slow tumor cell growth, through a combined action of induction of apoptosis and differentiation. Tumoricidal functions of P2X5/P2X7 and/or P2Y11 receptors have been documented in both hormone refractory prostate cancer (PC-3) and high-grade bladder cancer (HT-1376) cells [105,139,140]. P2Y receptor subtypes are also implicated in cancer biology. Namely, P2Y1 and P2Y2 signaling can both alter intracellular cAMP levels (*via* adenylyl cyclase) and elevate intracellular Ca^{2+} concentrations (*via* PLC pathway). Accordingly, these proteins can significantly impact cell proliferation [114]. Indeed, anti-proliferative activity has been observed for both P2Y1 and P2Y2 in melanoma A375, colon cancer and oesophageal cancer cells [106,141-144]. Collectively, cytotoxic effects of extracellular ATP on most neuroblastoma cell lines appear to be dominantly mediated *via* P2X7, P2Y2, and P2Y6 receptors.

Adenosine has also been shown to limit cell growth in some cancer types *via* activation of distinct P1 receptors. In human colon cancer (Caco-2) and hepatoma (HepG2) cells, A2A signaling decreases expression of anti-apoptotic protein BCL-XL while increasing pro-apoptotic factor BH3 interacting-domain (BID) which could ultimately lead to cancer cell death [118,145]. When combined with a phosphodiesterase inhibitor (to maximize intracellular cAMP levels), A2A receptor agonists provoke death of multiple myeloma and diffuse large cell lymphoma [146]. As to A1 and A3 receptors, considerable evidence directly links their activation to tumor cell killing [147]. Activation of A1 receptor induces apoptosis in CW2 human colon cancer cells (mediated *via* caspase-3, -8 and -9) [148] and mouse glioblastoma [149]. A3 signaling has been demonstrated to inhibit growth of a wide range of cancer cell types, including human lymphoma, melanoma, prostate, lung, colon, and neural cancer cells, and murine hepatocellular carcinoma (HCC) cells [150-160]. The underlying mechanisms suggested are varied: induction of G0 or G1 cell cycle arrest, upregulation of pro-apoptotic genes with concurrent reduction of anti-apoptotic genes, or inhibition of the Wnt pathway [150-160].

These ambiguities amongst purinoreceptor functionalities in tumor growth (summarized in Tables **1-2**) have yet to be fully explained. Further study is required and ought to consider the role of concurrent activation of distinct, yet opposite, intracellular signals, and/or relative differences in the levels of extracellular ATP/adenosine.

As the control nodes for extracellular ATP metabolism, ectonucleotidases CD39 and/or CD73 (to a lesser extent) play pivotal and direct roles in tumor growth. Both have been shown to be overexpressed on several types of cancer cells. For instance, CD39 is the dominant ectonucleotidase on human melanocytes, and melanoma cells and differentiated human

melanomas overexpress CD39 [161]. Heightened CD39 expression is also found in some newly developed human hematological malignant cell lines, such as BALM-25 (derived from acute lymphoblastic leukemia) [162], MedB-1 (derived from large B-cell lymphoma) [163], and chronic lymphocytic leukemia cell lines [164,165]. Cancer cells overexpressing CD73 include bladder cancer [166], leukemia [167], glioma [168], glioblastoma [169], melanoma [170], ovarian [165], thyroid [171], esophageal [172], gastric [173], colon [174], prostate, breast [175], and endometrial cancer [85]. Recently, Clayton *et al* discovered that exosomes from diverse cancer cell types (HT1376 bladder, Caco2 colon, and DU145 and PC3 prostate cancer cells) coexpress CD39 and CD73 and display high ectonucleotidase activity [176]. Coexpression of CD39 and CD73 on cancer cells or derived exosomes suggest that cancer cells could produce adenosine from extracellular substrates in an autocrine fashion.

Taken together, CD39 and/or CD73, expressed on both tumor and host cells, have been demonstrated to exhibit potent influences, directly or indirectly, on tumor growth *via* modulating the repertoire of pericellular ATP and adenosine, through multiple purinergic mechanisms that initiate different, sometimes opposing, cellular responses. The final outcome is dictated by the end result of these divergent cellular functions. This will be discussed in further detail throughout this section.

Host Adaptations and Responses

Purinergic elements expressed by almost all types of host cells also critically control the host adaptations and responses, *e.g.* angiogenic and immune responses in the local tumor microenvironment.

Angiogenesis

Tumor angiogenesis, *de novo* formation of tumor-associated blood vessels, is vitally important to the development, malignant transformation, rapid growth, and progression of solid tumors. It has also become a major target for cancer therapeutics [177-180]. The capacity for tumor growth above the size of 2-3 mm^3 is determined by the availability of oxygen and nutrients [181,182]. Enhanced angiogenesis can be noted during early carcinogenesis. Hypoxic conditions during proliferation drive cancer cells to produce pro-angiogenic proteins, including VEGF, basic fibroblast growth factor (bFGF), TGF-β, and interleukin-8 (IL-8) [183-185]. Besides the indispensable vascular ECs, several other cell types also greatly contribute to angiogenesis in supporting roles. Monocytes/macrophages can secrete angiogenic factors and metalloproteases to facilitate EC migration and new vessel growth [186]. Vascular smooth muscle cells facilitate vascular endothelial maturation and pericytes protect the nascent, *de novo* formed EC-lined tubes [187]. Accessory and stromal cells are also linked to tumor angiogenesis, yet the sources of these cells are still not clear.

When cancer cells undergo ischemic damage, intrinsic release of angiogenic mediators (*e.g.* ATP and other chemoattractants) promotes angiogenesis. Further intratumoral accumulation of adenosine is another key driver of tumor angiogenesis. Purinergic signaling modulates growth, function, and migration of all cell types associated with tumor angiogenesis, and is thus a fundamental player in this crucial process dictating cancer development. With the growing importance of angiogenesis in cancer biology, it is worthwhile to exploit mechanisms of extracellular nucleotide/nucleoside metabolism and action, in order to limit angiogenesis thereby blocking tumor growth and potentially preventing metastasis and associated cancer mortality [188].

Vascular Endothelial Cells (ECs)

Vascular ECs, the key player in angiogenesis, express a broad-spectrum P2 receptor including P2Y1, P2Y2, P2Y11, P2X4, and P2X7 [189] as well as A2A and A2B adenosine receptors [190-192].

P2Y receptors, well-established integral modulators of blood flow [193], have been recognized as important regulators of EC function [194,195]. Several elegant mechanistic studies demonstrate the abilities of endothelial P2Y receptors to drive angiogenesis vis-a-vis EC motility, mitogenic responses, and vascular permeability [196-198]. Extracellular ATP is considered as a vasoactive mediator. It can activate P2Y1/2 expressed on vascular ECs to promote release of additional vasoactive mediators including ATP, nitric oxide (NO), and prostacyclin [199-201]. Consequently, through an autocrine, positive feedback mechanism these effects boost vasoactive and angiogenic responses [202].

P2Y2 receptor contains an extracellular integrin-binding domain RGD (arginine-glycine-aspartic acid). RGD has been shown, in human K562 erythroleukemia cells, to interact with integrins $\alpha v\beta 3$ and $\alpha v\beta 5$, both key players in modulating vascular EC functionality and angiogenesis [203]. Activation of P2Y2 transactivates VEGFR-2, suggesting crosstalk among signals for extracellular nucleotides (specifically ATP and UTP *via* a GPCR) and growth factors (*via* receptor tyrosine kinase; RTK) [204]. Buvinic *et al* noted that synergy between P2Y1 and EGFR signaling enhances cell proliferation [205]. Rumjahn *et al* reported that extracellular nucleotide-initiated angiogenic responses are mediated through convergent elicitation of P2Y1 receptor and subsequent VEGFR-2 signaling in ECs [206]. Beldi *et al* explicitly delineate how, in the hepatic microenvironment, alterations in extracellular nucleotide fluxes (ATP, UTP, and ADP) tightly control the crosstalk between P2Y2 and VEGFR-2 signaling in both hepatocytes and liver sinusoidal endothelial cells (LSECs) to control the release of mitogenic factors. As critical mediators of LSEC and hepatocyte proliferation, these mitogenic factors (including VEGF, HGF, and IL-6) further govern the success of the well-regulated, multistep liver regeneration process. Moreover, colocalization of VEGFR-2 and P2Y receptors is also noted in endothelial caveolae [195,207-210], allowing for sufficient pro-angiogenic signaling activation, even with stimulation by small amounts of agonists.

P2X4 is a highly expressed P2X subtype in ECs [189]. P2X4 plays a key role in regulation of EC responses to changes in blood flow and sheer stress and thereby controls blood flow-dependent processes including angiogenesis and vascular remodeling [211]. In human umbilical vein endothelial cells (HUVECs), ATP-P2X4 signaling mediates biotinylated thymosin β-4 (Tβ4)-stimulated cell migration [212]. P2X7 also plays important, yet paradoxical, roles in angiogenesis. Persistent P2X7 activation drives primary human monocytes to vigorously release VEGF, dependent on calcium signals and linked to ROS production [213]. However, transient P2X7 activation by high ATP levels exerts direct cytotoxicity on ECs limiting tumor angiogenesis [65].

Finally, A2A and A2B receptor signaling has been extensively demonstrated to directly enhance EC proliferation and pro-angiogenic factor secretion, driving tumor angiogenesis [190-192].

Of note, owing to the complexity of signal transduction networks, suppression of an element often activates other compensatory pathways, chiefly by perturbing crucial negative feedback loops. Notably, genetic knock down/out systems could present strong compensatory effects, since knockdown of a specific receptor subtype could lead to a

compensatory activation of other subtype functions [214]. P2X7 deficiency is shown to induce compensatory elevations of P2X4 [127,215], and P2X1 [216].

Monocytes/Macrophages

Blood mononuclear cell population and their descendants, tissue macrophages, are also involved in modulation of angiogenesis [217]. Monocytes/macrophages possess several P2/P1 receptors. Upregulation of P2X7 receptor mRNA in monocytes has been observed upon their differentiation to macrophages [218]. In macrophages, activation of P2X7 receptor signaling by high levels of ATP has been linked to several downstream MAPKs (such as ERK1/2 and p38). These signals play important roles in numerous pathophysiological responses *e.g.* inflammation and tissue remodeling [218], possibly mediated through driving production of inflammatory mediators inclusive of tissue factor that has been implicated in thrombotic disorders, angiogenesis, and metastasis [219]. Also recently, Clark *et al* documented that A1 signaling transduction in peripherally-derived monocytes stimulates VEGF release to further impact EC function [220].

Vascular Smooth Muscle Cells, Accessory and Stromal Cells

Vascular smooth muscle cells express P2X receptors that mediate extracellular ATP-initiated vasoconstrictor responses [221]. These cells also possess a variety of P2Y and P1 receptors to control the vascular tonus [221,222]. In human coronary smooth muscle cells, ATP-induced growth suppression is mediated *via* the P2X1-transcription factor NR4A1 signaling axis [223]. Activation of A2A, A2B, and A3 receptors in these cells is able to drive release of pro-angiogenic mediators including growth factors and cytokines [224,225]. Paradoxically, adenosine is also shown to suppress proliferation of rat and human aortic smooth muscle cells *via* A2B receptor signaling transduction [226]. Additionally, accessory and stromal cells are also capable to metabolize pericellular ATP thereby participating in tumor angiogenesis.

In principle, CD39 and/or CD73 modulate these ATP- or adenosine-mediated angiogenic responses, through tightly controlling the local pericellular ATP/adenosine levels within the tumor microenvironment. Critical for EC growth and function, CD39 is absolutely requisite for angiogenesis [227,228]. Deletion of CD39 has been shown to abrogate growth of transplanted tumors in various types in mice, largely due to defective angiogenesis [72,229]. Vascular cells expressing CD39 also boost angiogenesis by scavenging cytotoxic extracellular ATP [65], leading to the production of adenosine that is able to promote tumor angiogenesis through activation of A2A receptor on ECs [21]. In addition, CD39 also protects macrophages from ATP-P2X7 elicited cell death [230]. Loss of CD39 leads to perturbed chemotactic and mitogenic responses of ECs, monocytes/macrophages, and pericytes, and impaired integrin expression, as well as defective release of angiogenic factors and mitogens. Each of these dysfunctions is the result of disordered P2Y receptor signaling [227,231,232]. Finally, CD39 expression on vascular smooth muscle cell also plays a critical role in cell migration [233].

More recently, host cell expression of CD73 has been shown to drive angiogenic responses *in vivo* such as tube formation and EC migration [234]. Furthermore, ENTPD2 activity appears to also play a critical role in tumor progression through ADP production which recruits platelets to the tumor site and thereby regulate angiogenesis and tumor growth [235].

Innate and Adaptive Immune Responses

An effective immune system, including both innate and adaptive immunity, is required for clearance of the invading tumor cells. At the same time, the inflammatory tumor microenvironment, which is endowed by both innate and adaptive immune cells, possesses a bifunctional feature: either stimulatory or inhibitory for cancer cells. The purinergic system, expressed literally on all types of inflammatory and immune cells, plays an indispensable role in inflammation [236] and antitumor immune responses [4,237].

Extracellular ATP in Innate Immunity

In general, high extracellular ATP levels have immune stimulatory effects by boosting innate responses. Accumulated pericellular ATP levels within the tumor site function as chemoattractants to recruit virtually all innate immune cells towards the tumor, and subsequently induce pro-inflammatory cytokine release by these cells.

Damaged cells/tissues release endogenous "danger signals" (called damage-associated molecular pattern molecules (DAMPs)). Innate immune cells sense these soluble molecules in order to be fully activated [238]. One unique feature shared by all DAMPs is their complete absence under physiological conditions and rapid accumulation in the extracellular milieu upon cell damage. The high levels of ATP released by necrotic cells [79] are actually potent, non-protein DAMPs [38]. Chemotherapy-induced ATP release by tumor cells is also able to trigger immunogenic cell death [17,239-241]. Beyond their role as a danger signal, high extracellular ATP levels perform other antitumor actions through multiple molecular mechanisms [1]. ATP can also act as a "find-me" signal to initiate and drive efficient clearance of apoptotic cells by phagocytes [242]. Based on persuasive *in vitro* and *in vivo* evidence, P2Y2 and P2X7 are the major P2 receptor subtypes mediating ATP-induced pro-inflammatory responses. Indeed, P2Y2 is essential for recruitment and function of inflammatory cells at the inflammation sites, namely the release of pro-inflammatory mediators (*e.g.* elastase, interleukin-33 (IL-33), and mononcyte chemoattractant protein-1 (MCP-1/CCL2)) [38]. In addition, upon binding P2X7 receptor expressed on monocytes/macrophages, ATP activates the NLRP3 inflammasome, and as a consequence, promotes production of pro-inflammatory cytokine interleukin-1β (IL-1β) [243]. Truthfully, as above, ATP's effects are complex and occasionally contradictory; ATP is also reported to block the synthesis of pro-inflammatory cytokines interleukin-12 (IL-12) and TNF-α in macrophages [82].

Extracellular ATP in Adaptive Immunity

As DCs that trigger T-cell costimulation and specific immune-cell activation are key players linking the host innate and adaptive responses against cancer, we will discuss them in more detail. The danger signals released by activated, injured, or dying tumor cells – including ATP – are perceived by DCs to elicit an effective antitumor response (*e.g.* adjunctive vaccine effect). Indeed, ATP modulates DC maturation [244], migration, and function [123], and thus plays a critical role in signal amplification during T-cell activation [245-247]. Killed tumor-derived ATP acts on the P2X7 receptor present on DCs to evoke the NLRP3/ASC/caspase-1 inflammasome, thereby driving IL-1β production. IL-1β subsequently fosters the maturation of IFNγ-producing $CD8^+$ cytotoxic T lymphocytes (CTLs), conferring them with maximal cytotoxicity towards tumor cells. These novel findings support the potential for ATP-P2X7 signaling as a potent adjuvant in cancer chemotherapy treatment [240].

As recently shown using the murine experimental autoimmune encephalomyelitis (EAE), binding of IL-27 to its receptor on DCs limits Th1 and Th17 cell generation and EAE development [248]. This is mediated in large part through

induction of intrinsic CD39 expression/activity that impedes ATP-dependent NLRP3 inflammasome activation *via* clearance of pericellular ATP [249]. Additionally, IL-27 released by DCs upon exposure to supernatants of apoptotic cancer cells has the capacity to activate $CD39^+CD69^+$ Tregs thereby decreasing myelotoxicity [250]. On the contrary, ATP is also able to perturb DC maturation and capability of recruiting and evoking Th1 cell responses [80,81].

Furthermore, ATP can also directly drive Th-cell differentiation towards highly pro-inflammatory populations Th1 and Th17 [1], thus augmenting antitumor immunity. A subset of human Th17 cells, so called “supTh17”, can also display immune suppressive function *in vitro*. We recently found that, in contrast to "pathogenic" Th17 cells, supTh17 cells contain high levels of CD39 expression and enzymatic activity, capable of metabolizing exogenous ATP to adenosine thereby exerting immunosuppressive activity [251]. Additionally, ATP-P2X7 signaling further boosts antitumor immune responses by inducing Treg apoptosis [252,253].

Adenosinergic Modulation of Antitumor Immunity

As a potent immunosuppressant, through multiple P1 receptor signaling pathways, adenosine converts the inflammatory and immune cell functions to confer a hostile tumor microenvironment [21,147,254]. Adenosine generation is further heightened under hypoxic conditions, as in the necrotic center of proliferating tumors. As a key molecule maintaining oxygen homeostasis, HIF-1 is mainly responsible for hypoxia-driven anti-inflammatory responses [255]. Hypoxia seems to regulate CD73 expression, given that the CD73 gene promoter contains a binding site for the HIF-1 transcription factor [256]. Recently, HIF-1 has been implicated in modulation of adenosine production and subsequent downstream signals [255,257].

Adenosine in general, has suppressive effects on the host innate and adaptive immunity [258,259]. As Gs-coupled receptors, A2A and A2B play essential roles in adenosine-induced immunosuppression in a cAMP-dependent manner. Adenosine-A2A signaling blocks function of activated NK cells, the first line in the tumor immune surveillance program against malignant cells [260]. Beyond that, binding of adenosine to A2A and A2B could control adaptive immune responses in many ways: induction of tolerogenic DC differentiation and secretion of inhibitory cytokines such as interleukin-10 (IL-10) and TGF-β [261]; and potent suppression of effector T-cell proliferation, cytokine production, and cytotoxic activity [262-266]. In addition, A2A signaling also promotes intratumor immune-suppressive Treg accumulation [21,254]. A2B activation stimulates the expansion of another important immune suppressor myeloid-derived suppressor cells (MDSCs) and intrinsic release of angiogenic factors [21,254], ultimately facilitating tumor growth. Moreover, adenosine also skews T-cell differentiation towards Th type 2 (Th2) cells and Tregs that are thought to suppress antitumor immunity [267].

