

## Triggering of Toll-like receptors in the elderly. A pilot study relevant for vaccination

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### Abstract

The impaired ability of the elderly to mount an efficient immune response after exposure to microbes or vaccines represents a major challenge in protection against pathogens in ageing. Recently studies have shown that stimulation of Toll-like receptors (TLRs), using stimulatory ligands, can enhance vaccine efficacy by a number of mechanisms, including the activation of innate immune cells and the consequent production of inflammatory cytokines. Since TLR stimulation is a key regulator of the type and magnitude of the immune response, we evaluated cytokine production in dendritic cell populations upon stimula-

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tion with two complementary TLR agonists, R848 and MPLA. Our preliminary results demonstrate that TLR activation by this combination of agonists can significantly enhance the activation of dendritic cells in the peripheral blood isolated from healthy elderly donors. This data suggest that the inclusion of appropriate combination of TLR agonists may enhance the efficacy of vaccination in the elderly.

**Key Words**

Ageing, Cytokines, TLR, Vaccination.

## Introduction

Increasing age is accompanied by a progressive decline of both the innate and acquired immune system, noted as “Immunosenescence” [Caruso & Vasto, 2016].

The impact of ageing on the immune system typically includes intrinsic defects within immune cells as well as alterations in number and activity, and possibly defects in the bone marrow and thymic stromal microenvironment. This phenomenon results in a reduction of naive T and B cells, leading to inefficient primary responses of immune effector cells to pathogens, as well as reduced T cell cytotoxicity, proliferation and cytokine production, and defective memory responses in the elderly population [Larbi et al., 2008; Caruso et al., 2009; Nikolich-Zugich & Rudd, 2010]. Consequently, aged individuals exhibit increased incidence of infectious diseases, cancer and autoimmune diseases [McElhaney et al., 2012]. In addition, their aged immune system does not respond to stimuli as efficiently as that of younger adults, therefore current vaccines are less effective in the elderly [Derhovanessian & Pawelec, 2012].

Research in immunological ageing seeks not only to understand the age-related disorders of immune regulation, but also to identify new efficient strategies for immune rejuvenation and for effective vaccination induced immunity in the elderly. One such strategy would be to develop vaccines comprising suitable adjuvants to enhance the impaired cellular immune responses [Wells et al., 2008].

Adjuvants are molecules that stimulate the non-specific, innate immune responses, inducing the activation of antigen presenting cells (APCs) and their recruitment to the site of vaccination. Dendritic cells (DCs) are the most potent APCs, specialized in the uptake, processing, transport and presentation of antigens to T cells [Collin et al., 2013]. After their activation in the periphery, DCs migrate to lymphoid tissues where they interact with T and B cells to initiate and shape the acquired immune responses.

DCs in human blood are defined as Lineage 1 (CD3, CD14, CD16, CD19, CD20, and CD56)-negative and HLA-DR-positive cells. DCs can be divided into three subsets according to the expression of various markers (CD123, CD1c, CD141): one subset of plasmacytoid DCs (pDCs), and two subsets of myeloid DCs (mDCs). pDCs are characterized by the expression of CD123 marker and possess the capacity to produce high levels of type I

Interferons (IFN- $\alpha/\beta$ ). In contrast, mDCs express the CD11c marker and are divided into two subsets: CD1c+ mDCs and CD141+ mDCs [Collin et al., 2013]. Upon stimulation, mDCs secrete mainly IL-6, IL-12, and TNF- $\alpha$ .

Both pDCs and mDCs express toll-like receptors (TLRs) that recognize conserved molecular patterns on microbes and are key regulators of antimicrobial host defence responses. Recognition of microbial components by TLRs culminates in the secretion of type I IFNs and pro-inflammatory cytokines that facilitate the linkage of innate to acquired immune responses. Deficiencies in human TLR signalling lead to increased severity of multiple immunological disorders, including sepsis, immunodeficiencies, atherosclerosis and asthma [Cook et al., 2004].

Recently, stimulation of TLRs by adjuvants has been shown to be a promising strategy to enhance vaccine efficacy against both foreign and self, tumour-associated, antigens in aged mice by activating innate immune cells and enhancing production of inflammatory cytokines [Tye et al., 2015].

On the basis of these promising results in mice, we have investigated the ability of combined TLR ligands to induce pro-inflammatory responses in the peripheral blood dendritic cells isolated from healthy donors with evidence of immunosenescence.

## Material and Methods

### *Samples*

A total of 23 samples, including five centenarians, five centenarian offspring (CO), six old donors and six young donors used as controls, were processed. All participants were in good health according to their clinical history and none of them had infectious, inflammatory, neoplastic or autoimmune diseases at the time of the study. The University Hospital Ethics Committee approved the study, and written informed consent was obtained from all participants according to Italian law. Whole blood was collected by venepuncture in vacutainer tubes containing ethylenediaminetetraacetic acid. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on Lympholyte® (Cedarlane, Canada, United States) and viably cryopreserved according to standard protocols.

