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Immunomodulator plasmid projected by systems biology as a candidate for the development of adjunctive therapy for respiratory syncytial virus infection

José Eduardo Vargas [a,*], Ana Paula Duarte de Souza [a], Bárbara Nery Porto [a], Tiago Fazolo [b], Fabiana Quoos Mayer [c], Paulo Márcio Pitrez [a], Renato Tetelbom Stein [a]

[a] Centro Infant – Pontifícia Universidade Católica do Rio Grande do Sul (PUCRS), Av. Ipiranga, 6690 2° andar, 90610-000 Porto Alegre, RS, Brazil
[b] Departamento de Biologia Celular e Molecular, FABIO, Instituto de Pesquisas Biomédicas, PUCRS, Av. Ipiranga, 6690 2° andar, 90610-000 Porto Alegre, RS, Brazil
[c] Laboratório de Biologia Molecular, Instituto de Pesquisas Veterinárias Desidério Finamor, Fundação Estadual de Pesquisa Agropecuária, Estrada do Conde, 6000, Eldorado do Sul, RS 92990-000, Brazil

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ABSTRACT

An imbalance in Th1/Th2 cytokine immune response has been described to influence the pathogenesis of respiratory syncytial virus (RSV) acute bronchiolitis and the severity of infection. Th2-driven response has been well described under first RSV vaccine (formalin-inactivated RSV vaccine antigens) and replicated in some conditions for RSV-infected mice, in which a Th2-dependent lung eosinophilia increases illness severity, accompanied of tissue damage. Currently, several prototypes of RSV vaccine are being tested, but there is no vaccine available so far. The advance of bioinformatics can help to solve this issue. Systems biology approaches based on network topological analysis may help to identify new genes in order to direct Th1 immune response during RSV challenge. For this purpose, network centrality analyses from high-throughput experiments were performed in order to select major genes enrolled in each T-helper immune response. Thus, genes termed Hub (B) and bottlenecks (H), which control the flow of biological information (Th1 or Th2 immune response, in this case) within the network, would be identified. As these genes possess high potential to promote Th1 immune response, they could be cloned under regulation of specific promoters in a plasmid, which will be available as a gene-transfer adjunctive to vaccines. Th1 immune response potentiated by our strategy may contribute to accelerate Th1/Th2 shift from neonatal immune system, which might favor protective immunity against RSV infection and reduce lung damage.

Introduction

Respiratory syncytial virus (RSV) is the single major cause of viral lower respiratory tract infection with high levels of hospitalization and mortality in children around the world [1–4]. It is estimated that all infants by two years of age have been infected by RSV and more than a half of them are re-infected [5]. Acute bronchiolitis is commonly associated with RSV infection in children under 1 year of age, which is associated with subsequent asthma diagnosis later in life [6–8]. Additional, in immunocompromised infants, RSV infection may also increase the risk of longer hospitalization periods [9].

The treatment of RSV infection is highly dependent on symptom presentation and severity. RSV treatment is based on supportive care, requiring adequate hydration, use of antipyretics and ventilator support. Other conventional treatments based on administration of bronchodilators and corticosteroids have not been shown to be effective for the majority of cases [10].

RSV preventive treatments would reduce morbidity and potential costs for patients and societies. However, many difficulties due to the complexity of anti-viral immune responses impair the development of cost-effectiveness therapies. Currently, the prophylactic therapy is based on the treatment with human monoclonal antibody palivizumab, which is a high-cost therapy for high-risk infants [11].

Natural RSV infection does not promote long-term protection [12], possibly due to the ability of RSV to interfere in host immunity [13]. A few trials were performed on vaccine development, but deaths occurred with intervention, and high eosinophilic

* Corresponding author at: Centro Infant – PUCRS, Av. Ipiranga, 6690, CEP 90610-000 Porto Alegre, RS, Brazil. Tel.: +55 51 3320 3000.
E-mail address: josevargas123@gmail.com (J.E. Vargas).

