EXPERT OPINION

Dual effect of Thymosin α 1 on human monocyte-derived dendritic cell *in vitro* stimulated with viral and bacterial toll-like receptor agonists

Elena Giacomini, Martina Severa, Melania Cruciani, Marilena Paola Etna, Fabiana Rizzo, Manuela Pardini, Carolina Scagnolari, Enrico Garaci & Eliana Marina Coccia[†]

[†]*Istituto Superiore di Sanità, Viale Regina Elena 299, 00161, Rome, Italy*

Objectives: Thymosin α 1 (Tα1) recently gained interest as immune adjuvant for vaccines because of its ability to modulate the T-cell/dendritic cell (DC) axis and to improve antibody production. The objective of this study was to determine whether Tα1 would address *in vitro* the response of human primary monocyte-derived DC, crucial regulators of vaccine-induced immunity, upon exposure to different toll-like receptor (TLR) agonists or infection with viruses or bacteria.

Methods: DC maturation and production of pro-inflammatory cytokines were analyzed.

Results: Our data revealed a dual effect of Tα1 on DC biology upon viral or bacterial stimulation. Interestingly, Tα1 enhanced human leukocyte antigen (HLA)-I and II surface expression and secretion of IL-6, TNF-α and IL-8 when DCs were treated with viral TLR3 and TLR7/8 agonists. Similarly, in pandemic H1N1 influenza A-infected DCs, Tα1 raised the expression of maturation markers and type I and III Interferon (IFN). In contrast, following bacterial TLR2 and 4 stimulation, as well as upon Bacillus Calmette–Guerin infection, the presence of Tα1 in DC cultures drastically lowered the analyzed cellular parameters.

Conclusion: The knowledge that Tα1 pleiotropic effect might ameliorate antiviral immune responses and, at the same time, dampen inflammation caused by bacterial infections could lay the groundwork for a more appropriate therapeutic application of this molecule.

Keywords: dendritic cell, human, Thymosin α 1, toll-like receptor

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1. Introduction

Thymosin α 1 (Tα1) is a naturally occurring thymic peptide first described and characterized by Goldstein [1]. In the form of a synthetic 28-mer amino-terminal acetylated peptide, this compound, displaying an excellent safety profile [2], has been used worldwide as an adjuvant or immunotherapeutic agent to treat the more disparate human diseases, including viral infections [3-5], immunodeficiencies [6] and malignancies [7,8].

Tα1 is a context-dependent molecule exceptionally capable of multi-targeted interactions, for which many molecular mechanisms have been described leading to promotion of innate and adaptive immunity [9,10]. In this regard, effects of Tα1 were shown on different immune cell subsets, most importantly on

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subpopulations of T cells [11] and on dendritic cells (DC) [12,13]. In particular, DC are widely distributed at various sites throughout the body and are considered sentinels of microbes, of which they can acquire and process antigens, thus, becoming activated and undergoing maturation [14]. Matured DC upregulate co-stimulatory molecules and secrete a plethora of pro-inflammatory cytokines thereby, upon migration to regional lymph nodes, interacting and instructing T-cell differentiation and initiating cellular and humoral immune responses.

In an effort to design effective immunotherapies and vaccines for treating cancer and/or infectious diseases, it is, thus, attractive to understand and manipulate the DC activation process and, in turn, to modulate immune responses. One of the most basic mechanisms for activation of the DC system is through toll-like receptors (TLRs), highly conserved pattern recognition receptors ubiquitously expressed from epithelial to immune cells. TLRs recognize conserved structures of either non-self- or self-identity, in particular pathogen-associated molecular patterns [15] but also endogenous molecules created upon tissue injury, called damage-associated molecular patterns [16,17]. In addition, TLRs sample different compartments within the cells. TLRs involved in the recognition of nucleic acids (TLR3, TLR7, TLR8 and TLR9) of self and non-self origin are intracellularly localized within endolysosomes, whereas other TLR family members (TLR1, TLR2, TLR4, TLR5 and TLR6), triggered by lipidic or proteic structures of microbial or danger source [17], are found at the cell surface [18]. Interestingly, cellular localization of different TLRs may have important consequences not only on ligand accessibility but also on downstream signaling events leading to diverse immune signatures.

In DC, TLR triggering by viral, bacterial or fungal pathogens stimulates the activation of a complex intracellular cascade ultimately leading to transcription of hundreds of genes, production of pro-inflammatory cytokines and induction of innate effector mechanisms.

Tα1, besides modulating directly DC differentiation and functional maturation [12], was shown to regulate TLR signaling at different levels mainly in murine DC, being at the intersection between canonical and noncanonical NF-κB and JNK/p38/AP-1 pathways on one side [13,19] and IFN-regulatory factors on the other side [20]. Interestingly, the immunomodulatory effects of Tα1 on DC correlated with a therapeutic effect in experimental fungal or viral infections [13,20].

Because of the key role of this cell type in the promotion of vaccine-induced immunity, we recently demonstrated the efficacy of a human primary DC-based *in vitro* setting to test the immunogenicity of vaccine candidates by studying the live-attenuated *Mycobacterium tuberculosis* SO2 strain, recently entered in clinical trial [21].

Collectively, these evidences provided a solid scientific ground for testing Tα1 as a DC-directed adjuvant for novel vaccines or therapies. This is also in light of the increasing interest gained in recent years by Tα1 as immune adjuvant for vaccines [22]. In particular, Tα1 therapeutic action was examined for its potential to enhance influenza virus (Flu) vaccine responses in difficult-to-treat individuals, such as those immune-suppressed due to age or hemodialysis, given that, despite a largely diffuse vaccination protocol, this virus still remains a prominent cause of morbidity and mortality in these populations [23]. Flu infection, directly or indirectly either by promoting bacterial super-infection leading to secondary pneumonia, principally caused by *Streptococcus pneumonia* or *Staphylococcus aureus*, or exacerbating cardiovascular diseases, is indeed considered a major threat worldwide.