Cellular sources of adenosine are many and include tumor cells, immunosuppressor cells, and the tumor-vasculature, each coexpressing CD39 and CD73. In order to promote the antitumor responses, it is of great importance to inhibit adenosine production by way of blocking CD39 and/or CD73 [268]. $CD39^+CD73^+$ Tregs, that mediate potent immunosuppression operational *via* adenosine-mediated pathways [73], are upregulated in most human malignancies. NK cell-mediated antitumor functions, both *in vitro* and *in vivo*, are also susceptible to direct Treg suppression [269]. Many well-conducted studies have illustrated the role of tumor-infiltrating $CD39^+$ Tregs in promoting immunosuppression by suppressing NK tumor-killing activity and scavenging tumoricidal ATP while supplying adenosine [56,72]. Genetic deletion of CD39 is shown to result in defective angiogenesis and slower tumor growth in several syngenic murine cancer models, concurrent with substantively decreased migratory potential of inflammatory

cells [72,229,232,270]. Hence, blockade of CD39 entails favorable outcomes in cancer patients by restoring antitumor immune responses.

Invasion and Metastasis

Besides local growth, tumor cells often spread, termed "metastasis". Basically, metastasis is the dissemination of a cancer from its primary tissue/organ to other distant location(s) through circulation or direct seeding. It is a complex, multistep process and, as such, less than 1 in 10,000 cells that have "escaped" from the primary tumor could survive to colonize another organ to form a metastatic tumor. Effective tumor-host interactions are vital during these processes.

In order to attain invasive and metastatic characteristics, tumor cells must first acquire the ability to secrete cytokines, chemokines, and matrix degrading enzymes. These drivers of metastasis are either intrinsic such as genetic mutations or extrinsic *e.g.* hypoxia. It has been reported that breast cancer cells can be driven to extrude nucleoside diphosphate kinase (sNDPK-B), locally converting ADP to ATP. ATP then further activates endothelial P2Y receptor signaling thereby modulating blood flow and vascular permeability and ultimately initiating the metastatic process [206,271-273]. In cancer cells, ATP-P2Y2-mediated MAPK ERK1/2 and p38 signaling activates epithelial-mesenchymal transition (EMT)/invasion-related genes to facilitate migratory, invasive, and metastatic capacities of these cells [274-276]. Additionally, in ECs, synergy between P2Y2 and integrin signaling confers maximal adhesive activity for circulating tumor cells to adhere [277]. Moreover, tumor cells can stimulate platelets to release adenine nucleotides, which also act on P2Y2 receptor expressed by ECs to promote cancer cell survival and breach the endothelial barrier to allow cancer cell transendothelial migration [278]. P2Y1 activation mediates glioma cell mobility and migration through the alteration of intracellular Ca^{2+} levels suggesting yet another mechanistic link between activated purinergic receptor and functional responses [279]. These findings imply that pharmacological modulation of P2Y2- or P2Y1-dependent signaling pathways may serve as novel therapeutic options to lower aggressiveness and slow metastasis of tumors.

P2X7, too, has been linked to tumor metastasis in many cancer types. A pro-invasive role of P2X7 signaling has been suggested in breast cancer where it increases cell motility and extracellular matrix infiltration as well as levels of cathepsins [280,281]. In patients with papillary thyroid carcinoma (PTC), upregulated P2X7 expression was suggested as a biomarker predicting lymph node metastasis [282]. P2X7-mediated signaling also modulates migration of PC-9 human lung cancer cells [283].

Elevated expression and activity of CD39 and/or CD73 has also been linked to more aggressive clinical behaviors, higher tumor grade, and higher metastatic potentials of several cancer types [118,171,175,270,284-287]. Tumor cells coexpressing CD39/CD73 can generate adenosine that promotes metastatic behavior through A2A and A2B receptors. A2A blocks incoming antitumor T lymphocytes to protect cancer cells and facilitate tumor metastasis [265]. Some tumors are endowed with high adenosine generation capability and abundant expression of A2B. In these cells, adenosine-provoked A2B signaling would cause PKA pathway-mediated reductions in prenylation and cell membrane localization of Rap1B, thereby increasing cell scattering and invasive potential [288]. On the contrary, actions by A3 receptor intrinsically expressed on tumor cells are anti-metastatic. Interestingly, muscle cells have the capacity to secrete natural/endogenous A3 receptor agonists. These molecules are stable, non-hydrolyzable, and exert systemic anticancer and chemoprotective activity, underlying the scarcity of muscle metastases [153].

The specific role of CD39 in tumor invasion and metastasis has been extensively evaluated. Tumor cell adhesion has been linked to ecto-ATPDases, either directly *via* intrinsic enzymatic activity, or indirectly *via* both integrin-dependent and-independent adhesion pathways. Pharmacological inhibition of CD39 is shown to enhance the adhesive interactions between neutrophils and ECs [161]. Interactions of CD39 with adhesion molecules influence P2 receptor activation thereby regulating cell adhesion. In human ECs, ATP-evoked P2 receptor signaling enhances intracellular calcium levels. This would lead to subsequent cytoskeletal and cell contact rearrangements as well as phosphorylation of several integrin signaling components, including focal adhesionkinase (FAK), paxillin, and proline-rich tyrosine kinase 2 (Pyk2) [55]. Wild type ECs exhibit robust phosphorylation of FAK, whereas the integrin-mediated FAK activation is defective in CD39 null cells. As such, deletion of CD39 results in differential P2 receptor desensitization, which impedes the activity and function of integrins [229]. Another study showed that RanBPM forms a molecular complex with CD39 on cell membrane, with the potential to regulate ENTPDase catalytic activity [48]. RanBPM is also associated with integrin and modulates integrin function [289]. Furthermore, within the "immunological synapse", CD39 on DCs is essential for the maintenance of proper synergies among signaling transduction for integrins, ATP, and chemokines [71].

CD73 generally promotes tumor metastasis, possibly by stimulating chemotaxis. Indeed, resistance to melanoma metastasis is noted with CD73 null mice [268,290]. This phenomenon is thought to be as a result of CD73 loss in both hematopoietic and nonhematopoietic cells. CD73 deficiency might limit lymphocyte trafficking to the distant sites by decreasing their adhesive and migratory abilities to/through the endothelium that are dependent of CD73-generated adenosine [290]. However, severe combined immunodeficiency (SCID) mice still preserve these CD73-mediated pro-metastatic functions [291]. Moreover, intrinsic CD73 expression by tumor cells is also found to facilitate metastasis, yet the underlying molecular mechanism remains unclear [292].

In vivo mechanistic studies on tumor metastasis overall are very limited, owing to difficulties in examining the complex multievent metastatic process using a single animal model. For example, in humans, dissemination through the lymphatic system is an important feature of breast cancer biology. Tumor cells within lymph nodes provide a cell reservoir for future lethal metastases, and therefore, lymph node status in breast cancer patients is a critical factor for clinical decision-making. By contrast, although mouse models for breast cancer metastasis can be easily induced by tumor cell injection and xenograft transplantation [293,294], unfortunately, metastatic target sites in these models are always lungs. This discrepancy may be due to much less efficient lymphatic system in mice. Meanwhile, for human lung cancer, metastatic target organs include the other lung, adrenal glands, bones, and brain. However, in animal models of lung cancer metastasis, irrespective of injection method, metastasis often occurs in mediastinal lymph nodes and systemic metastases are extremely uncommon [295].

The molecular underpinnings of why and how tumors undergo metastases are not fully understood. Recent studies have highlighted the importance of tumor microenvironment, in which communication between tumor cells and the body system occurs, in tumor metastasis [296,297]. Specifically, inflammation and host immune responses that chiefly contribute to the metastatic process are critical aspects that athymic nude mouse models lack [296,297]. To date, cancer drugs targeting metastasis are still lacking, in a large part, due to lack of clinically relevant preclinical animal models for drug development (*e.g.* testing the efficacy, effectiveness, and safety), which is always imperative before clinical trials.

In the past decade, our laboratory has been devoted to the development of innovative small animal models for studying tumor metastases in distinct organs. As for melanoma and colon cancers, the most common initial site of tumor spread is

the liver, causing substantive morbidity and mortality in these patients. Our strategy is to dissect the multistep metastatic process using separate models by way of different injection/implantation routes. For example, we directly implant/inject mouse colon tumor tissues and dispersed cells into mouse colonic wall and into the liver *via* portal vein infusion (or splenic injection), respectively. We can then investigate cancer metastasis at different steps, *i.e.* how the primary cancer cells grow locally and invade into circulation, as well as how the circulating cancer cells colonize and expand at their target organ – liver. And also by engineering tumor cell lines to express bioluminescence markers, we can better tracing the metastatic cancer cells using state-of-the-art noninvasive imaging techniques for small animals. These models closely resemble human colon cancer metastases [72,270] (and unpublished data in Robson lab).

Utilizing these unique, clinically relevant metastasis animal models, we have delineated the pro-metastatic roles of CD39 expressed on the vasculature and immune cells. Jackson *et al* showed that loss of CD39 markedly blocks melanoma growth and metastasis to the lungs [229]. More recently, Sun and Wu *et al* demonstrated that metastases of melanoma and colon cancer cells in the liver are abrogated in CD39 null mice [72]. Multiple CD39-dependent mechanisms have been established, including defective EC functions, increased EC death, and enhanced tumor clearance activity of NK cells secondary to decreased Treg suppressive function [65,72,229,270]. In addition, the reduced metastatic behavior of tumor cells in CD39 null liver has also been attributed to impaired EC-tumor crosstalk and decreased adhesive and invasive activities, accompanied by lowered expression of adhesion molecules such as integrins and cadherins (unpublished data in Robson lab). These in-depth *in vivo* data manifest the striking anti-metastatic growth effects of CD39 blockade, suggesting a promising molecular target in cancer therapy.

Perspectives and Future Therapies

Carcinogenesis is a very complicated dynamic multi-event process composed of occasionally opposing multilevel interactions depending on the stage of cancer development. These include: i) Tumor-host interactions inclusive of reciprocal crosstalk, *viz.*, tumor cells release several signaling molecules including ATP which is simultaneously capable of enhancing VEGF levels to facilitate angiogenesis, promote cell proliferation and inflammation, allow for immune system escape, assist in cell matrix infiltration, invasion, and metastasis to distant sites and neovascularization [298,299] and; inversely, ECs can also produce growth factors and inflammatory cytokines driving tumor cell growth and inflammation. ii) Low level of chronic inflammation is known to drive early tumor angiogenesis in premalignant tissues by promoting malignant transformation and enables invasive behavior; acute inflammation, on the other hand is required for efficient clearance of malignant cells. These may underscore the paradoxical finding recently noted of increased susceptibility to autochthonous liver cancer formation in aged CD39 null mice [97].

Furthermore, purinergic mechanisms in cancer are complex, and sometimes exert opposing cellular functions at distinct cancer stages. Given the broad-spectrum expression of purinoreceptors in the body, multiple receptors expressed on different cell types might be simultaneously elicited by a given extracellular nucleotide or nucleoside. Thus it is difficult to determine the end result of these complex cellular responses using any *in vitro* systems or animal models that are genetically modified for purinergic receptors. Our unique CD39 mutant murine model has provided a powerful and useful tool for the study of purinergic modulation in cancer biology. Our investigations utilizing these CD39 mutant mice have affirmed CD39 as a potential target in cancer therapy.

THERAPEUTIC APPROACH AND POSSIBLE TARGETS

Experimental data indicate that activity/function of ectonucleotidases and/or purinergic receptors can be modulated by bioactive pharmacological compounds, blocking monoclonal antibodies (mAbs), or genetic modifications, thereby altering a given purinergic response. Purine-based drugs, either alone or incombination with currently available anticancer modalities, have been exploited using preclinical animal models.

In this section, we will highlight the discovery, development, and potential clinical applications of purine-based drugs in cancer therapy. We will also discuss recent advances targeting purinergic modulation as innovative cancer therapeutics and the hurdles that need to be overcome.

Targeting Pericellular Nucleotide/Nucleoside Levels in Cancer Therapy

Though several clinical trials have been employed to evaluate the benefits of direct supplementation of exogenous ATP alone in treating patients with non-small cell lung carcinoma (NSCLC) during 2001 to 2003, the detailed safety and pharmacokinetics and pharmacodynamics profiles as well as precise mechanisms of action still remain largely elusive. They showed that intravenous injection of ATP fails to cause tumor regression, but successfully mitigates a range of systemic symptoms associated with advanced malignancies including weight loss [126,300-302]. The underlying mechanism is thought to be ATP's ability to curb the loss of both metabolically active and inactive tissues. However, these early clinical trials have not been followed up in the past decade.

There are some serious limits to systematic infusion of ATP. First, extracellular ATP metabolism is such a dynamic process generating multiple intermediates that may exert distinct/opposing functions on many types of cells/tissues. Several approaches, including both *in vitro* luciferase assays and *in vivo* imaging of luciferase indicator mice, have been established to measure the levels and depositions of ATP and derived nucleotides/nucleosides [124,303]. Nevertheless, currently, there are no non-invasive tools for monitoring these important attributes after ATP infusion in the human body. These largely preclude dissecting the exact mechanisms of action by infused ATP in humans.

Second, as ATP concentrations in local extracellular microenvironment or in circulation must be constantly and precisely controlled and sustained, achieving consistent tumoricidal levels is very difficult. Even the desired final ATP concentrations are very low, dilution effect *via* delivery route to the designated tissues/organs must be considered. It is unclear what original concentration is needed to achieve and maintain the desired levels in the final destination for a certain time period that is sufficient to exert their optimal activity/function. For instance, the typical extracellular fluid volume in a human body averages around 15 liters including 3 liters of plasma. For an 1 nM increase in effective circulating volume (ECV), 100 milliliters of 150 mM of ATP are needed, which is ample to cause dramatic pathologic changes in circulation, such as platelet aggregation and thrombosis at the infusion vein.

Accordingly, to harness purinergic signaling for adjunctive cancer therapy, we must focus on the intrinsic mechanisms inclusive of ectonucleotidase expression/function as well as ATP release. Control of the local extracellular ATP-adenosine repertoire through regulating expression/activity of ectonucleotidases, especially the master switch molecule CD39, to ultimately govern the purinergic signals within the designated path, is the key to development of successful anticancer purinergic modalities that would greatly benefit cancer patients.

Targeting Purinergic Receptors in Cancer Therapy

The anticancer therapeutic effects of drugs targeting purinergic receptors have been widely explored. As a key pro-apoptosis modulator, P2X7-mediated apoptosis provides a novel pharmacological modality for cancer therapy. Induction of intrinsic tumor P2X7 expression leads to tumor cell death, suggestive of the therapeutic utility of P2X7 agonists in cancer treatments [104]. Conversely, P2X7 inhibition by antagonist brilliant blue G (BBG) promotes glioma cell growth [135]. More recently, high ATP levels are reported to induce tumor cell death *via* P2X7 integrated AMPK-PRAS40-mTOR and PI3K-AKT signaling pathways [65,127]. These well-delineated molecular mechanisms provide further rationales for the potential utility of purine-based drugs in management of cancer. To the contrary, P2X7 receptor antagonism, *e.g.* by emodin (1,3,8-trihydroxy-6-methylanthraquinone; priorly shown to contain anticancer properties) or the cholesterol-lowing drug atorvastatin, is able to attenuate ATP-driven invasive and metastatic potentials of human cancer cells [304-306]. In addition, several selective agonists to P2Y1 (*e.g.* methylthioadenosine-5-diphosphate (2-methylthio-ADP) and the ADP analogue MRS2365) and P2Y2, and an antagonist to P2Y12 have been shown to inhibit tumor growth [106,144,307,308]. However, explorations of pharmacological modulation of P2 receptors as innovative approaches or neoadjuvants for anticancer therapy have been limited to *in vitro* studies, largely lacking *in vivo* evidence.

In parallel, the potential therapeutic values of anti-adenosinergic modalities targeting P1 receptors have also been tested in treating cancer, in a large part, *via* destroying the immune evasion mechanisms, using preclinical animal models. Suppression of A2A receptor in antitumor T cells by antagonists such as caffeine, or by gene silencing using small interfering RNAs (siRNAs), markedly improves the effectiveness in blocking tumor growth, limiting metastases, and preventing neovascularization of these cells [265]. Inhibition of A2A and/or A2B receptors on $CD73^{+}$ tumor cells drastically reduced their metastatic potential, *via* both immunological and non-immunological mechanisms [309,310]. And also, blockade of A2B on DCs enhances DC activation and chemokine receptor CXCR3-dependent antitumor responses, thereby hindering growth of bladder and breast cancers [311]. Moreover, hypoxia is able to increase A2B expression on colon cancer cells, seemingly promoting cell growth. In turn, A2B is responsible for the release of an array of pro-angiogenic cytokines under hypoxic conditions [312]. Inactivation of the immunosuppressive A2A and A2B receptors (*via* genetic deletion or pharmacological blockade) can prevent hypoxia-mediated inhibition of tumor-infiltrating antitumor T cells, and resultantly lead to complete tumor rejection [265,313,314].

Selective agonists for A3 receptor, specifically CF101 and CF102, were found to enhance anti-inflammatory and anticancer responses in both xenograft and syngenic mouse models. Orally administered CF101 (also known as IB-MECA) efficaciously suppresses tumor growth at the primary sites as well as liver metastases of CT-26 colon cancer cells [315]. Oral administration of Cl-IB-MECA, a synthetic selective A3 agonist with high affinity, to melanoma-bearing mice substantially slows the development of tumor metastases in th lungs [316]. CF102 blocks HCC Hep3B cell growth [317], *via* deregulation of Wnt and NF-κB signaling pathways [154]. Truncated thio-Cl-IB-MECA, a novel A3 antagonist, has been shown to effectively inhibit both T24 bladder and lung cancer cell growth *via* induction of growth arrest and apoptosis [318,319].