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infiltrates on lower respiratory tracts were observed [10,13]. Currently, several prototypes of vaccines are under study. The majority of vaccines are based on the use of RSV viral peptides [14,15], live attenuated viruses [15,16] and RSV subunit particles [17]. These new vaccines are focused to produce efficient humoral and cellular immune responses, which would result in lower lung inflammation and higher protection against RSV infection. However, until the present date there is no licensed RSV vaccine available, supporting the search for different approaches towards a more effective RSV-specific preventive therapy.

Expanding the knowledge of immunomodulation may enhance our capacity to develop novel therapies to direct a specific immune response. In this sense, systems biology, an interdisciplinary approach that analyses complex interactions as parts of a biological system, can be applied to elucidate new intracellular proteins that could promote or inhibit specific CD4 T cell responses. Thus, this approach may provide gene candidate to develop new adjuvants for use in humans.

Hypothesis

We hypothesize that an immunomodulator plasmid can be produced based on selection of key endogenous genes that direct the immune response to Th1 immune profile during RSV infection. Hence, the aim of this paper is to use systems biology strategies in combination with DNA recombinant technologies to generate hypothesis for preventive strategy to RSV acute bronchiolitis. Our hypothesis will be supported in two interrelated sections: Th1/Th2 balance explored by systems biology networks and development of an immunomodulatory plasmid for gene transfer.

Th1/Th2 balance explored from systems biology networks

Some questions must be performed to understand *in silico* strategy proposed in this section.

What is the importance of differentiation of CD4+ T cells during RSV infection?

When the host reacts against RSV infection, the ensuing immune response involves a complex set of cellular responses distributed across many different types of cells. However, CD4+ T cells differentiation plays a major role during the control of RSV infection. CD4+ T cells are responsible for RSV clearance (when Th1 immune response is induced) or disease (when Th2 immune response is induced).

Viral clearance requires Th1 polarization driven partially by IL-12-secreting mature dendritic cells, which promote activation of IFN-γ-producing CD4+ T cells. IFN-γ, in turn, promotes cytotoxic T cell function by stimulating CD8+ T cells and NK cells in order to clear virus-infected cells [18]. Additionally, these Th1 cells stimulate macrophage phagocytic activity to promote clearance of dead cells and induce production of neutralizing IgG antibodies by B cells [19,20]. However, during RSV infection, high Th2 immune response is reported, accompanied of excessive mucus production [21], IgE production [22], airway hypersensitivity and lung injury, after a second exposure to the virus (high numbers of eosinophils were detected and appear to be associated with Th2 memory response activation) [23]. This finding was reported during the development of a first model of RSV vaccine, in which FI-RSV was inefficient in inducing IFN-γ-secreting NK cells and CD8+ T lymphocytes [18]. Clinical evidences confirm an association between severe pediatric RSV disease and elevated ratios of Th2/Th1 response, in which Th2 cytokines are detected in higher levels in nasal secretions or peripheral blood mononuclear cells stimulated by RSV [24–26].

Are Th1 and Th2 signaling pathways related?

Several studies define cell signaling pathways for Th1 or Th2. Originally, CD4+ Th1 cells express STAT4, which induces the transcription factor T-bet (known as a master regulator of Th1 cells) [27]. T-bet is induced by TCR signaling and is strongly enhanced by STAT1 transcription factor activation, which occurs as a positive feedback loop in response to auto/paracrine produced IFN-γ [28]. One of the genes induced by T-bet encodes Runx3, which in association with T-bet, binds to several enhancers and the promoter of the *Ifng* gene, inducing its transcription [29]. Runx3 and T-bet also bind to a silencer in interleukin (IL)-4 gene, resulting in a transcriptional repression of this gene [29]. On the other hand, Th2 cells express Gata3 (Th2 master regulator) that inhibit Th1 response by IL-4 production [30]. Today, it is recognized that these cellular types possess plasticity, and the expression of a master regulator is insufficient to define cell phenotype. Several signal pathways and the cellular microenvironment are linked with this process. Novel systems biology tools may help to organize this complex information in hierarchical levels, leading to a co-evolution of interrelated complexity of Th1 and Th2 signaling and to develop a possible DNA immunomodulator.