Based on this knowledge, in this study we investigated whether Tα1 treatment would act as adjuvant by regulating human monocyte-derived DC responsiveness to TLRs triggered on one hand by viral nucleic acids (TLR3 and TLR7/8) and on the other hand by bacterial components (TLR2 and TLR4) by performing a comparative analysis. Our results identified an opposite effect of Tα1 on DC stimulated with viral or bacterial TLR agonists. In particular, Tα1 synergized with viral stimuli to trigger DC maturation and release pro-inflammatory mediators, while a dampened phenotype was observed upon bacterial TLR2 and TLR4 stimulation. These findings perfectly matched what was found upon DC infection with pandemic H1N1 2009 Flu A virus (also known as 'swine Flu') or live *Mycobacterium bovis* Bacillus Calmette–Guerin (BCG), with the implication that this aspect may have on clinical and vaccinal applications of this drug.

2. Materials and methods

2.1 DC preparation

Monocyte-derived DC were prepared as previously described [24] under a protocol approved by Istituto Superiore di Sanità Review Board (CE/13/387). Briefly, peripheral blood mononuclear cells were isolated from freshly collected buffy coats obtained from 12 different healthy, voluntary blood donors (Blood Bank of University 'La Sapienza,' Rome, Italy) by density gradient centrifugation using Lympholyte-H (Cedarlane, Hornby, Ontario, Canada). Monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi, Bergisch Gladbach, Germany). The recovered cells were > 95% $CD14^+$, as determined by flow cytometry with an anti-CD14 antibody (Ab) (clone #M5E2, BD Bioscience, San Jose, CA). DC were generated by culturing monocytes for 5 days in six-well tissue-culture plates (Costar Corp., Cambridge, MA) with a cytokine cocktail composed of 50 ng/ml GM-CSF and 1000 U/ml IL-4 (R&D Systems, Abingdon, GB). Cells were plated at 0.5×10^6 cells/ml in RPMI 1640 (Lonza, Verviers, Belgium), supplemented with 2 mM L-glutamine, and 15% fetal bovine serum (FBS) (Lonza, Verviers, Belgium). At day 5, the cells were tested for their differentiation status by evaluating CD1a expression (> 90% $CD1a^+$) and lack of CD14 (> 95% $CD14^-$).

2.2 Viral and bacterial preparation and DC infection

The *M. bovis* BCG (ATCC 27291) was grown as previously described [25]. Briefly, bacteria were grown with gentle agitation (80 rpm) in Middlebrook 7H9 broth (Difco Laboratories, Detroit, MI, USA), supplemented with 0.05% Tween 80 (Sigma-Aldrich) and 10% Middlebrook oleic acid albumin dextrose catalase enrichment (Becton Dickinson, Sparks, MD, USA) for ~ 1 week. Logarithmically growing cultures were centrifuged at 800 rpm for 10 min to eliminate clumped mycobacteria and then washed three times in RPMI 1640. Mycobacteria were resuspended in RPMI 1640 containing 15% FBS and then stored at -80°C. Bacterial frozen vials were thawed and bacterial viability was determined by counting the number of colony forming units. All bacterium preparations were tested for endotoxin contamination (< 1 endotoxin Unit/ml) by the Limulus lysate assay (Lonza). DC cultures were then infected with a multiplicity of infection (MOI) of one bacteria/cell as previously described [25].

Where indicated DC were also infected by the pandemic H1N1 2009 Flu A Virus at MOI of 1. The pandemic H1N1 (A/Milan/UHSR1/2009 strain) virus was propagated in embryonated chicken's eggs and titrated to determine the 50% tissue culture infection dose in Madin–Darby canine kidney cells using Reed and Muench's method [26].

2.3 Reagents

Lipopolysaccharide (LPS) (TLR4 agonist) from *Escherichia coli* 0111:B4 (Sigma Chemical Co., St. Louis, MO) was used at a concentration of 1 ng/ml. The combined synthetic TLR7/8 ligand 3M007 (R-848-Resiquimod analog) was a kind gift from Dr. Mark A. Tomai (3M Pharmaceutical, St. Paul, MN) and was used at 1 μM. Polyinosinic–polycytidylic acid, [poly (I:C)] (Amersham; Piscataway, NJ), a specific TLR3 ligand, was used at 50 μg/ml. The TLR2 agonist Pam3CSK4 (Alexis Corp., San Diego, CA) was used at 2 μg/ml. Dose and stimulation time used in this manuscript were selected by dose–response experiments performed in kinetic (data not shown).

Where indicated, a 2-h pre-treatment with Tα1 (100 ng/ml, Sigma-Tau, Italy) was performed on DC before infection or TLR treatment.

2.4 Flow cytometry analysis

DC (at least 10^5) were harvested, washed once in PBS containing 2% FBS and incubated with monoclonal Ab at 4°C for 30 min. To stain DC, monoclonal Abs specific for CD1a, CD14, CD86, human leukocyte antigen (HLA)-DR, HLA-I (ABC alleles) together with IgG1 and IgG2a isotype controls (BD Bioscience), were used as direct conjugates to different fluorochromes as needed. Fixable Viability Dye eFluor 780 (eBioscience, San Diego, CA) was also used to label dead cells. After washing, cells were fixed in 2% formaldehyde (PanreacQuimica, Castellar del Valles, Spain) and run on a FACSCanto (BD Bioscience, San Jose, CA). A total of 30000 cells were analyzed per sample. After gating viable DC and selecting singlets only, the expression of the analyzed surface molecules was evaluated by subtracting the mean fluorescence intensity (MFI) values from those of the isotype Ab controls. Data were analyzed by FlowJo software (TreeStar, Inc.). The full gating strategy used is shown in **Figure 1**.

2.5 Cytokine determination

Supernatants of DC cultures were harvested after 24 h of TLR treatment or infection, filtered with 0.2 μm filtering advices and stored at -80°C. The production of TNF-α, IL-6 and IL-8 was then measured by the human inflammation Cytometric Bead Array (CBA) kit (BD Bioscience) on a FACSCanto (BD Bioscience) and analyzed by using the FCAP Array™ Software (v. 3.0) (BD Bioscience).