Targeting Ectonucleotidases in Cancer Therapy

Blockade of CD39 and/or CD73 could improve targeted cancer therapies not only by specifically labeling overexpressed tumor antigens but also by blocking adenosine-dependent immune evasion in malignancies, providing novel anticancer

treatment options [320]. Several pharmacological inhibitors and blocking mAbs of ectonucleotidases have been developed and tested using both *in vitro* and *in vivo* experimentation systems. To name a few: the inhibitory compounds include ARL67156 (for CD39), polyoxometalate (POM)-1 (potent for CD39; much weaker for ENTPD3), POM-6 (for ENTPD2 and 3), PSB06126 (for ENTPD3), PSB069 (for ENTPD1-3), and adenosine 5'-(α, β-methylene)diphosphate (APCP; for CD73).

Inhibition of CD39 bioactivity using ARL67156 is capable of partially overcoming T cell hyporesponsiveness, thereby restoring antitumor immunity [262]. Amongst all these inhibitors, POM-1, a heavy metal, shows the highest selectivity for CD39 (with an IC50 at 2.6 μM) [321] and displays potent antitumor activity towards melanoma and colon cancers in a recent preclinical investigation [72]. The antitumor effect of POM-1 in CD39-expressing wild type mice was similar to that of CD39 deficiency. Moreover, administration of POM-1 in tumor-bearing CD39 null mice failed to further decrease metastatic tumor growth in the liver, suggesting that the tumor-killing actions of POM-1 in wild type mice were indeed mediated *via* specific inhibition of host CD39 functionality [72]. Minimal *in vivo* cytotoxicity was noted with the optimal short-term treatment strategy for POM-1 (daily intraperitoneal injection at 5 mg/kg for 10 days). In contrast, growth of hepatic metastases of MC-26 colon cancer cells was markedly increased in transgenic mice over-expressing CD39, as compared to wild type or CD39 null mice [270].

Immunotherapeutics targeting CD39 to boost antitumor immune responses have also been actively pursued. Two mAbs against human CD39 (clones BY40 and BA54G) have been recently developed by Bastid and colleagues. Both antibodies specifically bind human CD39 thereby efficiently blocking CD39 enzymatic activity [56]. The BY40 clone is able to restore $CD39^+$ Treg-suppressed CD4 and CD8 T cell proliferation *in vitro* [56], implicating their potential in re-establishing antitumor immunity. However, *in vivo* efficacy and safety of BY40 remains to be extensively tested in preclinical animal models. Although it fails to block ectonucleotidase activity *in vitro,* the 5F2 anti murine CD39 mAb produced in Robson laboratory exhibits potent anti-thrombotic effects *in vivo via* clearance of CD39 from cell surface by internalization (unpublished data in Robson lab). *In vivo* testing of this mAb clone in tumor models is of great interest and remains to be determined.

Owing to the availability of several blocking mAbs against both mouse and human CD73, many studies examined the impacts of CD73 inhibition on tumor biology in depth. CD73 blockade, by inhibitor APCP or mAbs effectively abrogates tumor growth and metastasis and is associated with increased survival of tumor-bearing mice [165,234,291,322-325]. Multiple underlying molecular mechanisms have been suggested: i) enhanced tumor antigen-specific T-cell homing to the tumors; ii) decreased apoptosis of and increased expansion of tumor-specific T cells; iii) enhanced intratumor Th1 and Th17 cell-associated cytokine release and; iv) reduced intratumor VEGF levels and impaired tumor angiogenesis. In addition, in animal models bearing subcutaneous MC38-OVA colon and RM-1 prostate cancers, antibody blockade of CD73 enhances the therapeutic efficiency of immunotherapies targeting immune checkpoint regulators programmed death (PD)-1 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) [326].

Stagg and Smyth further exploited the benefit of co-targeting the CD39-CD73-adenosine pathway in cancer therapy [21]. Concurrent blockade of CD39 and CD73 *via* mAbs (A1 for CD39 and 7G2 for CD73) substantially boosts the anticancer activity towards ovarian cancer (OvCA), principally by mitigating adenosine-mediated immune evasion mechanisms [320]. More recently, Hausler *et al* showed, in human primary ovarian cancer cells and cell lines, that inhibition of CD39 and/or CD73 using siRNAs or small-molecule inhibitors drives $CD4^+$ T cell proliferation and augments lytic activities of

NK cells and cytotoxic T lymphocytes [327]. These preclinical studies have established the proof-of-concept for anticancer therapeutic strategies targeting CD39 and/or CD73.

In summary, CD39 and/or CD73 insinuate novel therapeutic targets in broad-spectrum cancer types, with the aptitude to augment the treatment efficacy of other (conventional and/or emerging) therapies. Targeting CD39 holds greater promise in so far as CD39: i) tightly governs the very first step of extracellular ATP metabolism; ii) is the master control node for ATP signaling transduction and; iii) is an upstream regulator of CD73 function; thereby dictating the fate of cells exposed to alterations in extracellular ATP levels. Steering the tumor microenvironment to maintain high levels of ATP while preventing their phosphohydrolysis to immune-suppressive adenosine would augment antitumor activity. Specifically, blockade of CD39 on the host immune compartment and the bulk of the tumor would effectively hinder growth of metastatic tumors by ultimately conferring an optimal antitumor microenvironment. Collectively, the molecular underpinnings of the primary anticancer role of CD39 blockade thus far include: i) inhibiting tumor angiogenesis and motility; ii) limiting local immunosuppressant adenosine generation by $CD39^+$ cells (*e.g.* immune suppressor and tumor cells and tumor-associated vasculatures); 3) culminating high ATP levels that could directly kill tumor cells and associated ECs, induce Treg apoptosis, enhance cytotoxicity of DC-primed tumor specific CD8 CTLs, increase phagocytosis by functioning as a 'find me" signal, as well as skew Th cells towards the antitumor lineages Th1 and Th17.

Challenges with Clinical Applications of Purinergic Modulation in Cancer Therapy

Cancer is a very complex disease. Like any anticancer therapeutic approaches, purine-based drugs, inclusive of molecular compounds and monoclonal blockade antibodies, would encounter many hurdles that need to be circumvented to achieve optimal beneficial effects and to be successfully translated into the clinic.

Drug Selectivity and Specificity

The conventional therapeutic agents (chemo or immnuo) for cancer treatments, albeit valuable, have faced many problems. These problems include unsatisfactory specificity and efficacy, intrinsic toxicity, and development of resistance to drugs (intrinsic and acquired). Specificity and efficacy are key factors in cancer therapy. Non-selectivity and non-specificity, resulting in severe adverse effects, acute or chronic, associated with morbidities, largely limit cancer treatment outcomes. The activity of virtually all clinical chemotherapeutic agents is circumscribed by their non-selective cytotoxicity towards both proliferating cells (inclusive of cancer and activated antitumor immune cells) and normal cells. Cancer-killing compounds with distinct mechanisms of action have been developed for widespread clinical use, *e.g.* paclitaxel, trastuzumab, and taxane. However, majority of these drugs are associated with unacceptable off-targets and thus have not been clinically useful, mainly owing to their lack of cancer selectivity. As to the synthetic purine-based drugs, the selectivity and specificity are also problematic, limiting their potential clinical applications. As an example, ARL67156, the synthesized ATP analogue that is considered to selectively inhibit ecto-ATPase, has been shown to exert weaker antagonism for P2X and P2Y12 receptors. POM-1, the most selective CD39 inhibitor up to date, also exhibits inhibitory activity towards ENTPD3, albeit with much less potency. Moreover, elevated cytotoxicity has been observed with long-term usage of these inhibitory compounds, limiting their clinical utility.

Furthermore, due to the important roles of purinergic signals in physiological processes such as maintenance of homeostasis, systemic blockade of purinergic signaling may disrupt multiple biological processes, thereby tipping off the internal balance of cells that will cause further pathologic alterations in the body. For example, short-term blockade of

CD39 would efficiently inhibit metastatic tumor growth *via* elicitation of a series of antitumor mechanisms, as alluded above. However, long-term loss of CD39 function would lead to spontaneous formation of liver cancer in mice [97]. Therefore, targeted delivery of purine-based drugs is needed to circumvent these off-target effects, thereby achieving optimal anticancer outcomes.

Several novel and attractive targeted delivery strategies have been developed, *e.g.* direct intratumor injection of peptides for antigen-specific cancer immunotherapy [328]. This type of therapy only enhances tumor specific adaptive immune reactions but autoimmunity. Radiosensitizing chemotherapy is another novel approach that has been shown to improve the outcomes in comparison to radiation alone. The delivery of radiation therapy with yttrium90 (90Y) radioembolization, in combination with a radiosensitizing chemotherapeutic agent, provides the opportunity to enhance radiation effects on hepatic malignancies [329]. ATP has been shown to act synergistically with radiotherapy. Cotreatment of ATP with radiotherapy increases cell death of human glioma cells, when compared to ATP or radiotherapy treatment alone [129]. Some advantages exist for delivering drugs in a controlled manner. Anticancer agents can accumulate in the targeted tumor site, lowering drug doses. In addition, nanostructures, including magnetic nanoparticles, liposomes, and polymers, have been shown to improve drug efficiency by specifically targeting cancer cells [330,331]. Moreover, multiple surface markers provide a more efficient way to selectively detect cancer cells. Such cancer cell-specific strategies are anticipated to have enhanced selectivity against tumor cells [332].

Hence, mAbs that bind specific tumor cell surface markers exhibit less toxicity in cancer therapy. In the past decade, the utility of mAbs targeting tumor antigens as an alternative anticancer approach has been actively exploited, entailing great promise, *i.e.* mAbs targeting EGFR, HER2/Neu, VEGF, CTLA-4, CD20, CD30, and CD52 [333]. Recently, potent anticancer effect of immunomodulatory antibodies or antibody-conjugated drugs has been noted in cancer clinic. Preclinical assessments of blocking mAbs against CD39 have shown promising immunotherapeutic activity against cancer. However, difficulties with production of CD39 blocking mAbs have been noted. The possible explanations for this may be the following: i) CD39 activity is critical for B cell expansion and function; ii) extracellular domains of CD39 are highly conserved and are not immunogenic and; iii) optimal CD39 activity/function is conferred by formation of dimers or tetramers through the transmembrane regions [334], which is such a dynamic process making CD39 polymers as moving objects that are extremely difficult to target. An alternative strategy would be to generate mAbs that can clear CD39 from the cell surface, as shown by Terp *et al* that mAbs bind CD73 can result in internalization of the proteins [87].

Yet, even with superb CD39 blocking/clearing mAbs, another concern regarding clinical applications is how to specifically block tumor-associated CD39 without off-target endothelial effects, given its ubiquitous expression on normal vasculatures. Theoretically, disruption of vascular CD39 would be possibly linked to clotting defects, cardiac vasculature dysfunction, and fibrosis. Whether any unforeseen toxicity is associated with saturated CD39 mAbs remains to be extensively evaluated using preclinical cancer models.

Thus far, preclinical anticancer immunotherapeutic evaluations of CD39 and CD73 mAbs have mostly been carried out in cancer cell lines and in small animal models and are proven to be novel propitious approaches in cancer therapy. Precise delineation of the mechanisms of action and cautious assessments of safety for any given purine-based drugs using clinically relevant animal models are absolute requisite before their clinical translation. This would also greatly benefit from further optimization and personalization of treatment strategies that are largely dependent upon tumor

stages *e.g.* early (with local tumors) *vs.* advanced (with metastases), tumor types, and optimistically the patient's genetic and epigenetic information.

Drug Resistance

Cancer cells often develop insensitivity/resistance to targeted therapies. Drug insensitivity/resistance, either *de novo* or acquired, often leads to recurrence and relapse of treated cancer patients and is another major concern in the treatment of metastatic cancers. A better understanding of the underlying mechanisms prior to clinical trials is the key to improve drugs to overcome drug resistance. Intratumoral heterogeneity is a general trait of most tumors. Intrinsic resistance may activate a subpopulation of tumor cells *e.g.* cancer stem cells (CSCs), leading to recurrence after treatment [335]. The underpinnings for acquired resistance include genetic and epigenetic alternations of crucial genes in cancer cells, induction of immune suppressor cell expression/function, as well as activation of compensatory oncogenic signaling pathways due to disruption of the negative feedback mechanisms [336]. These are underscored by the findings that high dose IL-2 therapy in melanoma patients induces expansion and expression of CD39, CD73, and TGF-β of the inducible T cell costimulator $(ICOS)^+$ Tregs, causing rapid disease progression in non-responders [337]; and that metastases quickly develop in breast cancer patients lacking a P2X7 function allele, following anthracycline treatment [17,338].

It is well accepted in the cancer drug discovery field that one drug is never enough. Though combination therapies have been actively pursued and used in oncology clinics to enhance antitumor activity and prevent resistance, the clinical outcomes are still disappointing. Ideally, culminating drug efficacy of any targeted cancer therapy while obviating emergence of resistance, integration of major antitumor mechanisms would be necessitated. A good proof-of-principle study in this regard has recently been conducted by Dr. Yu's laboratory [339]. In trastuzumab-resistant breast cancer, maximal therapeutic activity is noted with multi-targeted combinational therapies: inhibiting tumor cells by an AKT inhibitor, and concomitantly augmenting antitumor T cell responses *via* mAb blockade of HER2/Neu and CTLA-4. With regard to purinergic modulation, theoretically, upon induction of ATP release (as by chemotherapy and/or radiotherapy), solo blockade of CD39 would achieve maximal antitumor efficacy: elicitation of antitumor immunity, enhancement of direct tumor-killing activity, and inhibition of tumor angiogenesis, *via* actions on distinct cell types including immune cells, tumor cells, and tumor-associated ECs.

Another new therapeutic avenue against cancer while overcoming drug resistance is targeting cancer stemness. As an intrinsic mechanism for development of resistance, these highly tumorigenic CSCs have been shown to play an essential role in cancer growth, metastasis, and resistance. CSCs are a unique subpopulation of tumor cells that can generate all types of cells in the tumor mass through the self-renewal process. CSCs are generally refractory to conventional cancer drugs that directly disrupt tumor cell growth. Targeting CSC pathways have the potential to kill not only CSCs but also the bulk of the tumor. Several CSC pathway inhibitors, developed by Boston Biomedical (www.bostonbiomedical.com), that target both CSCs and non-stem cancer cells have shown potent antitumor and anti-metastatic activity in a wide range of cancer types in preclinical studies. Multiple ongoing clinical trials testing these drugs in treating patients with solid tumors, alone or in combination with standard-of-care therapies, have entered different phases. CD39 is recently identified as a novel marker for hematopoietic stem cells (HSCs) in both mice and humans [340]. Expression and enzymatic activity of CD39 confer HSCs' mobilization and functions, *e.g.* limiting inflammation and promoting regeneration after liver resection. Role of CD39 in cancer stem cell biology is of great interest and importance and thus remains to be dissected.

Lack of Clinical Relevant Animal Models and Robust Preclinical Translational Approaches

The factor of animal models to completely recapitulate the full-spectrum of pathogenesis of human cancer is well established. However, owing to the complexity of carcinogenesis, ideal animal models for any given type of cancer are not available. Clinically relevant animal models provide an alternative strategy for preclinical development and testing of cancer drugs. Up to now, therapeutic evaluations of purine-based drugs have mostly been based on cancer cell lines and xenograft models. It has increasingly been acknowledged that most cancer drugs developed on the basis of cell culture and xenograft studies have not translated well into the clinic. Alternatively, genetically engineered mouse (GEM) models provide better options. Well-conducted cancer research studies using the global CD39 null mice have unraveled multiple critical purinergic mechanisms underpinning pathogenesis of malignancy, which are seminal contributions to the cancer drug discovery field. The recent generation of floxed CD39 mice would be valuable tools to dissect the discrete roles of CD39 and related purinergic signaling in distinct types of cell responses during multi-event tumorigenesis (unpublished data in Robson lab).

Another key element that has delayed cancer drug discovery is the lack of a robust platform for preclinical translational screening and testing. It is urgently required to develop robotic automation systems for large-scale combination screening to expedite the discovery of highly effective drug combinations and predict cell responses to drugs before clinical testing. Recently, Jaffe *et al* has demonstrated that global chromatin profiling using a high-information-content mass spectrometry approach is efficacious in identifying novel therapeutic target for acute lymphoblastic leukemia [341]. In addition, system biology utilizing computational models might also provide useful clues to the dynamics of a given oncogenic pathway component in response to cancer drugs [342]. We have also established a mathematical model to simulate how cells respond to various stimuli and/or pharmacological inhibitors. We note that this modeling system could accurately and robustly predict what the final biological consequences of different treatments, alone or in combination, on a given cell type would be (manuscript in preparation by Robson lab). None of the conventional *in vitro* culture systems has such powerful capacity. Moreover, with recent advances in next-generation sequencing and computer technology, multi-omics analyses (inclusive of genomics, transcriptomics, proteomics, and metabolomics) have been substantiated as useful tools for stratification and diagnosis and prognosis of cancer patients [343]. The magnitude of multi-omics science in personalized cancer medicine is further highlighted by the findings that genetic variations exist that control expression levels of CD39 [344] and P2X7 [345].

Taken together, most studies with regard to purinergic modulation have hitherto focused on tumor-associated immune responses. Nevertheless, nucleotides and nucleosides have exhibited strong direct effects on tumor cells per se, which implies another valuable approach in cancer therapy. The potentials of P2 and P1 receptor antagonists as anticancer drugs have opened favorable perspectives for the development of innovative purine-based therapeutics to treat cancer. Similarly, these purinergic receptor inhibitors also face many problems, as alluded for CD39 and CD73 above, *e.g.* lacking tumor specificity. The efficaciousness of purine-based therapeutic approaches targeting activity/function of ectonucleotidases, P2, or P1 receptors, alone or in combination with immune-stimulatory cytokines, immune cell therapies, cancer vaccines, or traditional or state-of-art chemotherapy and/or radiotherapy, remain to be extensively evaluated.

CONCLUSION AND FUTURE DIRECTIONS

Cancer is a complicated disease for which significant therapeutic advances require personalized medicine. In the past decade, identification and characterization of extracellular nucleotides and nucleosides in the tumor microenvironment as well as related signal elicitation in distinct cancer cell types have been important in advancing our knowledge of cancer. Understanding unique purinergic mechanisms underlying the pathogenesis of malignancy is fundamental for discovery and development of effective drugs to treat cancer. The molecular underpinnings of inflammation and immunity are of paramount importance. Approaches that limit or resolve chronic inflammation and augment antitumor immune responses might provide potent anticancer therapeutics precluding cancer development and progression.