How applied systems biology may develop an immunomodulator based on CD4+ T biology?

We suggest a series of logical steps to test this hypothesis, which is detailed in Fig. 1. Firstly, human or mouse naive CD4+ T cells would be isolated from peripheral blood mononuclear cells (PBMCs); alternatively, spleen cells could be the source of naive T cells. Several protocols and commercial kits are available for naive CD4+ T cell isolation [31–34]. A posterior step involves *in vitro* naïve CD4+ T differentiation into Th1 or Th2 phenotypes. Specific cytokines could be applied to direct selective Th differentiation, such as IL-12 for Th1 [35] and IL-4 for Th2 [36].

The next step includes sample preparation for high-throughput sequencing (RNA-seq or microarray) and proteomic (mass spectrometry) analysis, which will allow obtaining global cell signaling expression of each *in vitro*-differentiated T cell type. For microarrays, we suggest to employ DNA chips with a high-density of genes, not recommending commercial chips, including probes of specific cell-signaling pathways. This may limit the search for genes differentially expressed that would act as unexplored regulators of Th1 or Th2 cell signaling.

After processing high-throughput expression data, Th1 and Th2 protein–protein interacting (PPI) networks could be performed through bioinformatics meta-search servers [37,38]. Studying these PPI networks will help to understand the flow of information through the network by relevant nodes (proteins or genes). In this sense, a network centrality analysis allows us to identify genes that have a relevant position in the overall network architecture with high probability to be candidates in order to direct Th1 effector pathways. Two major network centralities can be evaluated: node degree and betweenness. Node degree correspond to the number of nodes adjacent to a given node, in which adjacent means directly connected [39]. According to this definition, highly connected nodes in a network are termed hubs. On the other hand, betweenness indicates in what extent a specific node is between the others within the network [40]. Bottleneck is a term applied to define all nodes with high betweenness values, indicating that they are central points that control the communication between other nodes within the network. Thus, H-Bs of Th1 PPI network could be used to develop a recombinant plasmid to stimulate