2.6 RNA isolation and real-time PCR quantification

Total RNA was extracted from untreated or treated DC with RNeasy Mini kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Reverse transcription was primed with oligo (dT) and performed with murine leukemia virus reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA) as previously described [27]. Quantitative PCR assays for glyceraldhehyde-3-phosphate dehydrogenase (gapdh), IFN-αs, IFN-β and IFN-λ1 transcripts were done in triplicates by using the Light Cycler Fast Start DNA SYBR Green I Master Mix in the presence of 3 mM $MgCl_2$ on a Light Cycler Instrument (Roche Diagnostics, Mannheim, Germany). Sample values were normalized by calculating the relative quantity of each mRNA to that of gapdh using the formula $2^{-\Delta Ct}$ and expressed as mean ± SEM.

2.7 Statistical analysis

Statistical analysis was calculated using the Wilcoxon test for nonparametric paired data. A p-value ≤ 0.05 was considered statistically significant.

3. Results

3.1 Opposite effects of Tα1 on human monocyte-derived DC response to stimulation of surface-bound or intracellular TLRs

Among other recognized ligands of self or non-self origin, viral and bacterial pathogens are sensed in human DC by different sets of TLRs. In particular, viral nucleic acids are detected by endolysosome-resident TLR3 and TLR7/8, while cell surface TLR2 and TLR4 recognize different bacterial components. We evaluated whether the immunomodulatory potential of Tα1 would regulate TLR-induced DC responses.

Monocyte-derived DC were differentiated *in vitro* and then stimulated with different TLR ligands for 24 h in the presence or absence of a pretreatment with Tα1. To trigger TLR3 and TLR7/8 pathways, DC were stimulated with the synthetic analog of double-stranded RNA, poly (I:C) or with imidazoquinoline compound 3M007 (analog of Resiquimod),

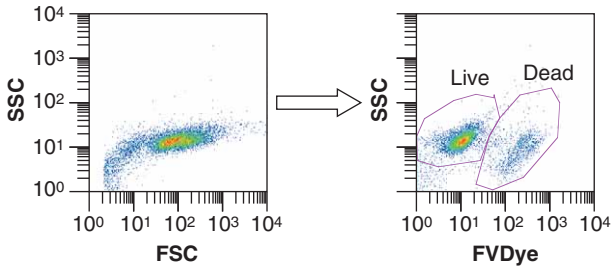

Figure 1. Gating strategy used for monocyte-derived DC flow cytometry analysis. Monocyte-derived DC were differentiated *in vitro* for 5 days in the presence of IL-4/GMCSF cytokine cocktail and then stimulated as described in the text. Cells were then collected for flow cytometry analysis. Full gating strategy used for monocyte-derived DC characterization is shown. Untreated or treated DC were identified by FSC/SSC gating (left panel) and dead cells excluded by means of FVDye staining (right panel). Dot plots derived from a representative individual are depicted.
DC: Dendritic cell; FSC: Forward-scattered light; FVDye: Fixable viability dye; SSC: Side-scattered light.

respectively; while TLR2 was activated by Pam3CSK4, a synthetic triacylated lipopeptide that mimics the acylated amino terminus of Gram-positive bacterial lipopeptide, and TLR4 by Gram-negative LPS.

The immune phenotype of infected DC was then analyzed by evaluating both the surface expression of co-stimulatory molecules, as well as the secretion of pro-inflammatory cytokines in culture supernatants.

Upon exposure of TLR3 and TLR7/8 to their ligands poly (I:C) and 3M007, respectively, DC induced the co-stimulatory marker CD86 and class I and II major histocompatibility complex (MHC) molecules, which mediate antigen presentation to T cells and activation of cellular immunity (**Figure 2A,B**), and produced high levels of TNF-α, IL-6 and IL-8, soluble factors critical to mediate inflammation (**Figure 2C**).

In line with previous findings, Tα1 alone did not represent a sufficient stimulus to induce the activation of immature DC [12,13], conversely the combined exposure to Tα1 and TLR3 or TLR7/8 ligands remarkably increased the expression of maturative markers (mainly MHC class I and II molecules) and the release of TNF-α, IL-6 and IL-8 (**Figure 2**).

To our surprise, we observed an opposite effect when DC were stimulated with bacterial components (**Figure 3**). The surface expression of CD86, HLA-I and HLA-DR was highly enhanced on DC upon both TLR2 and TLR4 triggering. However, their level was not enhanced by Tα1 co-treatment, but rather decreased when the drug was present in the cultures, as seen mainly for MHC II molecules (**Figure 3A,B**). This counterintuitive trend was even more pronounced when pro-inflammatory cytokine production was measured. Hence, TNF-α, IL-6 and IL-8 release was strikingly reduced in TLR2/4-treated DC in presence of Tα1 (**Figure 3C**).

Taken together, these findings may indicate that, while Tα1 reinforces the inflammatory state of DC upon viral stimuli, conversely this molecule may somehow reduce the release of pro-inflammatory cytokines during bacterial infections.

3.2 Tα1 dampens the response of human monocyte-derived DC to BCG infection

In light of this view, it is clear how the differential effect of Tα1 on viral versus bacterial TLR stimulation of DC, could be reflected in the regulation of vaccine-induced immunity, providing also that these cells are of key importance in this context. Thus, we tested whether Tα1 treatment would modulate the response of human DC also after infection with live viruses and bacteria, in particular the pandemic H1N1 Flu A virus or the attenuated mycobacterial vaccine strain BCG.

BCG is the only licensed tuberculosis (TB) vaccine in use at present and an estimated 100 million children receive BCG every year globally. However, this empirically attenuated vaccine strain imparts only a partial immune protection, thus, new TB vaccines are urgently needed [28].

Monocyte-derived DC were differentiated *in vitro* and then infected with BCG for 24 h in the presence or absence of a pre-treatment with Tα1. Expression of co-stimulatory molecules and pro-inflammatory cytokine secretion were then tested (**Figure 4**). After BCG infection, DC induced the co-stimulatory marker CD86 and both HLA-I and II molecules (**Figure 4A,B**), and produced high levels of the inflammatory cytokines TNF-α, IL-6 and IL-8 (**Figure 4C**). Interestingly and in line with what observed upon TLR2 and TLR4 stimulation, Tα1 treatment significantly dampened both expression of maturation markers (mainly HLA-I and II) (**Figure 4A,B**) and production of pro-inflammatory mediators (**Figure 4C**). Even if we do not know whether these differences are biologically relevant, we can hypothesize that BCG-infected DC, displaying decreased accessory signals for T-cell activation in the presence of Tα1, would have a reduced efficacy in driving protective vaccine-induced immune responses.