Pivotal roles of ectoenzyme-modulated purinerigic signaling have been established in virtually all aspects of tumor biology, notably tumor growth and metabolism, angiogenesis, inflammation, immunity, and metastasis. A major effort should be made to develop agents targeting ectoenzymes (specifically CD39) and purinergic mechanisms. Conceptually, together with chemotherapy and/or radiotherapy, this innovative therapeutic strategy has the capacity to concomitantly target distinct tumor biology, *viz.*, inhibition of angiogenesis, augmentation of antitumor immunity, and direct cytotoxicity towards tumor cells; in such a way that confers an immune-friendly tumor microenvironment, thereby achieving maximal anticancer activity (as illustrated in Fig.**1**).

Purinergic signaling plays distinct roles in tumor biology that is chiefly dependent upon context, *i.e.*, transplanted *vs.* autochthonous tumors; acute *vs.* chronic inflammation and; levels of and balance between pericellular nucleotides and nucleosides. It is noteworthy that, akin to several other key regulators of inflammation and angiogenesis *e.g.* NF-κB and DNA-binding protein inhibitor ID-1 [346,347], purinergic modulation also has dimorphic impacts according to the nature of the tumor biology targeted and the kinetics of responses observed. Hence, accurate cancer patient stratification and well-tailored individualized treatment strategy will be largely required for clinical applications of purine-based drugs to treat patients in a personalized manner.

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CONFLICT OF INTEREST

The authors disclose there is no conflict of interest.

Table 1: P2 receptors and impact on cancer cell growth.

Receptor	Cancer type	Cell line	Impact on cell growth	References
P2X5	Prostate cancer	PC-3	↓	[139]
	Bladder cancer	HT-1376	↓	[105]
P2X7	Colon cancer	MCA38, HCT8	↓	[65, 106,127,141,142]
	Melanoma	B16/F10, A375	↓	[65, 106,127,141,142]
	Squamous cell carcinoma	A431	↓	[128]
	Glioma	U-138MG, U-251MG	↓	[129,135]
	Cervical cancer	hECE, CaSki	↓	[130]
	Endometrial cancer	Tissue only	↓	[131]
	Prostate cancer	PC-3	↓	[139]
	Bladder cancer	HT-1376	↓	[105]
	Breast cancer	MDA-MB-231, MCF-7	↓	[132]
P2Y1	Melanoma	A375	↓	[106,141,142]
	Colon cancer	HCT8	↓	[106,141,142]
	Thyroid cancer	ARO	↑	[103]
P2Y2	Melanoma	A375	↓	[106]
	Colon cancer	HCT8, HT29	↓	[106,141,142,143]
	Oesophageal cancer	Kyse-140	↓	[144]
	Prostate cancer	HT-1376	↑	[105]
	Melanoma	A375	↑	[106]
	Squamous cell carcinoma	A431	↑	[107]
	Breast cancer	MCF-7	↑	[108]
	Lung cancer	A549	↑	[109]
	Breast cancer	MCF-7	↑	[110]
P2Y6	Lung cancer	A549	↑	[109,112]
	Astrocytoma	132N1	↑	[113]
P2Y11	Prostate cancer	PC-3	↓	[139]
	Bladder cancer	HT-1376	↓	[105]
P2Y12	Lung cancer	A549	↓	[111]

Note: ↓: Tumor-killing function. ↑: Tumor-promoting function.

Table 2: P1 adenosine receptors and impact on cancer cell growth.

Receptor	Cancer type	Cell line	Impact on cell growth	References
A1	Colon cancer	CW2	↓	[148]
	Glioblastoma	Gl261	↓	[149]
	Breast cancer	MDA-MB-468	↑	[119]
	Colon cancer	HT-29	↑	[120]
A2A	Lung adenocarcinoma	PC9	↑	[115]
	Pancreatic cancer	BON-1	↑	[116]
	Intestinal cancer	KRJ-I	↑	[116]
	Prostate cancer	PC-3, DU145, and LNCaP	↑	[117]
	Colon cancer	Caco-2	↓	[118,145]
	Hepatoma	HepG2	↓	[145]
	Multiple myeloma	MM.1S	↓	[146]
	Lymphoma	SU-DHL6	↓	[146]
A2B	Lung adenocarcinoma	PC9	↑	[115]
	Pancreatic cancer	BON-1	↑	[116]
	Intestinal cancer	KRJ-I	↑	[116]
	Prostate cancer	PC-3	↑	[117]
A3	Lymphoma	Nb2-11C	↓	[150]
	Melanoma	A375	↓	[151]
	Prostate cancer	DU-145, PC3, LNcap-FGC10	↓	[152]
	Colon cancer	HCT-116	↓	[153]
	Melanoma	B16-F10	↓	[153]
	Hepatoma	N1S1	↓	[154]

Note: ↓: Tumor-killing function. ↑: Tumor-promoting function.

REFERENCES

[1] Trautmann A. Extracellular ATP in the immune system: more than just a "danger signal". Sci Signal 2009; 2: pe6.

[2] Burnstock G, Campbell G, Satchell D, Smythe A. Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. Br J Pharmacol 1970; 40: 668-88.

[3] Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998; 50: 413-92.

[4] Eltzschig H K, Sitkovsky M V, Robson S C. Purinergic signaling during inflammation. N Engl J Med 2012; 367: 2322-33.

[5] Save S, Persson K. Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract. Infect Immun 2010; 78: 3609-15.

[6] MacDonald P E, Braun M, Galvanovskis J, Rorsman P. Release of small transmitters through kiss-and-run fusion pores in rat pancreatic beta cells. Cell Metab 2006; 4: 283-90.

[7] Zhang Z, Chen G, Zhou W, Song A, Xu T, Luo Q, Wang W, Gu X S, Duan S. Regulated ATP release from astrocytes through lysosome exocytosis. Nat Cell Biol 2007; 9: 945-53.

[8] Burnstock G. Physiology and pathophysiology of purinergic neurotransmission. Physiol Rev 2007; 87: 659-797.

[9] Gatof D, Kilic G, Fitz J G. Vesicular exocytosis contributes to volume-sensitive ATP release in biliary cells. Am J Physiol Gastrointest Liver Physiol 2004; 286: G538-46.

[10] Kang J, Kang N, Lovatt D, Torres A, Zhao Z, Lin J, Nedergaard M. Connexin 43 hemichannels are permeable to ATP. J Neurosci 2008; 28: 4702-11.

[11] Huang Y J, Maruyama Y, Dvoryanchikov G, Pereira E, Chaudhari N, Roper S D. The role of pannexin 1 hemichannels in ATP release and cell-cell communication in mouse taste buds. Proc Natl Acad Sci U S A 2007; 104: 6436-41.

[12] Liu H T, Toychiev A H, Takahashi N, Sabirov R ZOkada Y. Maxi-anion channel as a candidate pathway for osmosensitive ATP release from mouse astrocytes in primary culture. Cell Res 2008; 18: 558-65.

[13] Koyama T, Kimura C, Hayashi M, Watanabe M, Karashima Y, Oike M. Hypergravity induces ATP release and actin reorganization via tyrosine phosphorylation and RhoA activation in bovine endothelial cells. Pflugers Arch 2009; 457: 711-9.

[14] Suadicani S O, Brosnan C F, Scemes E. P2X7 receptors mediate ATP release and amplification of astrocytic intercellular Ca2+ signaling. J Neurosci 2006; 26: 1378-85.

[15] Eltzschig H K. Adenosine: an old drug newly discovered. Anesthesiology 2009; 111: 904-15.

[16] Selzner N, Selzner M, Graf R, Ungethuem U, Fitz J G, Clavien P A. Water induces autocrine stimulation of tumor cell killing through ATP release and P2 receptor binding. Cell Death Differ 2004; 11 Suppl 2: S172-80.

[17] Ghiringhelli F, Apetoh L, Tesniere A, Aymeric L, Ma Y, Ortiz C, Vermaelen K, Panaretakis T, Mignot G, Ullrich E, Perfettini J L, Schlemmer F, Tasdemir E, Uhl M, Genin P, Civas A, Ryffel B, Kanellopoulos J, Tschopp J, Andre F, Lidereau R, McLaughlin N M, Haynes N M, Smyth M J, Kroemer G, Zitvogel L. Activation of the NLRP3 inflammasome in dendritic cells induces IL-1beta-dependent adaptive immunity against tumors. Nat Med 2009; 15: 1170-8.

[18] Seminario-Vidal L, Lazarowski E R, Okada S F. Assessment of extracellular ATP concentrations. Methods Mol Biol 2009; 574: 25-36.

[19] Burnstock G. Historical review: ATP as a neurotransmitter. Trends Pharmacol Sci 2006; 27: 166-76.

[20] Burnstock G, Kennedy C. Is there a basis for distinguishing two types of P2-purinoceptor? Gen Pharmacol 1985; 16: 433-40.

[21] Stagg J, Smyth M J. Extracellular adenosine triphosphate and adenosine in ca7ncer. Oncogene 2010; 29: 5346-58.

[22] Volonte C, Amadio S, D'Ambrosi N, Colpi M, Burnstock G. P2 receptor web: complexity and fine-tuning. Pharmacol Ther 2006; 112: 264-80.

[23] Khakh B S, North R A. P2X receptors as cell-surface ATP sensors in health and disease. Nature 2006; 442: 527-32.

[24] Vaughn B P, Robson S C, Burnstock G. Pathological roles of purinergic signaling in the liver. J Hepatol 2012; 57: 916-20.

[25] Mulryan K, Gitterman D P, Lewis C J, Vial C, Leckie B J, Cobb A L, Brown J E, Conley E C, Buell G, Pritchard C A, Evans R J. Reduced vas deferens contraction and male infertility in mice lacking P2X1 receptors. Nature 2000; 403: 86-9.

[26] North R A. Molecular physiology of P2X receptors. Physiol Rev 2002; 82: 1013-67.

[27] Bleehen T, Keele C A. Observations on the algogenic actions of adenosine compounds on the human blister base preparation. Pain 1977; 3: 367-77.

[28] Hamilton S G, Warburton J, Bhattacharjee A, Ward J, McMahon S B. ATP in human skin elicits a dose-related pain response which is potentiated under conditions of hyperalgesia. Brain 2000; 123 (Pt 6): 1238-46.

[29] Lewis C, Neidhart S, Holy C, North R A, Buell G, Surprenant A. Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. Nature 1995; 377: 432-5.

[30] Allsopp R C, Lalo U, Evans R J. Lipid raft association and cholesterol sensitivity of P2X1-4 receptors for ATP: chimeras and point mutants identify intracellular amino-terminal residues involved in lipid regulation of P2X1 receptors. J Biol Chem 2010; 285: 32770-7.

[31] Garcia-Marcos M, Perez-Andres E, Tandel S, Fontanils U, Kumps A, Kabre E, Gomez-Munoz A, Marino A, Dehaye J P, Pochet S. Coupling of two pools of P2X7 receptors to distinct intracellular signaling pathways in rat submandibular gland. J Lipid Res 2006; 47: 705-14.

[32] Barth K, Weinhold K, Guenther A, Linge A, Gereke M, Kasper M. Characterization of the molecular interaction between caveolin-1 and the P2X receptors 4 and 7 in E10 mouse lung alveolar epithelial cells. Int J Biochem Cell Biol 2008; 40: 2230-9.

[33] Gonnord P, Delarasse C, Auger R, Benihoud K, Prigent M, Cuif M H, Lamaze C, Kanellopoulos J M. Palmitoylation of the P2X7 receptor, an ATP-gated channel, controls its expression and association with lipid rafts. FASEB J 2009; 23: 795-805.

[34] Deaglio S, Robson S C. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. Adv Pharmacol 2011; 61: 301-32.

[35] Abbracchio M P, Burnstock G, Verkhratsky A, Zimmermann H. Purinergic signalling in the nervous system: an overview. Trends Neurosci 2009; 32: 19-29.

[36] Hollopeter G, Jantzen H M, Vincent D, Li G, England L, Ramakrishnan V, Yang R B, Nurden P, Nurden A, Julius D, Conley P B. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature 2001; 409: 202-7.

[37] Marteau F, Le Poul E, Communi D, Communi D, Labouret C, Savi P, Boeynaems J M, Gonzalez N S. Pharmacological characterization of the human P2Y13 receptor. Mol Pharmacol 2003; 64: 104-12.

[38] Rayah A, Kanellopoulos J M, Di Virgilio F. P2 receptors and immunity. Microbes Infect 2012; 14: 1254-62.

[39] Lammer A B, Beck A, Grummich B, Forschler A, Krugel T, Kahn T, Schneider D, Illes P, Franke H, Krugel U. The P2 receptor antagonist PPADS supports recovery from experimental stroke in vivo. PLoS One 2011; 6: e19983.

[40] Jacobson K A, Boeynaems J M. P2Y nucleotide receptors: promise of therapeutic applications. Drug Discov Today 2010; 15: 570-8.

[41] Klinger M, Freissmuth M, Nanoff C. Adenosine receptors: G protein-mediated signalling and the role of accessory proteins. Cell Signal 2002; 14: 99-108.

[42] Fishman P, Bar-Yehuda S, Synowitz M, Powell J D, Klotz K N, Gessi S, Borea P A. Adenosine receptors and cancer. Handb Exp Pharmacol 2009; 399-441.

[43] Ohana G, Bar-Yehuda S, Barer F, Fishman P. Differential effect of adenosine on tumor and normal cell growth: focus on the A3 adenosine receptor. J Cell Physiol 2001; 186: 19-23.

[44] Hoskin D W, Mader J S, Furlong S J, Conrad D M, Blay J. Inhibition of T cell and natural killer cell function by adenosine and its contribution to immune evasion by tumor cells (Review). Int J Oncol 2008; 32: 527-35.

[45] Chen J F, Eltzschig H K, Fredholm B B. Adenosine receptors as drug targets--what are the challenges? Nat Rev Drug Discov 2013; 12: 265-86.

[46] Banz Y, Beldi G, Wu Y, Atkinson B, Usheva A, Robson S C. CD39 is incorporated into plasma microparticles where it maintains functional properties and impacts endothelial activation. Br J Haematol 2008; 142: 627-37.

[47] Robson S C, Wu Y, Sun X, Knosalla C, Dwyer K, Enjyoji K. Ectonucleotidases of CD39 family modulate vascular inflammation and thrombosis in transplantation. Semin Thromb Hemost 2005; 31: 217-33.

[48] Wu Y, Sun X, Kaczmarek E, Dwyer K M, Bianchi E, Usheva A, Robson S C. RanBPM associates with CD39 and modulates ecto-nucleotidase activity. Biochem J 2006; 396: 23-30.

[49] Heine P, Braun N, Sevigny J, Robson S C, Servos J, Zimmermann H. The C-terminal cysteine-rich region dictates specific catalytic properties in chimeras of the ectonucleotidases NTPDase1 and NTPDase2. Eur J Biochem 2001; 268: 364-73.

[50] Grinthal A, Guidotti G. Dynamic motions of CD39 transmembrane domains regulate and are regulated by the enzymatic active site. Biochemistry 2004; 43: 13849-58.

[51] Kukulski F, Levesque S A, Lavoie E G, Lecka J, Bigonnesse F, Knowles A F, Robson S C, Kirley T L, Sevigny J. Comparative hydrolysis of P2 receptor agonists by NTPDases 1, 2, 3 and 8. Purinergic Signal 2005; 1: 193-204.

[52] Bigonnesse F, Levesque S A, Kukulski F, Lecka J, Robson S C, Fernandes M J, Sevigny J. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. Biochemistry 2004; 43: 5511-9.

[53] Lavoie E G, Kukulski F, Levesque S A, Lecka J, Sevigny J. Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-3. Biochem Pharmacol 2004; 67: 1917-26.

[54] Vorhoff T, Zimmermann H, Pelletier J, Sevigny J, Braun N. Cloning and characterization of the ecto-nucleotidase NTPDase3 from rat brain: Predicted secondary structure and relation to other members of the E-NTPDase family and actin. Purinergic Signal 2005; 1: 259-70.

[55] Robson S C, Sevigny J, Zimmermann H. The E-NTPDase family of ectonucleotidases: Structure function relationships and pathophysiological significance. Purinergic Signal 2006; 2: 409-30.

[56] Bastid J, Cottalorda-Regairaz A, Alberici G, Bonnefoy N, Eliaou J F, Bensussan A. ENTPD1/CD39 is a promising therapeutic target in oncology. Oncogene 2013; 32: 1743-51.

[57] Ghiringhelli F, Bruchard M, Chalmin F, Rebe C. Production of adenosine by ectonucleotidases: a key factor in tumor immunoescape. J Biomed Biotechnol 2012; 2012: 473712.

[58] Camici M, Fini C, Ipata P L. Isolation and kinetic properties of 5'-nucleotidase from guinea-pig skeletal muscle. Biochim Biophys Acta 1985; 840: 6-12.

[59] Zimmermann H. 5'-Nucleotidase: molecular structure and functional aspects. Biochem J 1992; 285 (Pt 2): 345-65.

[60] Gordon E L, Pearson J D, Slakey L L. The hydrolysis of extracellular adenine nucleotides by cultured endothelial cells from pig aorta. Feed-forward inhibition of adenosine production at the cell surface. J Biol Chem 1986; 261: 15496-507.

[61] Robson S C. Role of CD73 and extracellular adenosine in disease : Presented by Maria P. Abbracchio. Purinergic Signal 2011; 7: 367-72.

[62] Visovatti S H, Hyman M C, Bouis D, Neubig R, McLaughlin V V, Pinsky D J. Increased CD39 nucleotidase activity on microparticles from patients with idiopathic pulmonary arterial hypertension. PLoS One 2012; 7: e40829.

[63] Sauer A V, Brigida I, Carriglio N, Hernandez R J, Scaramuzza S, Clavenna D, Sanvito F, Poliani P L, Gagliani N, Carlucci F, Tabucchi A, Roncarolo M G, Traggiai E, Villa A, Aiuti A. Alterations in the adenosine metabolism and CD39/CD73 adenosinergic machinery cause loss of Treg cell function and autoimmunity in ADA-deficient SCID. Blood 2012; 119: 1428-39.

[64] Colgan S P, Eltzschig H K, Eckle T, Thompson L F. Physiological roles for ecto-5'-nucleotidase (CD73). Purinergic Signal 2006; 2: 351-60.

[65] Feng L, Sun X, Csizmadia E, Han L, Bian S, Murakami T, Wang X, Robson S C, Wu Y. Vascular CD39/ENTPD1 directly promotes tumor cell growth by scavenging extracellular adenosine triphosphate. Neoplasia 2011; 13: 206-16.

[66] Niemela J, Ifergan I, Yegutkin G G, Jalkanen S, Prat A, Airas L. IFN-beta regulates CD73 and adenosine expression at the blood-brain barrier. Eur J Immunol 2008; 38: 2718-26.