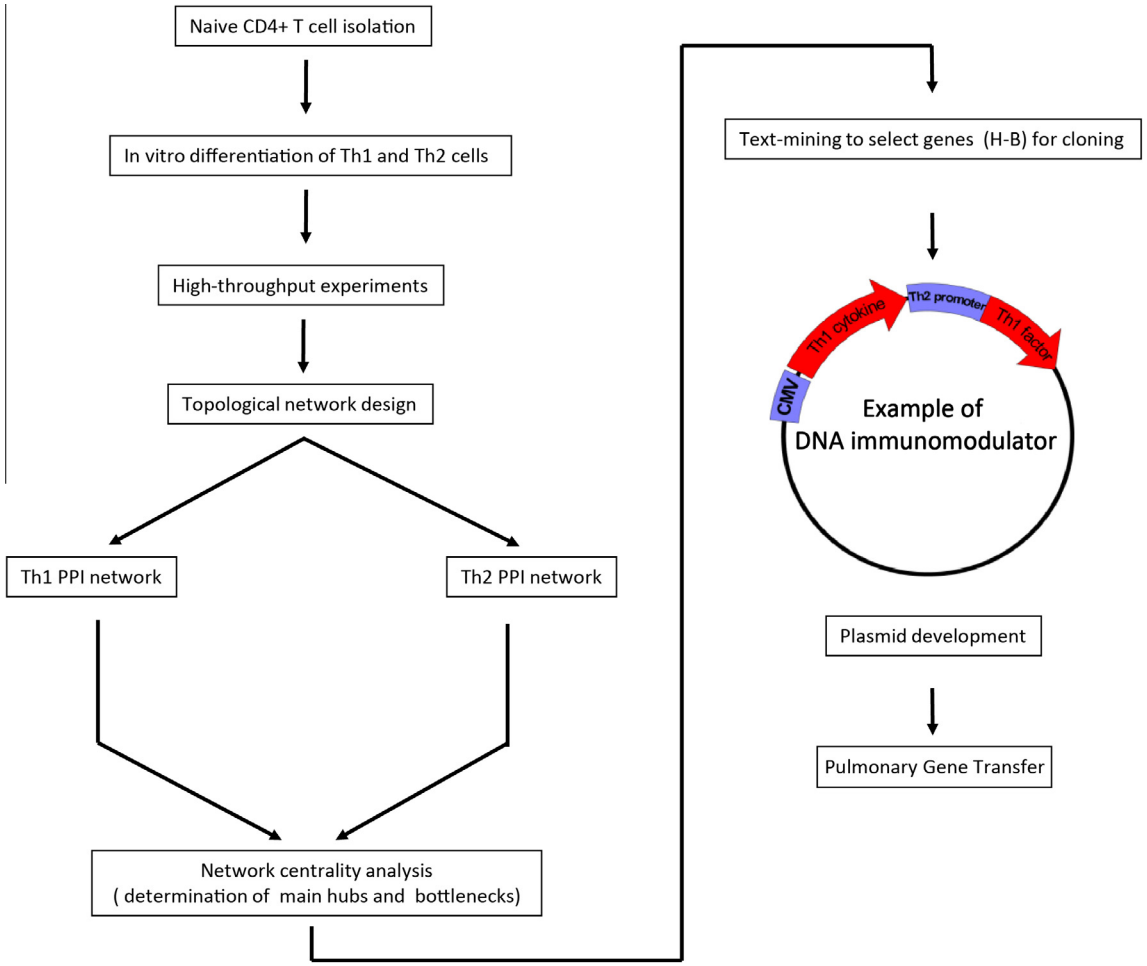

Fig. 1. Schematic representation of steps to immunomodulatory plasmid development. *In vitro* naïve CD4+ T cells are differentiated in Th1 or Th2. High throughput experiments can be then developed to obtain Th1 or Th2 signatures. These data can be used to PPI networks development and can be submitted to network topological analysis. Finally, text-mining can define hub-bottlenecks genes to be cloned in a plasmid backbone in order to promote Th1 immune response. The plasmid proposed in this figure is an example, where an H-B Th1 cytokine is under regulation of citomegalovirus promoter (CMV), which robustly drove transgene expression. Additionally, DNA Th2 promoter to drive H-B Th1 differentiation factors, in the same plasmid; thus, the immunomodulatory plasmid can be expressed in a selective manner, when transfected to activated Th2 cells during RSV infection.

Th1 response. However, topological analysis and the knowledge of Th2 network are necessary to an adequate choice of H-Bs to be cloned. In this sense, we suggest to use textmining tools to understand how H-Bs nodes of Th1 PPI network inhibit H-Bs nodes from PPI Th2 network (Fig. 2). Commonly, text-mining is applied to define stimulatory or inhibitory mechanisms between genes or proteins [41–45]. Thus, H-Bs candidates for cloning would be selected according to their inhibitory effect on Th2 PPI network.

Development of a DNA plasmid for gene transfer therapy

In order to generate a vector plasmid containing H-Bs, the knowledge on the lung biology and its immune microenvironment is essential. Thus, some fundamental questions must be answered to ensure the functioning of the plasmid proposed.

For generating functional immunomodulatory plasmid, what would be the minimum number of genes to be expressed or repressed?

Recent studies suggest that the phenotype of Th cells is not based on a single master regulator expression (above described). According it, the function of the 'master regulators' is different from the classic molecular cell biology ones (e.g. MyoD expression can promote muscle differentiation) [46]. In T cells, the master regulators are not sufficient to induce a phenotype. Expression of one master regulator, defined as classical for a specific Th cell, is frequently described in diverse T cell types. It may be more exact to think on co-expressed master regulators [47]. Hence, a minimal number of two H-B genes should be cloned into a plasmid to promote Th1 phenotype.

Is the size of plasmid a problem for a possible gene transfer therapy?