3.3 Tα1 amplifies human monocyte-derived DC response to Flu infection

Together with BCG, here, we also evaluated Tα1 effect on human DC response to infection with the pandemic H1N1 2009 Flu A virus (**Figure 5**).

H1N1 infection upregulated the co-stimulatory marker CD86 and both HLA-I and II molecules in DC (**Figure 5A, B**). However, while HLA-I was strongly induced by H1N1 and not further enhanced by Tα1, HLA-DR and CD86 were slightly expressed on DC upon H1N1 infection and the presence of Tα1 in culture enhanced the expression level of both these markers, as seen for TLR3 and TLR7/8 stimulation. Thus, Tα1 treatment was of key importance to drive a full DC maturation upon H1N1 infection.

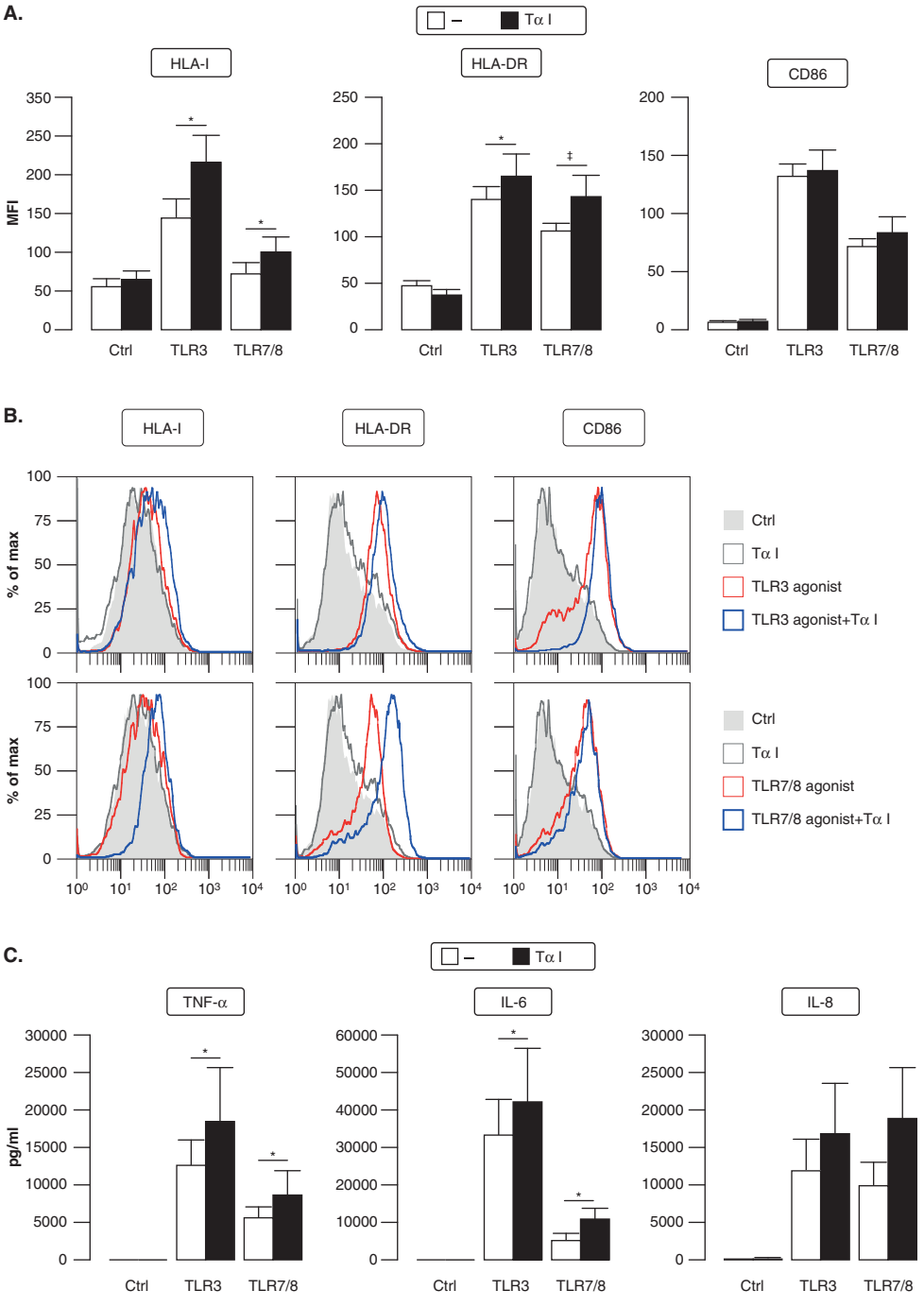

Figure 2. Effect of Tα1 on human monocyte-derived DC response to TLR3 and TLR7/8 triggering. DC were treated for 24 h with the TLR3 agonist poly (I:C) (50 μg/ml) and the TLR7/8 ligand 3M007 (1 μM) in the presence or absence of a 2 h pre-treatment with Tα1 (100 ng/ml). **(A)** Expression of the surface molecules HLA-I (ABC), HLA-DR and CD86 was calculated on live-gated DC by subtracting MFI values obtained from isotype control Abs to those derived from each different experimental condition. Data are reported as MFI mean ± SEM of six independent experiments. *p = 0.045; ‡p < 0.002. **(B)** Flow plots of HLA-I, HLA-DR and CD86 in live-gated monocyte-derived DC from one representative individual are shown. **(C)** TNF-α, IL-6 and IL-8 production was evaluated in DC culture supernatants by CBA analysis. The results represent means of pg/ml values ± SEM of six independent experiments. *p < 0.04.

ctrl: Untreated control culture; DC: Dendritic cell; MFI: Mean fluorescence intensity; TLR: Toll-like receptor.