[67] Algars A, Karikoski M, Yegutkin G G, Stoitzner P, Niemela J, Salmi M, Jalkanen S. Different role of CD73 in leukocyte trafficking via blood and lymph vessels. Blood 2011; 117: 4387-93.

[68] Fausther M, Lecka J, Soliman E, Kauffenstein G, Pelletier J, Sheung N, Dranoff J A, Sevigny J. Coexpression of ecto-5'-nucleotidase/CD73 with specific NTPDases differentially regulates adenosine formation in the rat liver. Am J Physiol Gastrointest Liver Physiol 2012; 302: G447-59.

[69] Corriden R, Chen Y, Inoue Y, Beldi G, Robson S C, Insel P A, Junger W G. Ecto-nucleoside triphosphate diphosphohydrolase 1 (E-NTPDase1/CD39) regulates neutrophil chemotaxis by hydrolyzing released ATP to adenosine. J Biol Chem 2008; 283: 28480-6.

[70] Doherty G A, Bai A, Hanidziar D, Longhi M S, Lawlor G O, Putheti P, Csizmadia E, Nowak M, Cheifetz A S, Moss A C, Robson S C. CD73 is a phenotypic marker of effector memory Th17 cells in inflammatory bowel disease. Eur J Immunol 2012; 42: 3062-72.

[71] Dwyer K M, Deaglio S, Gao W, Friedman D, Strom T B, Robson S C. CD39 and control of cellular immune responses. Purinergic Signal 2007; 3: 171-80.

[72] Sun X, Wu Y, Gao W, Enjyoji K, Csizmadia E, Muller C E, Murakami T, Robson S C. CD39/ENTPD1 expression by CD4+Foxp3+ regulatory T cells promotes hepatic metastatic tumor growth in mice. Gastroenterology 2010; 139: 1030-40.

[73] Deaglio S, Dwyer K M, Gao W, Friedman D, Usheva A, Erat A, Chen J F, Enjyoji K, Linden J, Oukka M, Kuchroo V K, Strom T B, Robson S C. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. J Exp Med 2007; 204: 1257-65.

[74] Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'Acqua M L, Rossini P M, Battistini L, Rotzschke O, Falk K. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007; 110: 1225-32.

[75] Zhou Q, Yan J, Putheti P, Wu Y, Sun X, Toxavidis V, Tigges J, Kassam N, Enjyoji K, Robson S C, Strom T B, Gao W. Isolated CD39 expression on CD4+ T cells denotes both regulatory and memory populations. Am J Transplant 2009; 9: 2303-11.

[76] Beldi G, Wu Y, Banz Y, Nowak M, Miller L, Enjyoji K, Haschemi A, Yegutkin G G, Candinas D, Exley M, Robson S C. Natural killer T cell dysfunction in CD39-null mice protects against concanavalin A-induced hepatitis. Hepatology 2008; 48: 841-52.

[77] Eltzschig H K, Kohler D, Eckle T, Kong T, Robson S C, Colgan S P. Central role of Sp1-regulated CD39 in hypoxia/ischemia protection. Blood 2009; 113: 224-32.

[78] Muller W A. Sorting the signals from the signals in the noisy environment of inflammation. Sci Signal 2011; 4: pe23.

[79] Zeh H J, 3rd, Lotze M T. Addicted to death: invasive cancer and the immune response to unscheduled cell death. J Immunother 2005; 28: 1-9.

[80] la Sala A, Sebastiani S, Ferrari D, Di Virgilio F, Idzko M, Norgauer J, Girolomoni G. Dendritic cells exposed to extracellular adenosine triphosphate acquire the migratory properties of mature cells and show a reduced capacity to attract type 1 T lymphocytes. Blood 2002; 99: 1715-22.

[81] la Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G. Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. J Immunol 2001; 166: 1611-7.

[82] Hasko G, Kuhel D G, Salzman A L, Szabo C. ATP suppression of interleukin-12 and tumour necrosis factor-alpha release from macrophages. Br J Pharmacol 2000; 129: 909-14.

[83] Martin C, Leone M, Viviand X, Ayem M L, Guieu R. High adenosine plasma concentration as a prognostic index for outcome in patients with septic shock. Crit Care Med 2000; 28: 3198-202.

[84] Sperlagh B, Doda M, Baranyi M, Hasko G. Ischemic-like condition releases norepinephrine and purines from different sources in superfused rat spleen strips. J Neuroimmunol 2000; 111: 45-54.

[85] Aliagas E, Vidal A, Texido L, Ponce J, Condom E, Martin-Satue M. High Expression of Ecto-Nucleotidases CD39 and CD73 in Human Endometrial Tumors. Mediators Inflamm 2014; 2014: 509027.

[86] Resta R, Yamashita Y, Thompson L F. Ecto-enzyme and signaling functions of lymphocyte CD73. Immunol Rev 1998; 161: 95-109.

[87] Terp M G, Olesen K A, Arnspang E C, Lund R R, Lagerholm B C, Ditzel H J, Leth-Larsen R. Anti-human CD73 monoclonal antibody inhibits metastasis formation in human breast cancer by inducing clustering and internalization of CD73 expressed on the surface of cancer cells. J Immunol 2013; 191: 4165-73.

[88] Coussens L M, Werb Z. Inflammation and cancer. Nature 2002; 420: 860-7.

[89] Grivennikov S I, Greten F R, Karin M. Immunity, inflammation, and cancer. Cell 2010; 140: 883-99.

[90] Mantovani A, Allavena P, Sica A, Balkwill F. Cancer-related inflammation. Nature 2008; 454: 436-44.

[91] Zou W, Restifo N P. T(H)17 cells in tumour immunity and immunotherapy. Nat Rev Immunol 2010; 10: 248-56.

[92] Van Meir E G, Hadjipanayis C G, Norden A D, Shu H K, Wen P Y, Olson J J. Exciting new advances in neuro-oncology: the avenue to a cure for malignant glioma. CA Cancer J Clin 2010; 60: 166-93.

[93] Vander Heiden M G, Cantley L C, Thompson C B. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; 324: 1029-33.

[94] Ellis B C, Graham L D, Molloy P L. CRNDE, a long non-coding RNA responsive to insulin/IGF signaling, regulates genes involved in central metabolism. Biochim Biophys Acta 2014; 1843: 372-86.

[95] Li M, Durbin K R, Sweet S M, Tipton J D, Zheng Y, Kelleher N L. Oncogene-induced cellular senescence elicits an anti-Warburg effect. Proteomics 2013; 13: 2585-96.

[96] Phan L M, Yeung S C, Lee M H. Cancer metabolic reprogramming: importance, main features, and potentials for precise targeted anti-cancer therapies. Cancer Biol Med 2014; 11: 1-19.

[97] Sun X, Han L, Seth P, Bian S, Li L, Csizmadia E, Junger W G, Schmelzle M, Usheva A, Tapper E B, Baffy G, Sukhatme V P, Wu Y, Robson S C. Disordered purinergic signaling and abnormal cellular metabolism are associated with development of liver cancer in Cd39/ENTPD1 null mice. Hepatology 2013; 57: 205-16.

[98] Baffy G, Derdak Z, Robson S C. Mitochondrial recoupling: a novel therapeutic strategy for cancer? Br J Cancer 2011; 105: 469-74.

[99] Li W, Nichols K, Nathan C A, Zhao Y. Mitochondrial uncoupling protein 2 is up-regulated in human head and neck, skin, pancreatic, and prostate tumors. Cancer Biomark 2013; 13: 377-83.

[100] Ayyasamy V, Owens K M, Desouki M M, Liang P, Bakin A, Thangaraj K, Buchsbaum D J, LoBuglio A F, Singh K K. Cellular model of Warburg effect identifies tumor promoting function of UCP2 in breast cancer and its suppression by genipin. PLoS One 2011; 6: e24792.

[101] Derdak Z, Mark N M, Beldi G, Robson S C, Wands J R, Baffy G. The mitochondrial uncoupling protein-2 promotes chemoresistance in cancer cells. Cancer Res 2008; 68: 2813-9.

[102] Fang M, Shen Z, Huang S, Zhao L, Chen S, Mak T W, Wang X. The ER UDPase ENTPD5 promotes protein N-glycosylation, the Warburg effect, and proliferation in the PTEN pathway. Cell 2010; 143: 711-24.

[103] Pines A, Perrone L, Bivi N, Romanello M, Damante G, Gulisano M, Kelley M R, Quadrifoglio F, Tell G. Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. Nucleic Acids Res 2005; 33: 4379-94.

[104] White N, Burnstock G. P2 receptors and cancer. Trends Pharmacol Sci 2006; 27: 211-7.

[105] Shabbir M, Ryten M, Thompson C, Mikhailidis D, Burnstock G. Purinergic receptor-mediated effects of ATP in high-grade bladder cancer. BJU Int 2008; 101: 106-12.

[106] White N, Ryten M, Clayton E, Butler P, Burnstock G. P2Y purinergic receptors regulate the growth of human melanomas. Cancer Lett 2005; 224: 81-91.

[107] Greig A V, Linge C, Healy V, Lim P, Clayton E, Rustin M H, McGrouther D A, Burnstock G. Expression of purinergic receptors in non-melanoma skin cancers and their functional roles in A431 cells. J Invest Dermatol 2003; 121: 315-27.

[108] Dixon C J, Bowler W B, Fleetwood P, Ginty A F, Gallagher J A, Carron J A. Extracellular nucleotides stimulate proliferation in MCF-7 breast cancer cells via P2-purinoceptors. Br J Cancer 1997; 75: 34-9.

[109] Schafer R, Sedehizade F, Welte T, Reiser G. ATP- and UTP-activated P2Y receptors differently regulate proliferation of human lung epithelial tumor cells. Am J Physiol Lung Cell Mol Physiol 2003; 285: L376-85.

[110] Scodelaro Bilbao P, Boland R. Extracellular ATP regulates FoxO family of transcription factors and cell cycle progression through PI3K/Akt in MCF-7 cells. Biochim Biophys Acta 2013; 1830: 4456-69.

[111] Nishimaki N, Tsukimoto M, Kitami A, Kojima S. Autocrine regulation of gamma-irradiation-induced DNA damage response via extracellular nucleotides-mediated activation of P2Y6 and P2Y12 receptors. DNA Repair (Amst) 2012; 11: 657-65.

[112] Ide S, Nishimaki N, Tsukimoto M, Kojima S. Purine receptor P2Y6 mediates cellular response to gamma-ray-induced DNA damage. J Toxicol Sci 2014; 39: 15-23.

[113] Kim S G, Gao Z G, Soltysiak K A, Chang T S, Brodie C, Jacobson K A. P2Y6 nucleotide receptor activates PKC to protect 1321N1 astrocytoma cells against tumor necrosis factor-induced apoptosis. Cell Mol Neurobiol 2003; 23: 401-18.

[114] Burnstock G, Di Virgilio F. Purinergic signalling and cancer. Purinergic Signal 2013; 9: 491-540.

[115] Mediavilla-Varela M, Luddy K, Noyes D, Khalil F K, Neuger A M, Soliman H, Antonia S. Antagonism of adenosine A2A receptor expressed by lung adenocarcinoma tumor cells and cancer associated fibroblasts inhibits their growth. Cancer Biol Ther 2013 July 17; 14(9).

[116] Kalhan A, Gharibi B, Vazquez M, Jasani B, Neal J, Kidd M, Modlin I M, Pfragner R, Rees D A, Ham J. Adenosine A2A and A2B receptor expression in neuroendocrine tumours: potential targets for therapy. Purinergic Signal 2012; 8: 265-74.

[117] Wei Q, Costanzi S, Balasubramanian R, Gao Z G, Jacobson K A. A2B adenosine receptor blockade inhibits growth of prostate cancer cells. Purinergic Signal 2013; 9: 271-80.

[118] Serra S, Horenstein A L, Vaisitti T, Brusa D, Rossi D, Laurenti L, D'Arena G, Coscia M, Tripodo C, Inghirami G, Robson S C, Gaidano G, Malavasi F, Deaglio S. CD73-generated extracellular adenosine in chronic lymphocytic leukemia creates local conditions counteracting drug-induced cell death. Blood 2011; 118: 6141-52.

[119] Mirza A, Basso A, Black S, Malkowski M, Kwee L, Pachter J A, Lachowicz J E, Wang Y, Liu S. RNA interference targeting of A1 receptor-overexpressing breast carcinoma cells leads to diminished rates of cell proliferation and induction of apoptosis. Cancer Biol Ther 2005; 4: 1355-60.

[120] Lelievre V, Muller J M, Falcon J. Adenosine modulates cell proliferation in human colonic adenocarcinoma. I. Possible involvement of adenosine A1 receptor subtypes in HT29 cells. Eur J Pharmacol 1998; 341: 289-97.

[121] Rapaport E. Treatment of human tumor cells with ADP or ATP yields arrest of growth in the S phase of the cell cycle. J Cell Physiol 1983; 114: 279-83.

[122] Weber F C, Esser P R, Muller T, Ganesan J, Pellegatti P, Simon M M, Zeiser R, Idzko M, Jakob T, Martin S F. Lack of the purinergic receptor P2X(7) results in resistance to contact hypersensitivity. J Exp Med 2010; 207: 2609-19.

[123] Wilhelm K, Ganesan J, Muller T, Durr C, Grimm M, Beilhack A, Krempl C D, Sorichter S, Gerlach U V, Juttner E, Zerweck A, Gartner F, Pellegatti P, Di Virgilio F, Ferrari D, Kambham N, Fisch P, Finke J, Idzko M, Zeiser R. Graft-versus-host disease is enhanced by extracellular ATP activating P2X7R. Nat Med 2010; 16: 1434-8.

[124] Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F. Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS One 2008; 3: e2599.

[125] Michaud M, Martins I, Sukkurwala A Q, Adjemian S, Ma Y, Pellegatti P, Shen S, Kepp O, Scoazec M, Mignot G, Rello-Varona S, Tailler M, Menger L, Vacchelli E, Galluzzi L, Ghiringhelli F, di Virgilio F, Zitvogel L, Kroemer G. Autophagy-dependent anticancer immune responses induced by chemotherapeutic agents in mice. Science 2011; 334: 1573-7.

[126] Shabbir M, Burnstock G. Purinergic receptor-mediated effects of adenosine 5'-triphosphate in urological malignant diseases. Int J Urol 2009; 16: 143-50.

[127] Bian S, Sun X, Bai A, Zhang C, Li L, Enjyoji K, Junger W G, Robson S C, Wu Y. P2X7 integrates PI3K/AKT and AMPK-PRAS40-mTOR signaling pathways to mediate tumor cell death. PLoS One 2013; 8: e60184.

[128] Deli T, Csernoch L. Extracellular ATP and cancer: an overview with special reference to P2 purinergic receptors. Pathol Oncol Res 2008; 14: 219-31.

[129] Gehring M P, Pereira T C, Zanin R F, Borges M C, Braga Filho A, Battastini A M, Bogo M R, Lenz G, Campos M M, Morrone F B. P2X7 receptor activation leads to increased cell death in a radiosensitive human glioma cell line. Purinergic Signal 2012; 8: 729-39.

[130] Wang Q, Wang L, Feng Y H, Li X, Zeng R, Gorodeski G I. P2X7 receptor-mediated apoptosis of human cervical epithelial cells. Am J Physiol Cell Physiol 2004; 287: C1349-58.

[131] Li X, Zhou L, Feng Y H, Abdul-Karim F W, Gorodeski G I. The P2X7 receptor: a novel biomarker of uterine epithelial cancers. Cancer Epidemiol Biomarkers Prev 2006; 15: 1906-13.

[132] Huang S, Chen Y, Wu W, Ouyang N, Chen J, Li H, Liu X, Su F, Lin L, Yao Y. miR-150 promotes human breast cancer growth and malignant behavior by targeting the pro-apoptotic purinergic P2X7 receptor. PLoS One 2013; 8: e80707.

[133] Zhang X J, Zheng G G, Ma X T, Yang Y H, Li G, Rao Q, Nie K, Wu K F. Expression of P2X7 in human hematopoietic cell lines and leukemia patients. Leuk Res 2004; 28: 1313-22.

[134] Gorodeski G I. P2X7-mediated chemoprevention of epithelial cancers. Expert Opin Ther Targets 2009; 13: 1313-32.

[135] Fang J, Chen X, Zhang L, Chen J, Liang Y, Li X, Xiang J, Wang L, Guo G, Zhang B, Zhang W. P2X7R suppression promotes glioma growth through epidermal growth factor receptor signal pathway. Int J Biochem Cell Biol 2013; 45: 1109-20.

[136] Mistafa O, Stenius U. Statins inhibit Akt/PKB signaling via P2X7 receptor in pancreatic cancer cells. Biochem Pharmacol 2009; 78: 1115-26.

[137] Mistafa O, Hogberg J, Stenius U. Statins and ATP regulate nuclear pAkt via the P2X7 purinergic receptor in epithelial cells. Biochem Biophys Res Commun 2008; 365: 131-6.

[138] Mistafa O, Ghalali A, Kadekar S, Hogberg J, Stenius U. Purinergic receptor-mediated rapid depletion of nuclear phosphorylated Akt depends on pleckstrin homology domain leucine-rich repeat phosphatase, calcineurin, protein phosphatase 2A, and PTEN phosphatases. J Biol Chem 2010; 285: 27900-10.

[139] Fang W G, Pirnia F, Bang Y J, Myers C E, Trepel J B. P2-purinergic receptor agonists inhibit the growth of androgen-independent prostate carcinoma cells. J Clin Invest 1992; 89: 191-6.

[140] Calvert R C, Shabbir M, Thompson C S, Mikhailidis D P, Morgan R J, Burnstock G. Immunocytochemical and pharmacological characterisation of P2-purinoceptor-mediated cell growth and death in PC-3 hormone refractory prostate cancer cells. Anticancer Res 2004; 24: 2853-9.

[141] White N, Butler P E, Burnstock G. Human melanomas express functional P2 X(7) receptors. Cell Tissue Res 2005; 321: 411-8.

[142] Coutinho-Silva R, Stahl L, Cheung K K, de Campos N E, de Oliveira Souza C, Ojcius D M, Burnstock G. P2X and P2Y purinergic receptors on human intestinal epithelial carcinoma cells: effects of extracellular nucleotides on apoptosis and cell proliferation. Am J Physiol Gastrointest Liver Physiol 2005; 288: G1024-35.