Cloning a high number of genes increase the size of plasmid DNA molecules, which may become a difficulty for some gene transfer protocols. However, plasmids with a size up to 20 kbp were successfully *in vivo* transferred [48], showing that it is not a limiting factor to test our hypothesis. In addition, molecular biology strategies are available to reduce plasmid size, if necessary, which allows the expression of a large number of genes, in a cap-independent form, under the regulation of a single promoter. For example, the use of internal ribosome entry site (IRES) sequence [49–51] or 2A sequence are based on this principle [52–54]. Polycistronic plasmids using these sequences showed efficient transgene expression in different lung models [55–57].

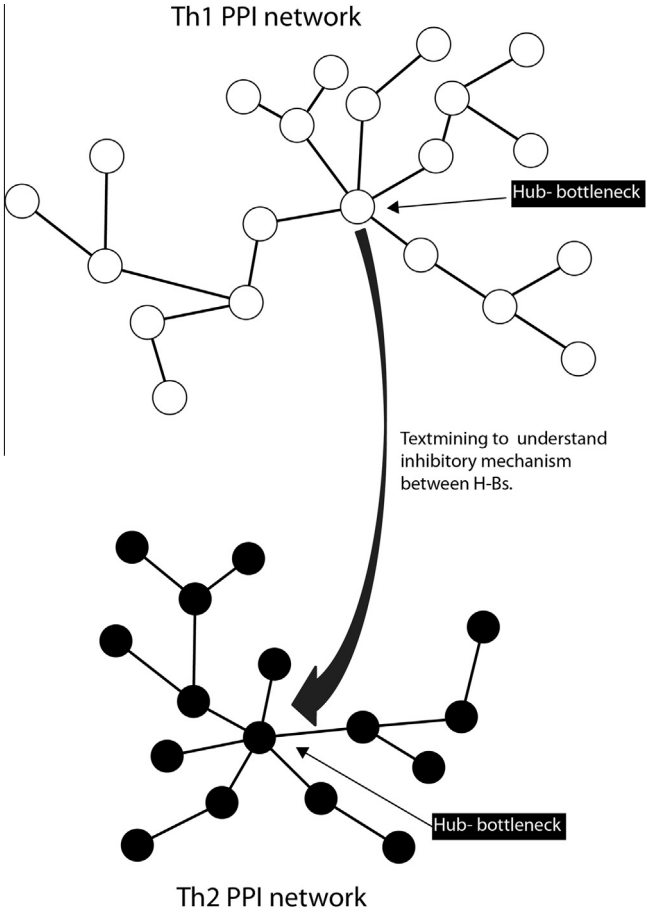

Fig. 2. Hypothetical Th PPI networks showing H-Bs nodes relation from single topological structures. Textmining can be performed in order to choose Th1 H-Bs genes with strong impact on Th2 PPI network.

Is our hypothesis a versatile strategy?

It is important to note that the immunomodulatory plasmid suggested in Fig. 1 is just an example. A variety of other immunomodulatory plasmids (monocystronic or polycistronic) may be developed using different promoters or H-B genes selected based on our strategy.

Which is a better route for gene transfer administration?

According to the literature, plasmid inhalation probably would be a better option for *in vivo* gene transfer. This is a non-invasive manner to rapidly deliver DNA directly into the airways. Two methods are frequently used in the airway gene transfer protocols, DNA aerolization and nebulization of liquid-suspended gene particles. The first option was used to correct specific genetic disorders, such as α-1 antitrypsin deficiency [58] and other non-genetic lung diseases, such as pulmonary hypertension [59] and acute lung injury [60]. The second alternative has low efficiency, but remains an alternative for inhaled-gene transfer in lungs, as demonstrated in cystic fibrosis [61].

What is the importance of our hypothesis?

The possibility to direct Th1 immune response using systems biology for driven plasmid design would be a faster way to guide therapeutic gene therapy against RSV infection. In conclusion, we propose a system that might help to maintain the integrity of the lung tissue affected by a Th2-biased immune response during RSV infection. If functional, similar therapies can be developed using systems biology strategy to develop new immunomodulatory plasmids to improve immune response against other viral infections. In addition, the flexibility of our strategy allows easy adapting to direct immune response of other CD4+ T cell types or a different lymphoid lineage, which can increase their potential for biotechnology and therapeutic applications.

Conflict of interest statement

The authors declare that no competing interests exist.

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