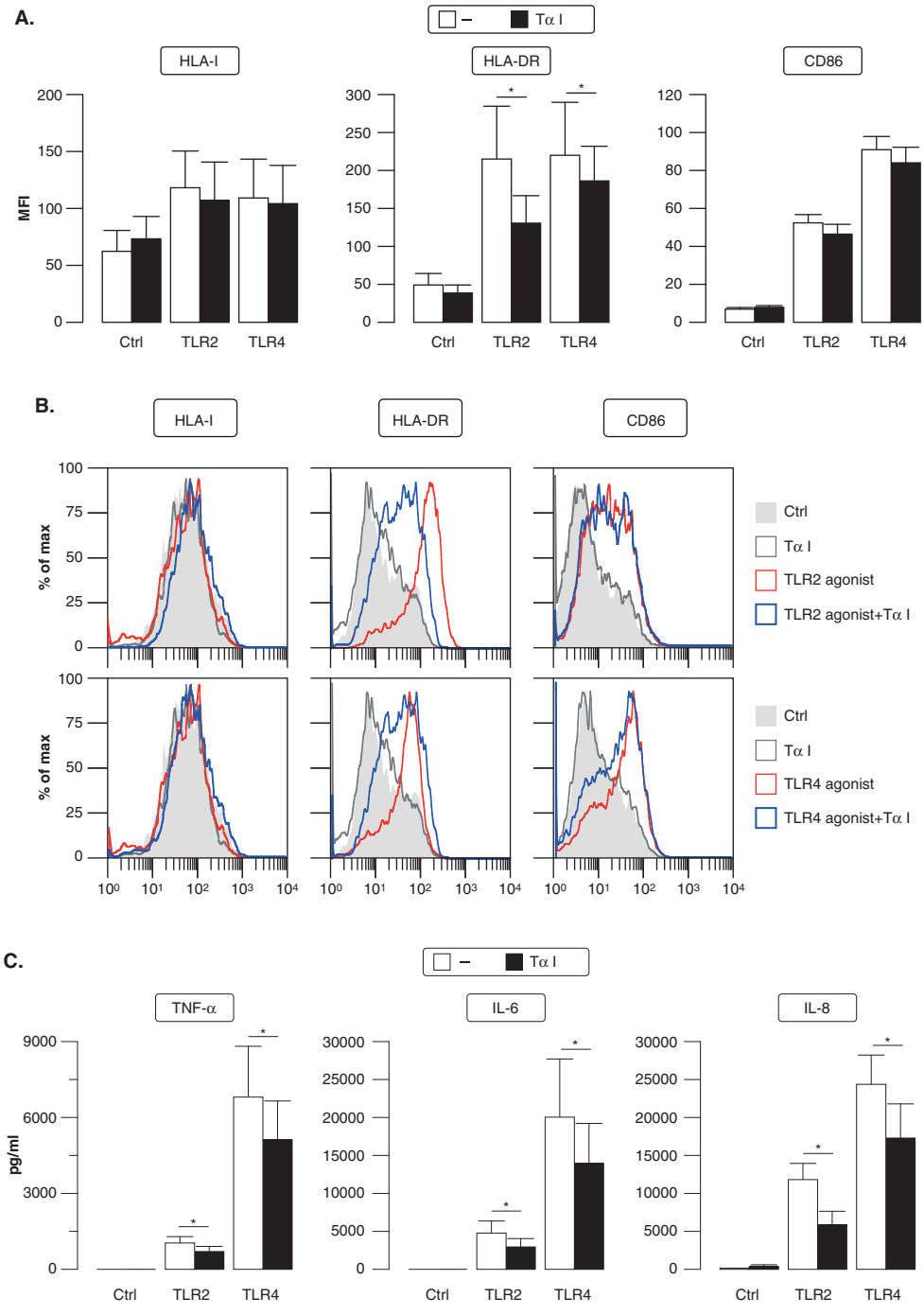

Figure 3. Effect of Tα1 on human monocyte-derived DC response to TLR2 and TLR4 triggering. DC were treated for 24 h with the TLR2 agonist Pam3CSK4 (2 μg/ml) and the TLR4 ligand LPS (1 ng/ml) in the presence or absence of a 2-h pre-treatment with Tα1 (100 ng/ml). **(A)** Expression of the surface molecules HLA-I (ABC), HLA-DR and CD86 was calculated on live-gated DC by subtracting MFI values obtained from isotype control Abs to those derived from each different experimental condition. Data are reported as MFI mean ± SEM of six independent experiments. *p = 0.012. **(B)** Flow plots of HLA-I, HLA-DR and CD86 in live-gated monocyte-derived DC from one representative individual are shown. **(C)** TNF-α, IL-6 and IL-8 production was evaluated in DC culture supernatants by CBA analysis. The results represent means of pg/ml values ± SEM of six independent experiments. *p < 0.04.

CBA: Cytometric bead array; Ctrl: Untreated control culture; DC: Dendritic cell; MFI: Mean fluorescence intensity; TLR: Toll-like receptor.