[143] Hopfner M, Lemmer K, Jansen A, Hanski C, Riecken E O, Gavish M, Mann B, Buhr H, Glassmeier G, Scherubl H. Expression of functional P2-purinergic receptors in primary cultures of human colorectal carcinoma cells. Biochem Biophys Res Commun 1998; 251: 811-7.

[144] Maaser K, Hopfner M, Kap H, Sutter A P, Barthel B, von Lampe B, Zeitz M, Scherubl H. Extracellular nucleotides inhibit growth of human oesophageal cancer cells via P2Y(2)-receptors. Br J Cancer 2002; 86: 636-44.

[145] Yasuda Y, Saito M, Yamamura T, Yaguchi T, Nishizaki T. Extracellular adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A(2a) adenosine receptors. J Gastroenterol 2009; 44: 56-65.

[146] Rickles R J, Tam W F, Giordano T P, 3rd, Pierce L T, Farwell M, McMillin D W, Necheva A, Crowe D, Chen M, Avery W, Kansra V, Nawrocki S T, Carew J S, Giles F J, Mitsiades C S, Borisy A A, Anderson K C, Lee M S. Adenosine A2A and beta-2 adrenergic receptor agonists: novel selective and synergistic multiple myeloma targets discovered through systematic combination screening. Mol Cancer Ther 2012; 11: 1432-42.

[147] Antonioli L, Blandizzi C, Pacher P, Hasko G. Immunity, inflammation and cancer: a leading role for adenosine. Nat Rev Cancer 2013; 13: 842-57.

[148] Saito M, Yaguchi T, Yasuda Y, Nakano T, Nishizaki T. Adenosine suppresses CW2 human colonic cancer growth by inducing apoptosis via A(1) adenosine receptors. Cancer Lett 2010; 290: 211-5.

[149] Synowitz M, Glass R, Farber K, Markovic D, Kronenberg G, Herrmann K, Schnermann J, Nolte C, van Rooijen N, Kiwit J, Kettenmann H. A1 adenosine receptors in microglia control glioblastoma-host interaction. Cancer Res 2006; 66: 8550-7.

[150] Fishman P, Bar-Yehuda S, Ohana G, Pathak S, Wasserman L, Barer F, Multani A S. Adenosine acts as an inhibitor of lymphoma cell growth: a major role for the A3 adenosine receptor. Eur J Cancer 2000; 36: 1452-8.

[151] Merighi S, Mirandola P, Milani D, Varani K, Gessi S, Klotz K N, Leung E, Baraldi P G, Borea P A. Adenosine receptors as mediators of both cell proliferation and cell death of cultured human melanoma cells. J Invest Dermatol 2002; 119: 923-33.

[152] Aghaei M, Panjehpour M, Karami-Tehrani F, Salami S. Molecular mechanisms of A3 adenosine receptor-induced G1 cell cycle arrest and apoptosis in androgen-dependent and independent prostate cancer cell lines: involvement of intrinsic pathway. J Cancer Res Clin Oncol 2011; 137: 1511-23.

[153] Bar-Yehuda S, Barer F, Volfsson L, Fishman P. Resistance of muscle to tumor metastases: a role for a3 adenosine receptor agonists. Neoplasia 2001; 3: 125-31.

[154] Bar-Yehuda S, Stemmer S M, Madi L, Castel D, Ochaion A, Cohen S, Barer F, Zabutti A, Perez-Liz G, Del Valle L, Fishman P. The A3 adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-kappaB signal transduction pathways. Int J Oncol 2008; 33: 287-95.

[155] Otsuki T, Kanno T, Fujita Y, Tabata C, Fukuoka K, Nakano T, Gotoh A, Nishizaki T. A3 adenosine receptor-mediated p53-dependent apoptosis in Lu-65 human lung cancer cells. Cell Physiol Biochem 2012; 30: 210-20.

[156] Aghaei M, Karami-Tehrani F, Panjehpour M, Salami S, Fallahian F. Adenosine induces cell-cycle arrest and apoptosis in androgen-dependent and -independent prostate cancer cell lines, LNcap-FGC-10, DU-145, and PC3. Prostate 2012; 72: 361-75.

[157] Vincenzi F, Targa M, Corciulo C, Gessi S, Merighi S, Setti S, Cadossi R, Borea P A, Varani K. The anti-tumor effect of A3 adenosine receptors is potentiated by pulsed electromagnetic fields in cultured neural cancer cells. PLoS One 2012; 7: e39317.

[158] Madi L, Bar-Yehuda S, Barer F, Ardon E, Ochaion A, Fishman P. A3 adenosine receptor activation in melanoma cells: association between receptor fate and tumor growth inhibition. J Biol Chem 2003; 278: 42121-30.

[159] Fishman P, Bar-Yehuda S, Ohana G, Barer F, Ochaion A, Erlanger A, Madi L. An agonist to the A3 adenosine receptor inhibits colon carcinoma growth in mice via modulation of GSK-3 beta and NF-kappa B. Oncogene 2004; 23: 2465-71.

[160] Fishman P, Madi L, Bar-Yehuda S, Barer F, Del Valle L, Khalili K. Evidence for involvement of Wnt signaling pathway in IB-MECA mediated suppression of melanoma cells. Oncogene 2002; 21: 4060-4.

[161] Dzhandzhugazyan K N, Kirkin A F, thor Straten P,Zeuthen J. Ecto-ATP diphosphohydrolase/CD39 is overexpressed in differentiated human melanomas. FEBS Lett 1998; 430: 227-30.

[162] Matsuo Y, Drexler H G, Harashima A, Takenaka K, Ishihara T, Ishimaru F, Orita K. A novel ALL-L3 cell line, BALM-25, expressing both immunoglobulin light chains. Leuk Lymphoma 2004; 45: 575-81.

[163] Moller P, Bruderlein S, Strater J, Leithauser F, Hasel C, Bataille F, Moldenhauer G, Pawlita M, Barth T F. MedB-1, a human tumor cell line derived from a primary mediastinal large B-cell lymphoma. Int J Cancer 2001; 92: 348-53.

[164] Bennett F, Rawstron A, Plummer M, de Tute R, Moreton P, Jack A, Hillmen P. B-cell chronic lymphocytic leukaemia cells show specific changes in membrane protein expression during different stages of cell cycle. Br J Haematol 2007; 139: 600-4.

[165] Jin D, Fan J, Wang L, Thompson L F, Liu A, Daniel B J, Shin T, Curiel T J, Zhang B. CD73 on tumor cells impairs antitumor T-cell responses: a novel mechanism of tumor-induced immune suppression. Cancer Res 2010; 70: 2245-55.

[166] Stella J, Bavaresco L, Braganhol E, Rockenbach L, Farias P F, Wink M R, Azambuja A A, Barrios C H, Morrone F B, Oliveira Battastini A M. Differential ectonucleotidase expression in human bladder cancer cell lines. Urol Oncol 2010; 28: 260-7.

[167] Mikhailov A, Sokolovskaya A, Yegutkin G G, Amdahl H, West A, Yagita H, Lahesmaa R, Thompson L F, Jalkanen S, Blokhin D, Eriksson J E. CD73 participates in cellular multiresistance program and protects against TRAIL-induced apoptosis. J Immunol 2008; 181: 464-75.

[168] Bavaresco L, Bernardi A, Braganhol E, Cappellari A R, Rockenbach L, Farias P F, Wink M R, Delgado-Canedo A, Battastini A M. The role of ecto-5'-nucleotidase/CD73 in glioma cell line proliferation. Mol Cell Biochem 2008; 319: 61-8.

[169] Ludwig H C, Rausch S, Schallock K, Markakis E. Expression of CD 73 (ecto-5'-nucleotidase) in 165 glioblastomas by immunohistochemistry and electronmicroscopic histochemistry. Anticancer Res 1999; 19: 1747-52.

[170] Sadej R, Spychala J, Skladanowski A C. Expression of ecto-5'-nucleotidase (eN, CD73) in cell lines from various stages of human melanoma. Melanoma Res 2006; 16: 213-22.

[171] Kondo T, Nakazawa T, Murata S I, Katoh R. Expression of CD73 and its ecto-5'-nucleotidase activity are elevated in papillary thyroid carcinomas. Histopathology 2006; 48: 612-4.

[172] Fukuda K, Sakakura C, Miyagawa K, Kuriu Y, Kin S, Nakase Y, Hagiwara A, Mitsufuji S, Okazaki Y, Hayashizaki Y, Yamagishi H. Differential gene expression profiles of radioresistant oesophageal cancer cell lines established by continuous fractionated irradiation. Br J Cancer 2004; 91: 1543-50.

[173] Durak I, Cetin R, Canbolat O, Cetin D, Yurtarslani Z, Unal A. Adenosine deaminase, 5'-nucleotidase, guanase and cytidine deaminase activities in gastric tissues from patients with gastric cancer. Cancer Lett 1994; 84: 199-202.

[174] Eroglu A, Canbolat O, Demirci S, Kocaoglu H, Eryavuz Y, Akgul H. Activities of adenosine deaminase and 5'-nucleotidase in cancerous and noncancerous human colorectal tissues. Med Oncol 2000; 17: 319-24.

[175] Spychala J, Lazarowski E, Ostapkowicz A, Ayscue L H, Jin A, Mitchell B S. Role of estrogen receptor in the regulation of ecto-5'-nucleotidase and adenosine in breast cancer. Clin Cancer Res 2004; 10: 708-17.

[176] Clayton A, Al-Taei S, Webber J, Mason M D, Tabi Z. Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. J Immunol 2011; 187: 676-83.

[177] Bautch V L, Ambler C A. Assembly and patterning of vertebrate blood vessels. Trends Cardiovasc Med 2004; 14: 138-43.

[178] Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. Endocr Rev 2004; 25: 581-611.

[179] Jain R K. Molecular regulation of vessel maturation. Nat Med 2003; 9: 685-93.

[180] Hicklin D J, Ellis L M. Role of the vascular endothelial growth factor pathway in tumor growth and angiogenesis. J Clin Oncol 2005; 23: 1011-27.

[181] Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971; 285: 1182-6.

[182] Holmgren L, O'Reilly M S, Folkman J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. Nat Med 1995; 1: 149-53.

[183] Bergers G, Benjamin L E. Tumorigenesis and the angiogenic switch. Nat Rev Cancer 2003; 3: 401-10.

[184] Kesisis G, Broxterman H, Giaccone G. Angiogenesis inhibitors. Drug selectivity and target specificity. Curr Pharm Des 2007; 13: 2795-809.

[185] Jain R K, Duda D G, Clark J W, Loeffler J S. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. Nat Clin Pract Oncol 2006; 3: 24-40.

[186] Sunderkotter C, Steinbrink K, Goebeler M, Bhardwaj RSorg C. Macrophages and angiogenesis. J Leukoc Biol 1994; 55: 410-22.

[187] Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000; 6: 389-95.

[188] Buxton I L, Yokdang N, Matz R M. Purinergic mechanisms in breast cancer support intravasation, extravasation and angiogenesis. Cancer Lett 2010; 291: 131-41.

[189] Wang L, Karlsson L, Moses S, Hultgardh-Nilsson A, Andersson M, Borna C, Gudbjartsson T, Jern S, Erlinge D. P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol 2002; 40: 841-53.

[190] Takagi H, King G L, Robinson G S, Ferrara N, Aiello L P. Adenosine mediates hypoxic induction of vascular endothelial growth factor in retinal pericytes and endothelial cells. Invest Ophthalmol Vis Sci 1996; 37: 2165-76.

[191] Grant M B, Tarnuzzer R W, Caballero S, Ozeck M J, Davis M I, Spoerri P E, Feoktistov I, Biaggioni I, Shryock J C, Belardinelli L. Adenosine receptor activation induces vascular endothelial growth factor in human retinal endothelial cells. Circ Res 1999; 85: 699-706.

[192] Feoktistov I, Goldstein A E, Ryzhov S, Zeng D, Belardinelli L, Voyno-Yasenetskaya T, Biaggioni I. Differential expression of adenosine receptors in human endothelial cells: role of A2B receptors in angiogenic factor regulation. Circ Res 2002; 90: 531-8.

[193] Dorsam R T, Kunapuli S P. Central role of the P2Y12 receptor in platelet activation. J Clin Invest 2004; 113: 340-5.

[194] Kaiser R A, Buxton I L. Nucleotide-mediated relaxation in guinea-pig aorta: selective inhibition by MRS2179. Br J Pharmacol 2002; 135: 537-45.

[195] Kaiser R A, Oxhorn B C, Andrews G, Buxton I L. Functional compartmentation of endothelial P2Y receptor signaling. Circ Res 2002; 91: 292-9.

[196] Kaczmarek E, Erb L, Koziak K, Jarzyna R, Wink M R, Guckelberger O, Blusztajn J K, Trinkaus-Randall V, Weisman G A, Robson S C. Modulation of endothelial cell migration by extracellular nucleotides: involvement of focal adhesion kinase and phosphatidylinositol 3-kinase-mediated pathways. Thromb Haemost 2005; 93: 735-42.

[197] Satterwhite C M, Farrelly A M, Bradley M E. Chemotactic, mitogenic, and angiogenic actions of UTP on vascular endothelial cells. Am J Physiol 1999; 276: H1091-7.

[198] Tanaka N, Nejime N, Kagota S, Kubota Y, Yudo K, Nakamura K, Kunitomo M, Takahashi K, Hashimoto M, Shinozuka K. ATP participates in the regulation of microvessel permeability. J Pharm Pharmacol 2006; 58: 481-7.

[199] Yang S, Cheek D J, Westfall D P, Buxton I L. Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. Circ Res 1994; 74: 401-7.

[200] Buxton I L, Kaiser R A, Oxhorn B C, Cheek D J. Evidence supporting the Nucleotide Axis Hypothesis: ATP release and metabolism by coronary endothelium. Am J Physiol Heart Circ Physiol 2001; 281: H1657-66.

[201] Burnstock G. Pathophysiology and therapeutic potential of purinergic signaling. Pharmacol Rev 2006; 58: 58-86.

[202] Kashiwagi S, Izumi Y, Gohongi T, Demou Z N, Xu L, Huang P L, Buerk D G, Munn L L, Jain R K, Fukumura D. NO mediates mural cell recruitment and vessel morphogenesis in murine melanomas and tissue-engineered blood vessels. J Clin Invest 2005; 115: 1816-27.

[203] Erb L, Liu J, Ockerhausen J, Kong Q, Garrad R C, Griffin K, Neal C, Krugh B, Santiago-Perez L I, Gonzalez F A, Gresham H D, Turner J T, Weisman G A. An RGD sequence in the P2Y(2) receptor interacts with alpha(V)beta(3) integrins and is required for G(o)-mediated signal transduction. J Cell Biol 2001; 153: 491-501.

[204] Seye C I, Yu N, Gonzalez F A, Erb L, Weisman G A. The P2Y2 nucleotide receptor mediates vascular cell adhesion molecule-1 expression through interaction with VEGF receptor-2 (KDR/Flk-1). J Biol Chem 2004; 279: 35679-86.

[205] Buvinic S, Bravo-Zehnder M, Boyer J L, Huidobro-Toro J P, Gonzalez A. Nucleotide P2Y1 receptor regulates EGF receptor mitogenic signaling and expression in epithelial cells. J Cell Sci 2007; 120: 4289-301.

[206] Rumjahn S M, Yokdang N, Baldwin K A, Thai J, Buxton I L. Purinergic regulation of vascular endothelial growth factor signaling in angiogenesis. Br J Cancer 2009; 100: 1465-70.

[207] Cho C H, Lee C S, Chang M, Jang I H, Kim S J, Hwang I, Ryu S H, Lee C O, Koh G Y. Localization of VEGFR-2 and PLD2 in endothelial caveolae is involved in VEGF-induced phosphorylation of MEK and ERK. Am J Physiol Heart Circ Physiol 2004; 286: H1881-8.

[208] Labrecque L, Royal I, Surprenant D S, Patterson C, Gingras D, Beliveau R. Regulation of vascular endothelial growth factor receptor-2 activity by caveolin-1 and plasma membrane cholesterol. Mol Biol Cell 2003; 14: 334-47.

[209] Ikeda S, Ushio-Fukai M, Zuo L, Tojo T, Dikalov S, Patrushev N A, Alexander R W. Novel role of ARF6 in vascular endothelial growth factor-induced signaling and angiogenesis. Circ Res 2005; 96: 467-75.

[210] Massimino M L, Griffoni C, Spisni E, Toni M, Tomasi V. Involvement of caveolae and caveolae-like domains in signalling, cell survival and angiogenesis. Cell Signal 2002; 14: 93-8.

[211] Yamamoto K, Sokabe T, Matsumoto T, Yoshimura K, Shibata M, Ohura N, Fukuda T, Sato T, Sekine K, Kato S, Isshiki M, Fujita T, Kobayashi M, Kawamura K, Masuda H, Kamiya A, Ando J. Impaired flow-dependent control of vascular tone and remodeling in P2X4-deficient mice. Nat Med 2006; 12: 133-7.

[212] Freeman K W, Bowman B R, Zetter B R. Regenerative protein thymosin beta-4 is a novel regulator of purinergic signaling. FASEB J 2011; 25: 907-15.

[213] Hill L M, Gavala M L, Lenertz L Y, Bertics P J. Extracellular ATP may contribute to tissue repair by rapidly stimulating purinergic receptor X7-dependent vascular endothelial growth factor release from primary human monocytes. J Immunol 2010; 185: 3028-34.

[214] Baroja-Mazo A, Barbera-Cremades M, Pelegrin P. The participation of plasma membrane hemichannels to purinergic signaling. Biochim Biophys Acta 2013; 1828: 79-93.

[215] Weinhold K, Krause-Buchholz U, Rodel G, Kasper M, Barth K. Interaction and interrelation of P2X7 and P2X4 receptor complexes in mouse lung epithelial cells. Cell Mol Life Sci 2010; 67: 2631-42.

[216] Vergani A, Tezza S, D'Addio F, Fotino C, Liu K, Niewczas M, Bassi R, Molano R D, Kleffel S, Petrelli A, Soleti A, Ammirati E, Frigerio M, Visner G, Grassi F, Ferrero M E, Corradi D, Abdi R, Ricordi C, Sayegh M H, Pileggi A, Fiorina P. Long-term heart transplant survival by targeting the ionotropic purinergic receptor P2X7. Circulation 2013; 127: 463-75.

[217] Moldovan N I. Role of monocytes and macrophages in adult angiogenesis: a light at the tunnel's end. J Hematother Stem Cell Res 2002; 11: 179-94.

[218] Aga M, Johnson C J, Hart A P, Guadarrama A G, Suresh M, Svaren J, Bertics P J, Darien B J. Modulation of monocyte signaling and pore formation in response to agonists of the nucleotide receptor P2X(7). J Leukoc Biol 2002; 72: 222-32.