### *DCs stimulation assay*

Total PBMCs were cultured at  $1 \times 10^6$  cells/well on a 96 U-bottom plate. Cells were plated with a combination of two adjuvants, chosen

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based on our preliminary experiments, in the presence of 1x Golgi Plug solution (BD Biosciences, Erembodegem, Belgium). Specifically, we tested the following TLR agonists: the TLR7/8 agonist R848, at 3 µg/ml, and the TLR4 agonist MPLA, at 10 µg/ml (InvivoGen, San Diego, California). For each donor, cells were also left unstimulated and served as controls. After 6 hours of incubation cells were transferred to labelled FACS tubes and stained with antibodies. We chose to study total PBMCs, rather than purified DCs, to minimize manipulations that might result in partial activation of DCs, and because purification procedures would substantially decrease the yield of cells for analysis.

#### *Flow cytometry and cell sorting*

Cells were stained with antibodies to human CD3, CD14, CD16, CD19, CD20, CD56 (Lineage 1, BD Biosciences, Erembodegem, Belgium), HLA-DR (clone L243, Biolegend, San Diego, California), CD123 (clone 6H6, Biolegend), CD1c (clone L161, Biolegend), CD11c (clone 3.9, Biolegend), and CD141 (clone 1A4, BD Biosciences) for extracellular staining. For intracellular staining, cells were then washed, fixed with permeabilization/fixation buffer (BD Biosciences) and stained with antibodies to human IL-6 (clone MQ2-13A5, Biolegend), TNF- $\alpha$  (clone Mab11, Biolegend), and the p40 subunit of IL-12/23 (clone C11.5, Biolegend). Samples were analysed by flow cytometry on a LSR Fortessa™ (BD Biosciences). FACS data were analysed and plotted using FlowJo software (Tree Star).

#### *Statistics*

Statistical analyses were performed using GraphPad Prism software; the significant level of p value was determined using Student t test. (\*  $p < 0.05$ , \*\*  $p \leq 0.01$ , \*\*\*  $p \leq 0.001$ )

## Results and Discussion

Given that DC activation is a key regulator of the magnitude and nature of the elicited adaptive immune responses, we evaluated whether TLR ligands could effectively activate naturally occurring, circulating DCs. To this end, we stimulated total PBMCs, derived from young, aged, centenarian and CO participants, with a combination of two TLR ligands. The nature and magnitude of cytokine production was assessed using multi-parameter flow cytometry.

Our preliminary *in vitro* screening experiments suggest that from the various TLR agonists tested, the condition that most effectively activated human leukocytes was the combination of TLR7/TLR8 with TLR4. This TLR agonist combination induces significantly greater cytokine production than that induced by each of the individual agonist. This greater stimulation is probably due to the combined activation of both Myd88 and TRIF-dependent signal transduction pathways.

Figure 1 shows an example of our analysis; after excluding doublets, we gated the Lineage (consisting of a cocktail of antibodies against CD3, CD14, CD16, CD19, and CD56)-negative, HLA-DR-positive population, with mDCs and pDCs identified as CD11c and CD123 positive cells, respectively. Subsequently, using intracellular cytokine staining, we evaluated the selected populations for the production of TNF- $\alpha$ , IL-6 and IL-12p40.

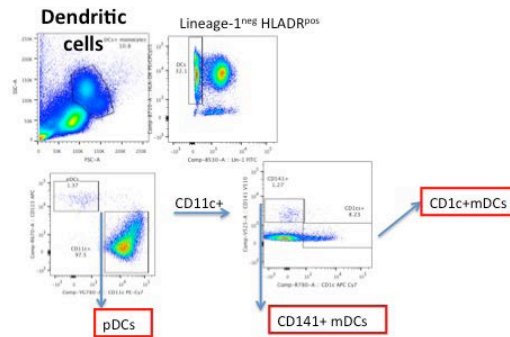


Figure 1. Characterization of human dendritic cells and their subsets. Phenotype of human DCs analysed by flow cytometry. Cells were gated on Lineage-1 negative HLA-DR-positive population by a cocktail of antibodies against CD3, CD14, CD16, CD19, and CD56. Subsequently, mDCs and pDCs were identified using staining against CD11, CD123, CD1c and CD141.

First, we report that the stimulation of cells with the combination of agonists for TLR7/8 and TLR4 is *de facto* an excellent inducer of TNF- $\alpha$  and IL-12/p40 cytokines in the CD141+ mDCs from young, old and CO subjects, with significantly high levels of cytokines compared to unstimulated samples ( $p < 0.05$ ,  $p \leq 0.01$ , and  $p \leq 0.001$ ; respectively). No significant variation was observed in centenarians (Figure 2A). This, probably, is due to the age-related lower viability and low number of centenarian samples. Nevertheless, we have reported that the difficulties to study centenarians can be overcome by studying CO that are one generation (about 20-30 years) younger than centenarians and are representative of an elderly cohort, characterised by a better functional status and a reduced risk for several age-related pathologies [Balistreri et al., 2014]. Focusing on old people, we observed that both Italian CO and elderly, showed elevated percentage of IL-12/p40 and TNF- $\alpha$  after treatment with the TLR ligands R848 (TLR7/8) and MPLA (TLR4); these differences were highly statistically significant compared to unstimulated cells.