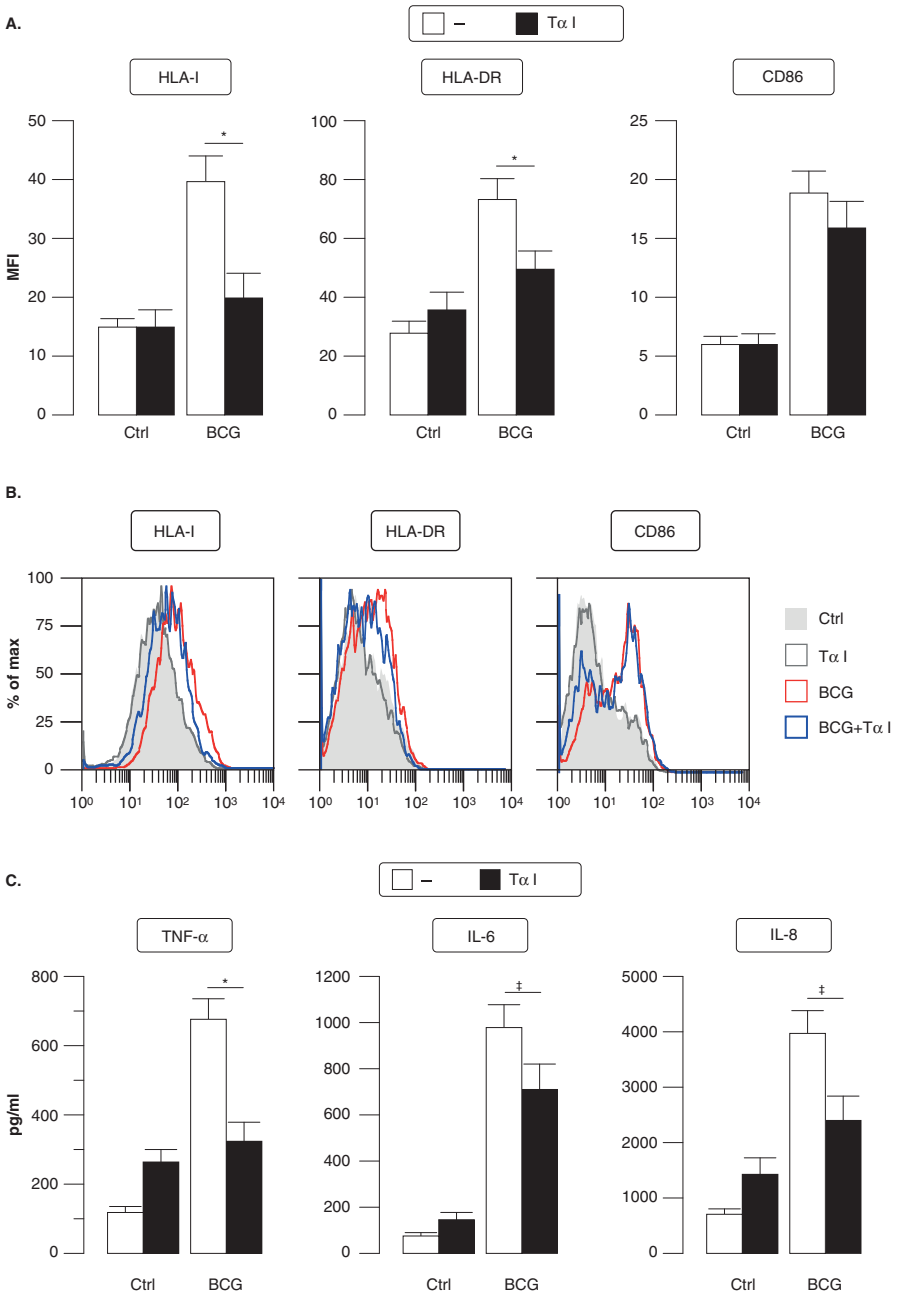

Figure 4. Tα1 dampens the activation of human monocyte-derived DC to BCG infection. DC were left untreated or infected for 24 h with BCG (MOI = 1) in the presence or absence of a 2-h pre-treatment with Tα1 (100 ng/ml). **(A)** Expression of the surface molecules HLA-I (ABC), HLA-DR and CD86 was calculated on live-gated DC by subtracting MFI values obtained from isotype control Abs to those derived from each different experimental condition. Data are reported as MFI mean ± SEM of six independent experiments. *$p < 0.05$. **(B)** Flow plots of HLA-I, HLA-DR and CD86 in live-gated monocyte-derived DC from one representative individual are shown. **(C)** TNF-α, IL-6 and IL-8 production was evaluated in DC culture supernatants by CBA analysis. The results represent means of pg/ml values ± SEM of six independent experiments. *$p = 0.036$; ‡$p < 0.04$.
BCG: Bacillus calmette–guerin; CBA: Cytometric bead array; Ctrl: Untreated control culture; DC: Dendritic cell; MFI: Mean fluorescence intensity.

In line with previous data showing that the pandemic strain induces a weak pro-inflammatory cytokine response in DC likely to escape host immune defense [29], we found an almost undetectable level of TNF-α, IL-6 and IL-8 in culture supernatants of H1N1-infected DC, that was not further enhanced upon Tα1 treatment (data not shown). Furthermore, it was also reported that the H1N1 strains are very sensitive to the anti-viral action of type I and III IFNs, IFN-α /β