[219] Guha A, Bach R, Konigsberg W, Nemerson Y. Affinity purification of human tissue factor: interaction of factor VII and tissue factor in detergent micelles. Proc Natl Acad Sci U S A 1986; 83: 299-302.

[220] Clark A N, Youkey R, Liu X, Jia L, Blatt R, Day Y J, Sullivan G W, Linden J, Tucker A L. A1 adenosine receptor activation promotes angiogenesis and release of VEGF from monocytes. Circ Res 2007; 101: 1130-8.

[221] Erlinge D, Burnstock G. P2 receptors in cardiovascular regulation and disease. Purinergic Signal 2008; 4: 1-20.

[222] Schuchardt M, Tolle M, van der Giet M. P2Y purinoceptors as potential emerging therapeutical target in vascular disease. Curr Pharm Des 2012; 18: 6169-80.

[223] Hinze A V, Mayer P, Harst A, von Kugelgen I. P2X1 receptor-mediated inhibition of the proliferation of human coronary smooth muscle cells involving the transcription factor NR4A1. Purinergic Signal 2013; 9: 677-86.

[224] Leibovich S J, Chen J F, Pinhal-Enfield G, Belem P C, Elson G, Rosania A, Ramanathan M, Montesinos C, Jacobson M, Schwarzschild M A, Fink J S, Cronstein B. Synergistic up-regulation of vascular endothelial growth factor expression in murine macrophages by adenosine A(2A) receptor agonists and endotoxin. Am J Pathol 2002; 160: 2231-44.

[225] Feoktistov I, Ryzhov S, Goldstein A E, Biaggioni I. Mast cell-mediated stimulation of angiogenesis: cooperative interaction between A2B and A3 adenosine receptors. Circ Res 2003; 92: 485-92.

[226] Jackson E K, Ren J, Gillespie D G, Dubey R K. Extracellular 2',3'-cyclic adenosine 5'-monophosphate is a potent inhibitor of preglomerular vascular smooth muscle cell and mesangial cell growth. Hypertension 2010; 56: 151-8.

[227] Beldi G, Wu Y, Sun X, Imai M, Enjyoji K, Csizmadia E, Candinas D, Erb L, Robson S C. Regulated catalysis of extracellular nucleotides by vascular CD39/ENTPD1 is required for liver regeneration. Gastroenterology 2008; 135: 1751-60.

[228] Sheth S, Bleibel W, Thukral C, Y A R, Beldi G, Csizmadia E, Robson S C. Heightened NTPDase-1/CD39 expression and angiogenesis in radiation proctitis. Purinergic Signal 2009; 5: 321-6.

[229] Jackson S W, Hoshi T, Wu Y, Sun X, Enjyoji K, Cszimadia E, Sundberg C, Robson S C. Disordered purinergic signaling inhibits pathological angiogenesis in cd39/Entpd1-null mice. Am J Pathol 2007; 171: 1395-404.

[230] Levesque S A, Kukulski F, Enjyoji K, Robson S C, Sevigny J. NTPDase1 governs P2X7-dependent functions in murine macrophages. Eur J Immunol 2010; 40: 1473-85.

[231] Goepfert C, Imai M, Brouard S, Csizmadia E, Kaczmarek E, Robson S C. CD39 modulates endothelial cell activation and apoptosis. Mol Med 2000; 6: 591-603.

[232] Goepfert C, Sundberg C, Sevigny J, Enjyoji K, Hoshi T, Csizmadia E, Robson S. Disordered cellular migration and angiogenesis in cd39-null mice. Circulation 2001; 104: 3109-15.

[233] Behdad A, Sun X, Khalpey Z, Enjyoji K, Wink M, Wu Y, Usheva A, Robson S C. Vascular smooth muscle cell expression of ectonucleotidase CD39 (ENTPD1) is required for neointimal formation in mice. Purinergic Signal 2009; 5: 335-42.

[234] Allard B, Turcotte M, Spring K, Pommey S, Royal I, Stagg J. Anti-CD73 therapy impairs tumor angiogenesis. Int J Cancer 2014; 134: 1466-73.

[235] Braganhol E, Morrone F B, Bernardi A, Huppes D, Meurer L, Edelweiss M I, Lenz G, Wink M R, Robson S C, Battastini A M. Selective NTPDase2 expression modulates in vivo rat glioma growth. Cancer Sci 2009; 100: 1434-42.

[236] Kunzli B M, Berberat P O, Giese T, Csizmadia E, Kaczmarek E, Baker C, Halaceli I, Buchler M W, Friess H, Robson S C. Upregulation of CD39/NTPDases and P2 receptors in human pancreatic disease. Am J Physiol Gastrointest Liver Physiol 2007; 292: G223-30.

[237] Di Virgilio F, Robson S C. Identification of novel immunosuppressive pathways paves the way for drug discovery. Curr Opin Pharmacol 2009; 9: 445-6.

[238] Janeway C A, Jr. How the immune system works to protect the host from infection: a personal view. Proc Natl Acad Sci U S A 2001; 98: 7461-8.

[239] Martins I, Tesniere A, Kepp O, Michaud M, Schlemmer F, Senovilla L, Seror C, Metivier D, Perfettini J L, Zitvogel L, Kroemer G. Chemotherapy induces ATP release from tumor cells. Cell Cycle 2009; 8: 3723-8.

[240] Aymeric L, Apetoh L, Ghiringhelli F, Tesniere A, Martins I, Kroemer G, Smyth M J, Zitvogel L. Tumor cell death and ATP release prime dendritic cells and efficient anticancer immunity. Cancer Res 2010; 70: 855-8.

[241] Mattarollo S R, Loi S, Duret H, Ma Y, Zitvogel L, Smyth M J. Pivotal role of innate and adaptive immunity in anthracycline chemotherapy of established tumors. Cancer Res 2011; 71: 4809-20.

[242] Elliott M R, Chekeni F B, Trampont P C, Lazarowski E R, Kadl A, Walk S F, Park D, Woodson R I, Ostankovich M, Sharma P, Lysiak J J, Harden T K, Leitinger N, Ravichandran K S. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 2009; 461: 282-6.

[243] Huang T T, Ojcius D M, Young J D, Wu Y H, Ko Y F, Wong T Y, Wu C Y, Lu C C, Lai H C. The anti-tumorigenic mushroom Agaricus blazei Murill enhances IL-1beta production and activates the NLRP3 inflammasome in human macrophages. PLoS One 2012; 7: e41383.

[244] Idzko M, Dichmann S, Ferrari D, Di Virgilio F, la Sala A, Girolomoni G, Panther E, Norgauer J. Nucleotides induce chemotaxis and actin polymerization in immature but not mature human dendritic cells via activation of pertussis toxin-sensitive P2y receptors. Blood 2002; 100: 925-32.

[245] Yip L, Woehrle T, Corriden R, Hirsh M, Chen Y, Inoue Y, Ferrari V, Insel P A, Junger W G. Autocrine regulation of T-cell activation by ATP release and P2X7 receptors. FASEB J 2009; 23: 1685-93.

[246] Schenk U, Westendorf A M, Radaelli E, Casati A, Ferro M, Fumagalli M, Verderio C, Buer J, Scanziani E, Grassi F. Purinergic control of T cell activation by ATP released through pannexin-1 hemichannels. Sci Signal 2008; 1: ra6.

[247] Bours M J, Swennen E L, Di Virgilio F, Cronstein B N, Dagnelie P C. Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 2006; 112: 358-404.

[248] Wang S, Miyazaki Y, Shinozaki Y, Yoshida H. Augmentation of antigen-presenting and Th1-promoting functions of dendritic cells by WSX-1(IL-27R) deficiency. J Immunol 2007; 179: 6421-8.

[249] Mascanfroni I D, Yeste A, Vieira S M, Burns E J, Patel B, Sloma I, Wu Y, Mayo L, Ben-Hamo R, Efroni S, Kuchroo V K, Robson S C, Quintana F J. IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. Nat Immunol 2013; 14: 1054-63.

[250] Sekar D, Hahn C, Brune B, Roberts E, Weigert A. Apoptotic tumor cells induce IL-27 release from human DCs to activate Treg cells that express CD69 and attenuate cytotoxicity. Eur J Immunol 2012; 42: 1585-98.

[251] Longhi M S, Moss A, Bai A, Wu Y, Huang H, Cheifetz A, Quintana F J, Robson S C. Characterization of Human CD39(+) Th17 Cells with Suppressor Activity and Modulation in Inflammatory Bowel Disease. PLoS One 2014; 9: e87956.

[252] Aswad F, Kawamura H, Dennert G. High sensitivity of CD4+CD25+ regulatory T cells to extracellular metabolites nicotinamide adenine dinucleotide and ATP: a role for P2X7 receptors. J Immunol 2005; 175: 3075-83.

[253] Schenk U, Frascoli M, Proietti M, Geffers R, Traggiai E, Buer J, Ricordi C, Westendorf A M, Grassi F. ATP inhibits the generation and function of regulatory T cells through the activation of purinergic P2X receptors. Sci Signal 2011; 4: ra12.

[254] Beavis P A, Stagg J, Darcy P K, Smyth M J. CD73: a potent suppressor of antitumor immune responses. Trends Immunol 2012; 33: 231-7.

[255] Sitkovsky M, Lukashev D. Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. Nat Rev Immunol 2005; 5: 712-21.

[256] Synnestvedt K, Furuta G T, Comerford K M, Louis N, Karhausen J, Eltzschig H K, Hansen K R, Thompson L F, Colgan S P. Ecto-5'-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 mediates permeability changes in intestinal epithelia. J Clin Invest 2002; 110: 993-1002.

[257] Morote-Garcia J C, Rosenberger P, Kuhlicke J, Eltzschig H K. HIF-1-dependent repression of adenosine kinase attenuates hypoxia-induced vascular leak. Blood 2008; 111: 5571-80.

[258] Di Virgilio F, Boeynaems J M, Robson S C. Extracellular nucleotides as negative modulators of immunity. Curr Opin Pharmacol 2009; 9: 507-13.

[259] Sitkovsky M, Lukashev D, Deaglio S, Dwyer K, Robson S C, Ohta A. Adenosine A2A receptor antagonists: blockade of adenosinergic effects and T regulatory cells. Br J Pharmacol 2008; 153 Suppl 1: S457-64.

[260] Raskovalova T, Huang X, Sitkovsky M, Zacharia L C, Jackson E K, Gorelik E. Gs protein-coupled adenosine receptor signaling and lytic function of activated NK cells. J Immunol 2005; 175: 4383-91.

[261] Novitskiy S V, Ryzhov S, Zaynagetdinov R, Goldstein A E, Huang Y, Tikhomirov O Y, Blackburn M R, Biaggioni I, Carbone D P, Feoktistov I, Dikov M M. Adenosine receptors in regulation of dendritic cell differentiation and function. Blood 2008; 112: 1822-31.

[262] Hilchey S P, Kobie J J, Cochran M R, Secor-Socha S, Wang J C, Hyrien O, Burack W R, Mosmann T R, Quataert S A, Bernstein S H. Human follicular lymphoma CD39+-infiltrating T cells contribute to adenosine-mediated T cell hyporesponsiveness. J Immunol 2009; 183: 6157-66.

[263] Kobie J J, Shah P R, Yang L, Rebhahn J A, Fowell D J, Mosmann T R. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. J Immunol 2006; 177: 6780-6.

[264] Mandapathil M, Hilldorfer B, Szczepanski M J, Czystowska M, Szajnik M, Ren J, Lang S, Jackson E K, Gorelik E, Whiteside T L. Generation and accumulation of immunosuppressive adenosine by human CD4+CD25highFOXP3+ regulatory T cells. J Biol Chem 2010; 285: 7176-86.

[265] Ohta A, Gorelik E, Prasad S J, Ronchese F, Lukashev D, Wong M K, Huang X, Caldwell S, Liu K, Smith P, Chen J F, Jackson E K, Apasov S, Abrams S, Sitkovsky M. A2A adenosine receptor protects tumors from antitumor T cells. Proc Natl Acad Sci U S A 2006; 103: 13132-7.

[266] Sitkovsky M V, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A, Thiel M. Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. Annu Rev Immunol 2004; 22: 657-82.

[267] Scheibner K A, Boodoo S, Collins S, Black K E, Chan-Li Y, Zarek P, Powell J D, Horton M R. The adenosine a2a receptor inhibits matrix-induced inflammation in a novel fashion. Am J Respir Cell Mol Biol 2009; 40: 251-9.

[268] Stagg J, Divisekera U, Duret H, Sparwasser T, Teng M W, Darcy P K, Smyth M J. CD73-deficient mice have increased antitumor immunity and are resistant to experimental metastasis. Cancer Res 2011; 71: 2892-900.

[269] Smyth M J, Teng M W, Swann J, Kyparissoudis K, Godfrey D I, Hayakawa Y. CD4+CD25+ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. J Immunol 2006; 176: 1582-7.

[270] Kunzli B M, Bernlochner M I, Rath S, Kaser S, Csizmadia E, Enjyoji K, Cowan P, d'Apice A, Dwyer K, Rosenberg R, Perren A, Friess H, Maurer C A, Robson S C. Impact of CD39 and purinergic signalling on the growth and metastasis of colorectal cancer. Purinergic Signal 2011; 7: 231-41.

[271] Rumjahn S M, Javed M A, Wong N, Law W E, Buxton I L. Purinergic regulation of angiogenesis by human breast carcinoma-secreted nucleoside diphosphate kinase. Br J Cancer 2007; 97: 1372-80.

[272] Anzinger J, Malmquist N A, Gould J, Buxton I L. Secretion of a nucleoside diphosphate kinase (Nm23-H2) by cells from human breast, colon, pancreas and lung tumors. Proc West Pharmacol Soc 2001; 44: 61-3.

[273] Rumjahn S M, Baldwin K A, Buxton I L. P2y receptor-mediated angiogenesis via vascular endothelial growth factor receptor 2 signaling. Proc West Pharmacol Soc 2007; 50: 58-60.

[274] Chadet S, Jelassi B, Wannous R, Angoulvant D, Chevalier S, Besson P, Roger S. The activation of P2Y2 receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. Carcinogenesis 2014; 35(6): 1238-47.

[275] Li W H, Qiu Y, Zhang H Q, Liu Y, You J F, Tian X X, Fang W G. P2Y2 receptor promotes cell invasion and metastasis in prostate cancer cells. Br J Cancer 2013; 109: 1666-75.

[276] Chen L, He H Y, Li H M, Zheng J, Heng W J, You J F, Fang W G. ERK1/2 and p38 pathways are required for P2Y receptor-mediated prostate cancer invasion. Cancer Lett 2004; 215: 239-47.

[277] Reymond N, d'Agua B B, Ridley A J. Crossing the endothelial barrier during metastasis. Nat Rev Cancer 2013; 13: 858-70.

[278] Schumacher D, Strilic B, Sivaraj K K, Wettschureck N, Offermanns S. Platelet-derived nucleotides promote tumor-cell transendothelial migration and metastasis via P2Y2 receptor. Cancer Cell 2013; 24: 130-7.

[279] Jantaratnotai N, McLarnon J G. Calcium dependence of purinergic subtype P2Y(1) receptor modulation of C6 glioma cell migration. Neurosci Lett 2011; 497: 80-4.

[280] Slater M, Danieletto S, Pooley M, Cheng Teh L, Gidley-Baird A, Barden J A. Differentiation between cancerous and normal hyperplastic lobules in breast lesions. Breast Cancer Res Treat 2004; 83: 1-10.

[281] Jelassi B, Chantome A, Alcaraz-Perez F, Baroja-Mazo A, Cayuela M L, Pelegrin P, Surprenant A, Roger S. P2X(7) receptor activation enhances SK3 channels- and cystein cathepsin-dependent cancer cells invasiveness. Oncogene 2011; 30: 2108-22.

[282] Gu L Q, Li F Y, Zhao L, Liu Y, Chu Q, Zang X X, Liu J M, Ning G, Zhao Y J. Association of XIAP and P2X7 receptor expression with lymph node metastasis in papillary thyroid carcinoma. Endocrine 2010; 38: 276-82.

[283] Takai E, Tsukimoto M, Harada H, Kojima S. Autocrine signaling via release of ATP and activation of P2X7 receptor influences motile activity of human lung cancer cells. Purinergic Signal 2014 March 14.

[284] Sadej R, Spychala J, Skladanowski A C. Ecto-5'-nucleotidase (eN, CD73) is coexpressed with metastasis promoting antigens in human melanoma cells. Nucleosides Nucleotides Nucleic Acids 2006; 25: 1119-23.

[285] Wang L, Zhou X, Zhou T, Ma D, Chen S, Zhi X, Yin L, Shao Z, Ou Z, Zhou P. Ecto-5'-nucleotidase promotes invasion, migration and adhesion of human breast cancer cells. J Cancer Res Clin Oncol 2008; 134: 365-72.

[286] Leth-Larsen R, Lund R, Hansen H V, Laenkholm A V, Tarin D, Jensen O N, Ditzel H J. Metastasis-related plasma membrane proteins of human breast cancer cells identified by comparative quantitative mass spectrometry. Mol Cell Proteomics 2009; 8: 1436-49.

[287] Mandapathil M, Szczepanski M J, Szajnik M, Ren J, Lenzner D E, Jackson E K, Gorelik E, Lang S, Johnson J T, Whiteside T L. Increased ectonucleotidase expression and activity in regulatory T cells of patients with head and neck cancer. Clin Cancer Res 2009; 15: 6348-57.

[288] Linden J. Adenosine promotes tumor metastasis. Sci Signal 2013; 6: pe20.

[289] Denti S, Sirri A, Cheli A, Rogge L, Innamorati G, Putignano S, Fabbri M, Pardi R, Bianchi E. RanBPM is a phosphoprotein that associates with the plasma membrane and interacts with the integrin LFA-1. J Biol Chem 2004; 279: 13027-34.

[290] Wang L, Fan J, Thompson L F, Zhang Y, Shin T, Curiel T J, Zhang B. CD73 has distinct roles in nonhematopoietic and hematopoietic cells to promote tumor growth in mice. J Clin Invest 2011; 121: 2371-82.

[291] Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer K M, Smyth M J. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. Proc Natl Acad Sci U S A 2010; 107: 1547-52.

[292] Allard B, Turcotte M, Stagg J. CD73-generated adenosine: orchestrating the tumor-stroma interplay to promote cancer growth. J Biomed Biotechnol 2012; 2012: 485156.