Notably, the combination of R848 and MPLA induce 5–10 fold higher production of IL-12/p40 in CD141+ mDCs isolated from old and CO samples compared with their young counterparts ( $p \leq 0.01$ ) (Figure 2A).

In addition, increased amounts of TNF- $\alpha$ , were also observed in CD1c+ mDCs and pDCs from older and CO subjects, in response to R848 and MPLA stimulation. These differences were statistically significant when compared to their unstimulated counterparts (Figure 2B).

Taken together, the data presented suggest that the combination of R848 and MPLA effectively promotes *in vitro* cytokine production in human DCs isolated from elderly, despite their immunosenescent phenotype.

To date, data regarding the influence of ageing on human DCs activity and cytokine production, in response to *in vitro* stimulation, has been inconsistent, showing either comparable or reduced DC function in the elderly [Lung et al., 2000; Pietschmann et al., 2000; Shurin et al., 2007]. Tan et al., [2012] report that human DCs isolated from both young and aged individuals exhibit comparable activation in response to most TLR ligands, and are equally capable of direct and cross-presentation of antigens to T cells *in vitro*. On the contrary, You et al., [2013] demonstrated a reduced production of TNF- $\alpha$  by DCs from old people in response to LPS stimulation.

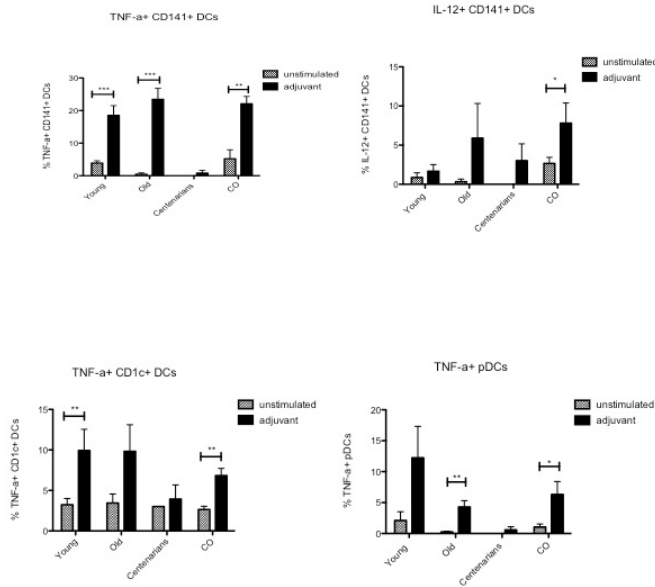


Figure 2. Cytokine secretion by DCs. (A) TNF- $\alpha$  and IL-12 secretion from CD141+ mDCs in response to TLR7/8 and TLR4 stimulation in vitro. PBMCs were cultured in the absence or presence of combined TLR7/8 and TLR4 ligands (unstimulated and adjuvants, respectively). After incubation, CD141+ mDCs were identified by flow cytometry analysis. TNF- $\alpha$  and IL-12 were evaluated using intracellular staining and analysed by FlowJo. The data report the percentage of cytokines produced from young, elderly, CO and centenarian samples after incubation. Statistical significance between the groups has been reported as \*p, 0.05, \*\*p, 0.01, \*\*\*p, 0.001. (B) TNF- $\alpha$  secretion from pDCs and CD1c+ mDCs in response to TLR7/8 and TLR4 stimulation in vitro. PBMCs were cultured in the absence or presence of combined TLR7/8 and TLR4 ligands (unstimulated and adjuvants, respectively). After incubation, pDCs and CD1c+ mDCs were identified by flow cytometry analysis. The data report the percentage of TNF- $\alpha$  produced from each sample group after incubation. Statistical significance between the groups has been reported as \*p, 0.05, \*\*p, 0.01, \*\*\*p, 0.001.



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In our study, stimulation with the specific combination of TLR agonists, R848 and MPLA, induced significantly higher cytokine secretion by mDCs and pDCs from both elderly and CO subjects. This has potentially important implications, since it has been reported that reduced production of TNF- $\alpha$  by pDCs from old people, caused by defects in TLR signalling pathways, is associated with an ineffective antibody response to influenza vaccination [Panda et al., 2010].

The involvement of TNF- $\alpha$  in DC-induced T cell proliferation is also evident from clinical data of rheumatoid arthritis patients, showing that treatment with anti-TNF- $\alpha$  antibodies cause poor stimulation of T cell activity by DCs [Baldwin et al., 2010; Liu et al., 2012]. Thus, impaired production of TNF- $\alpha$  by older DCs could result in a weak response to vaccination and may contribute to the dysregulation of DC-induced T cell proliferation in the elderly subjects.

## Conclusion

Our findings highlight the efficient effect of adjuvant in stimulation of cytokine production, and point towards the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for the elderly.

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