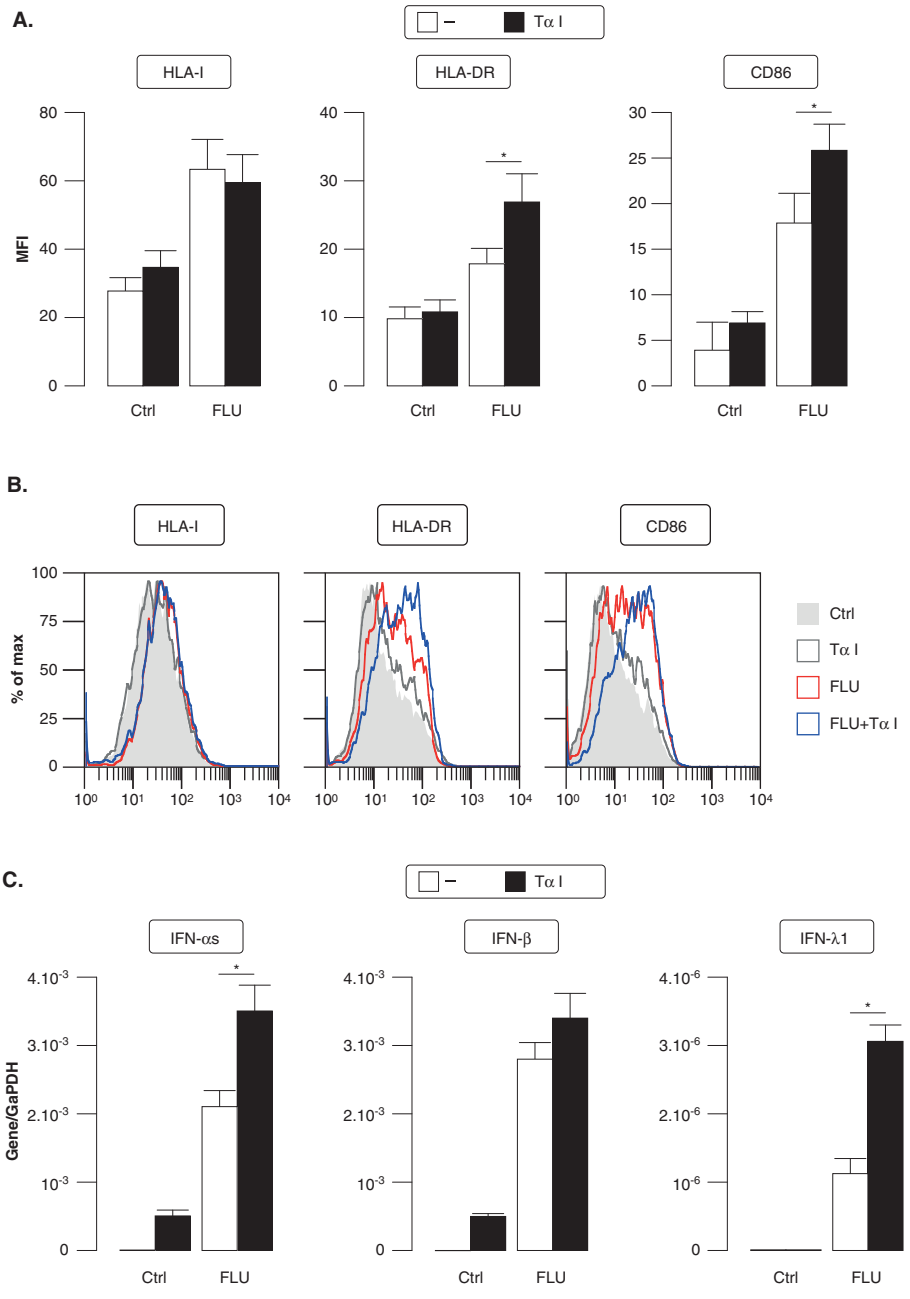

Figure 5. Tα1 amplifies human monocyte-derived DC response to H1N1 infection. DC were left untreated or infected for 24 h with H1N1 influenza A strain (MOI = 1) in the presence or absence of a 2-h pretreatment with Tα1 (100 ng/ml). **A.** Expression of the surface molecules HLA-I (ABC), HLA-DR and CD86 was calculated on live-gated DC by subtracting MFI values obtained from isotype control Abs to those derived from each different experimental condition. Data are reported as MFI mean ± SEM of six independent experiments. *$p < 0.04$. **B.** Flow plots of HLA-I, HLA-DR and CD86 in live-gated monocyte-derived DC derived from one representative individual are shown. **C.** The transcription of ifn-αs (multiple subtypes), ifn-β and ifn-λ 1 was analyzed by real-time quantitative RT–PCR. Quantification data are normalized to the level of the housekeeping gene gapdh by using the $2^{-\Delta Ct}$ equation. Data are shown as mean ± SEM of six independent experiments. *$p < 0.04$.

BCG: Bacillus calmette–guerin; Ctrl: Untreated control culture; DC: Dendritic cell; MFI: Mean fluorescence intensity; MOI: Multiplicity of infection.

and IFN-λs respectively [29], even if with different specificities [26]. It was of great interest to find that Tα1 treatment, conversely to what found for IFN-β, specifically and significantly promoted IFN-αs and IFN-λ 1 transcription (**Figure 5C**), two cytokines that display a great anti-viral potential on the pandemic Flu A strain, thus amplifying host-destroying mechanisms to contain the infection process.

4. Discussion

Several clinical applications and conditions demand progress in adjuvant research and development. In particular, new vaccine candidates, such as those comprising poorly immunogenic antigens of HIV, or vaccines for TB, requiring effective T-cell responses, will take advantage from adjuvants that enable these responses. Vaccines targeting antigenically diverse pathogens (such as Flu virus) can benefit from adjuvants that enhance immune response broadening, enabling vaccines to induce immunity to antigenic variations of a particular pathogen. In the aging population, adjuvanted vaccines may play a role in boosting protective immune responses. Last but not least, the emerging field of therapeutic vaccines, both for cancer and infectious diseases, will require adjuvants or pharmacological enhancers to amplify and direct effective immune responses.

In this context, TLR ligands has long been considered as immune response modifiers for their ability to address and regulate key immune cell types such as DC, B cells and T lymphocytes and increasing interest has been growing to use natural ligands or synthetic agonists for well-defined TLRs as adjuvants, either alone or with various formulations [30]. In particular, TLRs, upon recognition of pathogen-derived molecular patterns, initiate a series of signaling programs that execute the first line of host defense necessary for killing infectious microbes. In addition, their signaling simultaneously induces maturation of DC, responsible for alerting induction of the second line of host defense, the so-called adaptive immunity [15]. The plasticity of DC has brought this cell type to a center stage as promising targets for intervention for immunotherapy and vaccine development and shift the emphasis from the 'antigen or epitope' toward the 'adjuvant.'

In this regard, the synthetic peptide Tα1 has been examined worldwide as immunomodulatory agent unveiling its highly pleiotropic properties acting as regulator of inflammation, immunity and tolerance [31].

In patients with lung cancer undergoing radiotherapy Tα1 showed immune-restorative effects [32,33]. Since then, numerous experimental and clinical studies have tested the antitumor activity of Tα1, either alone or in combination with cytokines and/or chemotherapeutic agents [23,30].

Tα1 was also shown to regulate differentiation of the so-called suppressor or regulatory T cells (Treg) [10]. Current knowledge indicates that Treg constitute a pivotal mechanism of immunological tolerance and their manipulation may boost responses in cancer and microbial diseases or suppress those unwanted in autoimmunity and transplantation [34]. Tα1 induced the indoleamine 2,3-dioxygenase activity in plasmacytoid DC in a TLR9 and type I IFN-dependent manner, thus, promoting the generation of FoxP3-expressing $CD4^+CD25^+$ Treg [35].