[293] Whiteside T L, Mandapathil M, Schuler P. The role of the adenosinergic pathway in immunosuppression mediated by human regulatory T cells (Treg). Curr Med Chem 2011; 18: 5217-23.

[294] Surprenant A, North R A. Signaling at purinergic P2X receptors. Annu Rev Physiol 2009; 71: 333-59.

[295] Hasko G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nat Rev Drug Discov 2008; 7: 759-70.

[296] Arias J I, Aller M A, Arias J. Cancer cell: using inflammation to invade the host. Mol Cancer 2007; 6: 29.

[297] Rakoff-Nahoum S. Why cancer and inflammation? Yale J Biol Med 2006; 79: 123-30.

[298] Adinolfi E, Raffaghello L, Giuliani A L, Cavazzini L, Capece M, Chiozzi P, Bianchi G, Kroemer G, Pistoia V, Di Virgilio F. Expression of P2X7 receptor increases in vivo tumor growth. Cancer Res 2012; 72: 2957-69.

[299] Adinolfi E, Amoroso F, Giuliani A L. P2X7 Receptor Function in Bone-Related Cancer. J Osteoporos 2012; 2012: 637863.

[300] Agteresch H J, Burgers S A, van der Gaast A, Wilson J H, Dagnelie P C. Randomized clinical trial of adenosine 5'-triphosphate on tumor growth and survival in advanced lung cancer patients. Anticancer Drugs 2003; 14: 639-44.

[301] Agteresch H J, Dagnelie P C, Rietveld T, van den Berg J W, Danser A H, Wilson J H. Pharmacokinetics of intravenous ATP in cancer patients. Eur J Clin Pharmacol 2000; 56: 49-55.

[302] Agteresch H J, Rietveld T, Kerkhofs L G, van den Berg J W, Wilson J H, Dagnelie P C. Beneficial effects of adenosine triphosphate on nutritional status in advanced lung cancer patients: a randomized clinical trial. J Clin Oncol 2002; 20: 371-8.

[303] Falzoni S, Donvito G, Di Virgilio F. Detecting adenosine triphosphate in the pericellular space. Interface Focus 2013; 3: 20120101.

[304] Jelassi B, Anchelin M, Chamouton J, Cayuela M L, Clarysse L, Li J, Gore J, Jiang L H, Roger S. Anthraquinone emodin inhibits human cancer cell invasiveness by antagonizing P2X7 receptors. Carcinogenesis 2013; 34: 1487-96.

[305] Ghalali A, Wiklund F, Zheng H, Stenius U, Hogberg J. Atorvastatin prevents ATP-driven invasiveness via P2X7 and EHBP1 signaling in PTEN-expressing prostate cancer cells. Carcinogenesis Feb 17, 2014.

[306] Ren S, Zhang Y, Wang Y, Lui Y, Wei W, Huang X, Mao W, Zuo Y. Targeting P2X(7) receptor inhibits the metastasis of murine P388D1 lymphoid neoplasm cells to lymph nodes. Cell Biol Int 2010; 34: 1205-11.

[307] Wei Q, Costanzi S, Liu Q Z, Gao Z G, Jacobson K A. Activation of the P2Y1 receptor induces apoptosis and inhibits proliferation of prostate cancer cells. Biochem Pharmacol 2011; 82: 418-25.

[308] Sarangi S, Pandey A, Papa A L, Sengupta P, Kopparam J, Dadwal U, Basu S, Sengupta S. P2Y12 receptor inhibition augments cytotoxic effects of cisplatin in breast cancer. Med Oncol 2013; 30: 567.

[309] Beavis P A, Divisekera U, Paget C, Chow M T, John L B, Devaud C, Dwyer K, Stagg J, Smyth M J, Darcy P K. Blockade of A2A receptors potently suppresses the metastasis of CD73+ tumors. Proc Natl Acad Sci U S A 2013; 110: 14711-6.

[310] Beavis P A, Milenkovski N, Stagg J, Smyth M J, Darcy P K. A blockade enhances anti-metastatic immune responses. Oncoimmunology 2013; 2: e26705.

[311] Cekic C, Sag D, Li Y, Theodorescu D, Strieter R M, Linden J. Adenosine A2B receptor blockade slows growth of bladder and breast tumors. J Immunol 2012; 188: 198-205.

[312] Merighi S, Benini A, Mirandola P, Gessi S, Varani K, Simioni C, Leung E, Maclennan S, Baraldi P G, Borea P A. Caffeine inhibits adenosine-induced accumulation of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and interleukin-8 expression in hypoxic human colon cancer cells. Mol Pharmacol 2007; 72: 395-406.

[313] Lukashev D, Sitkovsky M, Ohta A. From "Hellstrom Paradox" to anti-adenosinergic cancer immunotherapy. Purinergic Signal 2007; 3: 129-34.

[314] Ma D F, Kondo T, Nakazawa T, Niu D F, Mochizuki K, Kawasaki T, Yamane T, Katoh R. Hypoxia-inducible adenosine A2B receptor modulates proliferation of colon carcinoma cells. Hum Pathol 2010; 41: 1550-7.

[315] Ohana G, Bar-Yehuda S, Arich A, Madi L, Dreznick Z, Rath-Wolfson L, Silberman D, Slosman G, Fishman P. Inhibition of primary colon carcinoma growth and liver metastasis by the A3 adenosine receptor agonist CF101. Br J Cancer 2003; 89: 1552-8.

[316] Fishman P, Bar-Yehuda S, Barer F, Madi L, Multani A S, Pathak S. The A3 adenosine receptor as a new target for cancer therapy and chemoprotection. Exp Cell Res 2001; 269: 230-6.

[317] Cohen S, Stemmer S M, Zozulya G, Ochaion A, Patoka R, Barer F, Bar-Yehuda S, Rath-Wolfson L, Jacobson K A, Fishman P. CF102 an A3 adenosine receptor agonist mediates anti-tumor and anti-inflammatory effects in the liver. J Cell Physiol 2011; 226: 2438-47.

[318] Kim H, Kang J W, Lee S, Choi W J, Jeong L S, Yang Y, Hong J T, Yoon do Y. A3 adenosine receptor antagonist, truncated Thio-Cl-IB-MECA, induces apoptosis in T24 human bladder cancer cells. Anticancer Res 2010; 30: 2823-30.

[319] Kim S J, Min H Y, Chung H J, Park E J, Hong J Y, Kang Y J, Shin D H, Jeong L S, Lee S K. Inhibition of cell proliferation through cell cycle arrest and apoptosis by thio-Cl-IB-MECA, a novel A3 adenosine receptor agonist, in human lung cancer cells. Cancer Lett 2008; 264: 309-15.

[320] Hausler S F, Del Barrio I M, Diessner J, Stein R G, Strohschein J, Honig A, Dietl J, Wischhusen J. Anti-CD39 and anti-CD73 antibodies A1 and 7G2 improve targeted therapy in ovarian cancer by blocking adenosine-dependent immune evasion. Am J Transl Res 2014; 6: 129-39.

[321] Muller C E, Iqbal J, Baqi Y, Zimmermann H, Rollich A, Stephan H. Polyoxometalates--a new class of potent ecto-nucleoside triphosphate diphosphohydrolase (NTPDase) inhibitors. Bioorg Med Chem Lett 2006; 16: 5943-7.

[322] Zhang B. Opportunities and challenges for anti-CD73 cancer therapy. Immunotherapy 2012; 4: 861-5.

[323] Forte G, Sorrentino R, Montinaro A, Luciano A, Adcock I M, Maiolino P, Arra C, Cicala C, Pinto A, Morello S. Inhibition of CD73 improves B cell-mediated anti-tumor immunity in a mouse model of melanoma. J Immunol 2012; 189: 2226-33.

[324] Stagg J, Beavis P A, Divisekera U, Liu M C, Moller A, Darcy P K, Smyth M J. CD73-deficient mice are resistant to carcinogenesis. Cancer Res 2012; 72: 2190-6.

[325] Zhang B. CD73 promotes tumor growth and metastasis. Oncoimmunology 2012; 1: 67-70.

[326] Allard B, Pommey S, Smyth M J, Stagg J. Targeting CD73 enhances the antitumor activity of anti-PD-1 and anti-CTLA-4 mAbs. Clin Cancer Res 2013; 19: 5626-35.

[327] Hausler S F, Montalban del Barrio I, Strohschein J, Anoop Chandran P, Engel J B, Honig A, Ossadnik M, Horn E, Fischer B, Krockenberger M, Heuer S, Seida A A, Junker M, Kneitz H, Kloor D, Klotz K N, Dietl J, Wischhusen J. Ectonucleotidases CD39 and CD73 on OvCA cells are potent adenosine-generating enzymes responsible for adenosine receptor 2A-dependent suppression of T cell function and NK cell cytotoxicity. Cancer Immunol Immunother 2011; 60: 1405-18.

[328] Nobuoka D, Yoshikawa T, Fujiwara T, Nakatsura T. Peptide intra-tumor injection for cancer immunotherapy: enhancement of tumor cell antigenicity is a novel and attractive strategy. Hum Vaccin Immunother 2013; 9: 1234-6.

[329] Hickey R, Mulcahy M F, Lewandowski R J, Gates V L, Vouche M, Habib A, Kircher S, Newman S, Nimeiri H, Benson A B, Salem R. Chemoradiation of hepatic malignancies: prospective, phase 1 study of full-dose capecitabine with escalating doses of yttrium-90 radioembolization. Int J Radiat Oncol Biol Phys 2014; 88: 1025-31.

[330] Wilczewska A Z, Niemirowicz K, Markiewicz K H, Car H. Nanoparticles as drug delivery systems. Pharmacol Rep 2012; 64: 1020-37.

[331] Sofou S. Surface-active liposomes for targeted cancer therapy. Nanomedicine (Lond) 2007; 2: 711-24.

[332] Bae Y H, Park K. Targeted drug delivery to tumors: myths, reality and possibility. J Control Release 2011; 153: 198-205.

[333] Scott A M, Wolchok J D, Old L J. Antibody therapy of cancer. Nat Rev Cancer 2012; 12: 278-87.

[334] Wang T F, Ou Y, Guidotti G. The transmembrane domains of ectoapyrase (CD39) affect its enzymatic activity and quaternary structure. J Biol Chem 1998; 273: 24814-21.

[335] Abdullah L N, Chow E K. Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med 2013;2: 3.

[336] Szakacs G, Paterson J K, Ludwig J A, Booth-Genthe C, Gottesman M M. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006; 5: 219-34.

[337] Sim G C, Martin-Orozco N, Jin L, Yang Y, Wu S, Washington E, Sanders D, Lacey C, Wang Y, Vence L, Hwu P, Radvanyi L. IL-2 therapy promotes suppressive ICOS+ Treg expansion in melanoma patients. J Clin Invest 2014; 124: 99-110.

[338] Sluyter R, Stokes L. Significance of P2X7 receptor variants to human health and disease. Recent Pat DNA Gene Seq 2011; 5: 41-54.

[339] Wang Q, Li S H, Wang H, Xiao Y, Sahin O, Brady S W, Li P, Ge H, Jaffee E M, Muller W J, Hortobagyi G N, Yu D. Concomitant targeting of tumor cells and induction of T-cell response synergizes to effectively inhibit trastuzumab-resistant breast cancer. Cancer Res 2012; 72: 4417-28.

[340] Schmelzle M, Duhme C, Junger W, Salhanick S D, Chen Y, Wu Y, Toxavidis V, Csizmadia E, Han L, Bian S, Furst G, Nowak M, Karp S J, Knoefel W T, Schulte Esch J, Robson S C. CD39 modulates hematopoietic stem cell recruitment and promotes liver regeneration in mice and humans after partial hepatectomy. Ann Surg 2013; 257: 693-701.

[341] Jaffe J D, Wang Y, Chan H M, Zhang J, Huether R, Kryukov G V, Bhang H E, Taylor J E, Hu M, Englund N P, Yan F, Wang Z, Robert McDonald E, 3rd, Wei L, Ma J, Easton J, Yu Z, deBeaumount R, Gibaja V, Venkatesan K, Schlegel R, Sellers W R, Keen N, Liu J, Caponigro G, Barretina J, Cooke V G, Mullighan C, Carr S A, Downing J R, Garraway L A, Stegmeier F. Global chromatin profiling reveals NSD2 mutations in pediatric acute lymphoblastic leukemia. Nat Genet 2013; 45: 1386-91.

[342] Purvis J E, Karhohs K W, Mock C, Batchelor E, Loewer A, Lahav G. p53 dynamics control cell fate. Science 2012; 336: 1440-4.

[343] Miao R, Luo H, Zhou H, Bu D, Yang X, Zhao X, Zhang H, Liu S, Zhong Y, Zou Z, Zhao Y, Yu K, He L, Sang X, Zhong S, Huang J, Wu Y, Miksad R A, Robson S C, Jiang C, Zhao Y, Zhao H. Identification of prognostic biomarkers in hepatitis B virus-related hepatocellular carcinoma and stratification by integrative multi-omics analysis. J Hepatol May 22, 2014.

[344] Friedman D J, Kunzli B M, YI A R, Sevigny J, Berberat P O, Enjyoji K, Csizmadia E, Friess H, Robson S C. From the Cover: CD39 deletion exacerbates experimental murine colitis and human polymorphisms increase susceptibility to inflammatory bowel disease. Proc Natl Acad Sci U S A 2009; 106: 16788-93.

[345] Mitsiades C S, Mitsiades N. P2X7 polymorphism and chronic lymphocytic leukaemia. Lancet 2003; 361: 1478; author reply 1478-9.

[346] Pikarsky E, Ben-Neriah Y. NF-kappaB inhibition: a double-edged sword in cancer? Eur J Cancer 2006; 42: 779-84.

[347] Sikder H, Huso D L, Zhang H, Wang B, Ryu B, Hwang S T, Powell J D, Alani R M. Disruption of Id1 reveals major differences in angiogenesis between transplanted and autochthonous tumors. Cancer Cell 2003; 4: 291-9.

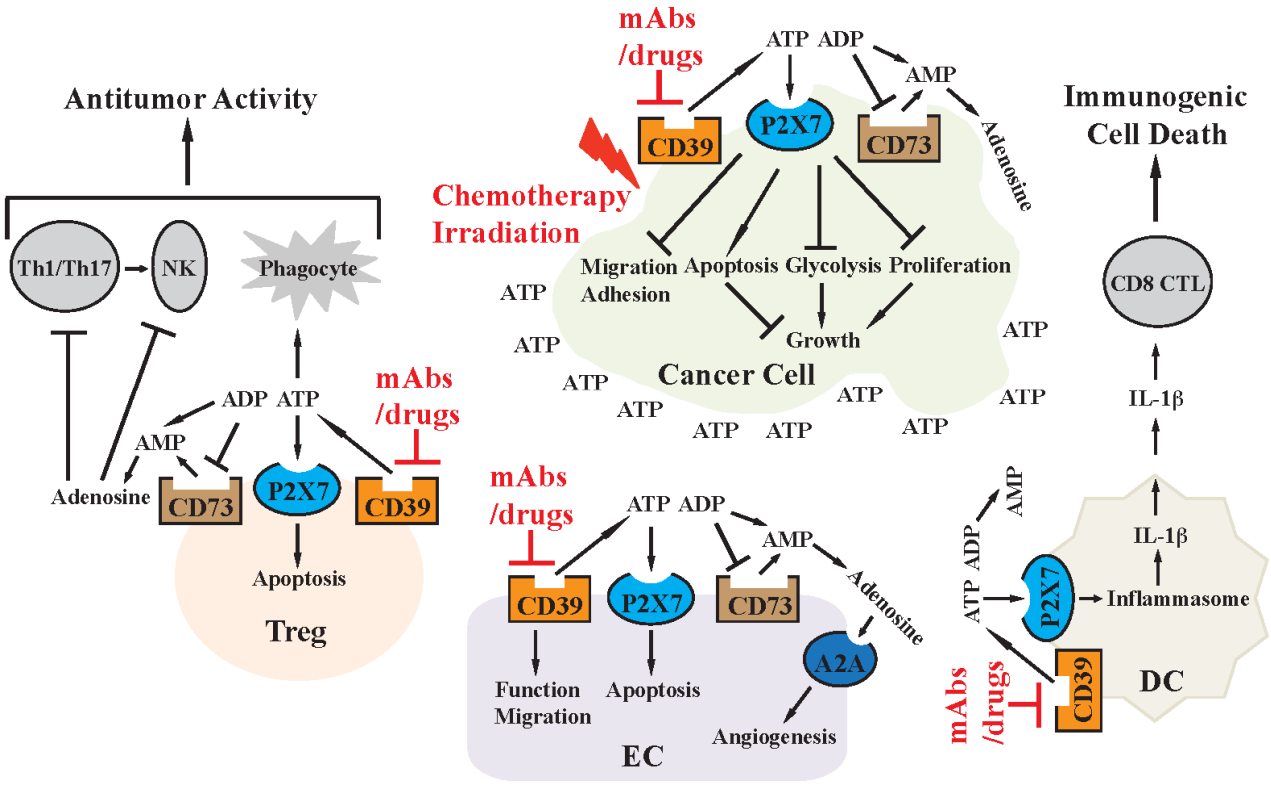

Figure 1: Proposed impacts of CD39 blockade in combination with chemotherapy and/or radiotherapy. Chemotherapy and/or radiotherapy will induce ATP release by cancer cells. Concurrent blockade of CD39 (*via* monoclonal antibodies (mAbs)) or pharmacological inhibitors) will alter the tumor microenvironment by boosting ATP levels and precluding subsequent phosphohydrolysis to tumor-promoting adenosine. Theoretically, this combinational therapeutic approach addresses several distinct aspects of tumor biology, *viz.*, limiting angiogenesis, augmenting antitumor immune responses, and directly killing cancer cells per se, thereby achieving maximal anticancer activity.

Pathway key: →: Stimulation; ⊥: Inhibition.

ANEXO II

Marina Gehring <marinapgehring@gmail.com>

Successful Submission of a Manuscript to Science

1 message

science_editors@aaas.org <science_editors@aaas.org> Wed, Sep 2, 2015 at 7:00 PM
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Manuscript Title: Cabozantinib eradicates advanced murine prostate cancer by activating anti-tumor innate immunity
Author: Gehring
Manuscript Number: aad3138

Dear Dr. Gehring:

You are listed as a coauthor on the above manuscript, which has recently been submitted to Science. According to Science policy, all authors must have seen and approved the submission of their manuscript. If you have seen the manuscript and approved its submission, no action is necessary.

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