The immunotherapeutic potential of Tα1 was evaluated to treat different viral infectious diseases, such as chronic Hepatitis C virus [4] and HIV [5] infections. A recent randomized controlled clinical trial showed also a trend toward improved survival in patients affected by bacterial sepsis offering new hopes in the treatment of this fatal disease [36].

A very good experience for Tα1 came also from the use of this drug as adjuvant in the Flu vaccine in preclinical studies and then in large clinical trials, in which Tα1 enhanced Ab response and lead to a decrease of infections in difficult-to-treat populations, such as those immunosuppressed due to age or hemodialysis [23]. Furthermore, during the 2009 pandemic H1N1 outbreak, Tα1 was also successfully used as Flu vaccine enhancer [22].

Investigation of the mechanisms of action of Tα1 at the cellular level has implicated a number of intracellular signaling pathways in a variety of cells leading to stimulation of the immune system that can explain the effectiveness of this biologic; however, one of its central role seems to reside in the modulation of DC functions. Hence, many studies, mainly conducted in mice, highlighted how Tα1 modulates maturation, differentiation and functions of DC [13,35,37].

DC have a crucial role in determining immune outcomes becoming cells that are either stimulators or suppressors of T cell responses [38] unveiling their key role in instructing vaccine-induced immunity. Thus, we recently developed a novel biological platform based on human primary DC for the preclinical assessment of novel vaccine strains to predict their immunological potential within a human context [21]. This system may also represent a good model to stimulate *in vitro* the generation of inflammatory DC, a distinct subset of monocyte-derived DC present in inflammatory conditions as recently reported by Segura *et al.* [39].

By using this experimental setting, we tested the capacity of the Mtb vaccine prototype SO2 to stimulate the immunomodulatory properties of human monocyte-derived DC [21]. Indeed, despite BCG being the most widely used vaccine in human history with over 100 million doses administered annually, TB still remains a global health emergency with around two million new deaths caused by this disease each year [40].

Meta-analysis studies have confirmed that BCG protects children, providing > 80% efficacy against severe forms of TB, including tuberculous meningitis and miliary TB. In contrast, evidence for protection against pulmonary TB in adolescents and adults remains contentious with very variable efficacy as reported by clinical trials, observational case–control studies and contact studies [41]. Thus, improving the efficacy of BCG would be of key importance for cost-effective and sustainable long-term intervention programs.

Based on these assumptions, here we tested the capacity of Tα1 as vaccine enhancer by studying the regulation of human

monocyte-derived DC functions. Our study investigated Tα1 action at two levels: first, by evaluating whether this drug would directly modulate viral and bacterial TLR-triggered pathways leading to modifications of DC immune phenotype; then, by exploring whether these immune changes would occur also in the presence of live viruses or bacteria such as H1N1 Flu A and BCG, respectively.

Our experimental setting demonstrated that Tα1 is a very efficient promoter of anti-viral immune responses. Indeed, here we observed that upon triggering of TLR3 and TLR7/8, sensing viral nucleic acids, monocyte-derived DC showed a remarkable increase in the synthesis of TNF-α, IL-6 and IL-8, pro-inflammatory cytokines critical to stimulate a protective Th1 response, when Tα1 was present in culture (**Figure 2**). The combined exposure to Tα1 and TLR3 or TLR7/8 ligands significantly upregulated on DC surface the expression of HLA-I and II molecules, event that is desirable for adjuvants since the MHC system is critical for the presentation of specific antigens to T cells.

In accordance with what observed upon stimulation with synthetic TLR ligands mimicking intermediates of viral replication, Tα1 amplified DC responses during infection with the live pandemic H1N1 2009 Flu A virus. It is known that the pandemic H1N1 virus exhibits a weak ability to induce DC maturation and the release of pro-inflammatory cytokines and type I and III IFN, a classical mechanism of virus-induced immune escape to dampen host responses [29]. Here, we confirmed these findings and we further found that, while Tα1 has no effect on the almost absent Flu-driven TNF-α, IL-6 and IL-8 production (data not shown), however this peptide promoted the maturation process in DC and the expression of IFN-αs and IFN-λ1 transcripts (**Figure 5**).

Surprisingly, however, Tα1 displayed an opposite trend when DC were stimulated with bacterial components. Indeed, differently to what found upon stimulation with viral TLR ligands or Flu infection, the presence of Tα1 dampened DC immune phenotype upon stimulation with either TLR2 and TLR4 ligands or live BCG bacteria (**Figures 3** and **4**). In particular, in these experimental conditions, the presence of Tα1 significantly dropped the expression of MHC class I and II molecules and the release of the pro-inflammatory mediators TNF-α, IL-6 and IL-8 indicating that Tα1 may rather reduce than augment the efficacy in driving protective vaccine-induced immune responses in the context of BCG and/or bacterial infection.

5. Conclusions and future perspectives

All together, these results indicate that the usage of Tα1 might have controversial effects depending on the context in which this molecule is used. This is probably due to the highly pleiotropic properties of this protein, working on many components of the inflammatory/anti-inflammatory cascade at a time, leading to the more various and sometimes opposite trends in immune-regulation. Using a preclinical setting such as that of human primary monocyte-derived DC to test or screen adjuvant or immunotherapeutic agents alone or in conjunction with the target pathogen might be of key importance to evaluate the immunological outcome of the chosen compounds in an *in vitro* model as closest as possible to the final vaccine recipient.

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Declaration of interest

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Affiliation

Elena Giacomini[1], Martina Severa[1], Melania Cruciani[1], Marilena Paola Etna[1], Fabiana Rizzo[1], Manuela Pardini[1], Carolina Scagnolari[1,2], Enrico Garaci[3] & Eliana Marina Coccia[†1]

[†]Author for correspondence

[1]Istituto Superiore di Sanità of Infectious, Parasitic and Immune-mediated Diseases, Viale Regina Elena, Rome, 00151, Italy

Tel: +39 06 49903638;

Fax: +39 06 49903638;

E-mail: eliana.coccia@iss.it

[2]Pasteur Institute-Cenci Bolognetti Foundation, Department of Molecular Medicine, Sapienza UniversityRome, Italy

[3]University of Rome Tor Vergata, Department of Experimental Medicine and Surgery, Rome, Italy