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Pidotimod promotes functional maturation of dendritic cells and displays adjuvant properties at the nasal mucosa level

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ABSTRACT

Mucosal dendritic cells (DCs) are very important in the process of antigen presentation to T cells, playing a key role in the induction of primary and secondary immune responses. Pidotimod is a synthetic substance capable of modulating immune cell functions, but the effect of pidotimod on human DCs has not been investigated yet. Here we demonstrate the ability of pidotimod to induce DC maturation and up-regulate the expression of HLA-DR and co-stimulatory molecules CD83 and CD86, which are fundamental for communication with adaptative immunity cells. Pidotimod also stimulated DCs to release high amounts of pro-inflammatory molecules such as MCP-1 and TNF-α cytokines and to drive T cell proliferation and differentiation towards a Th1 phenotype. Moreover, we demonstrate that pidotimod *in vivo* promotes strong and specific humoral and cellular immune response when co-administered intranasally with a model antigen.
Taken together our data suggest the possibility to use pidotimod as adjuvant molecule to facilitate the activation of the innate immune system as well as to promote an effective mucosal and systemic immune response.

1. Introduction

Mucosa constitutes the main entry site for the majority microbial pathogens. A large and highly specialized innate and adaptative mucosal immune system protects the mucosal surfaces and the body interior from potential injuries. The cells of local immune system are accumulated in or transit between various mucosa-associated lymphoid tissues (MALT). The MALT represents a highly compartmentalized immunological system which acts almost independently from the systemic one. Immune responses in mucosal tissues are governed by the nature of the antigen, the type of antigen-presenting cells (APCs) involved and the local environment. In mucosal sites the antigens can be directly captured by professional APCs, which include dendritic cells, B lymphocytes and macrophages, for presentation to T cell. Mucosal dendritic cells are very important in the process of antigen presentation, serve as a link between the innate and adaptive immune system [1,2] and govern both initiation and polarization of adaptive immunity [3]. Immature DCs patrol as sentinels in almost every non-lymphoid tissue and organ, have a highly developed capacity to capture antigens, but a poor T cell-stimulatory activity [4,5]. Upon recognition of pathogens, DCs undergo phenotypic and functional changes to differentiate into mature stage resulting in the up-regulation of specific proteins for antigen presentation and T cell activation, including the major histocomplex (MHC) and the co-stimulatory molecules CD40, CD80, and CD86. They also up-regulate CCR7, a chemotactic receptor that induces DCs to travel through the blood stream to the spleen or through the lymphatic system to a lymph node. Here they act as antigen-presenting cells, prime naive antigen-specific T cells and drive their differentiation. The results of clinical application indicate that maturation status of DCs plays a pivotal role in initiating and guiding the immune response [6]. Therefore, it is important to identify reagents to use in promoting DCs maturation and differentiation.

For this reason it is important to understand how to mobilize mucosal DCs to prime robust protective immunity against mucosally acquired infections especially for children and elderly people, who may present with a poor or even deficient immune response.

In the first 2 years of life children haven't got a completely formed immune system yet and, especially in early childhood, they are also subjected to recurrent infections, essentially in the respiratory tract, and they need prophylactic and therapeutic immunomodulant treatments of diseases. Also elderly people show a higher susceptibility to infections [7,8] and a reduced response to vaccination, particularly against influenza virus [9,10]. This is essentially due to a reduced ability of the innate immune system to properly respond to dangerous signals and to organize a correct antigen processing and presentation [11]. The result is a decreased production of cytokines and chemokines, which are able to create a suitable microenvironment to generate a crosstalk between innate and adaptive immunity [12,13].

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The co-administration of immune modulatory drugs represents a widely used strategy to increase mucosal — and systemic — immune response [14–16] and to favour the development of stronger and broader immune responses engaging both the humoral and cellular immunity. Molecules with immunomodulatory activity and adjuvant properties at the mucosal level are few to date and there is an urgent need for new drugs displaying such features.

Pidotimod (3-L-pyroglutamyl-L-thiaziolidine-4-carboxylic acid) is a synthetic substance with important immunomodulatory properties *in vitro* and *in vivo* [17,18]. Pidotimod is able to induce a protective effect against infectious processes as demonstrated in many controlled clinical trials of experimental bacterial and viral infection. In particular, administration of pidotimod by oral route increases resistance to viral infections [19] and makes the activity of antiviral drugs more effective for recurrent respiratory infections (RRI) in pediatric patients [20–22]. However the effects of pidotimod on cellular response are still poorly characterized, and there is little information concerning the general mechanism of action of this synthetic compound.

In this study, we investigated whether pidotimod could induce the maturation of human monocyte-derived immature DCs, influence the cytokines production from MDDCs and induce proliferation and differentiation of $CD4^+$ naïve T cells towards a Th1-polarizing phenotype. Moreover, we investigated the ability of pidotimod to act as adjuvant in mice challenged intranasally with a model antigen.

2. Materials and methods

2.1. Monocyte-derived DC (MDDCs) generation and culture

Peripheral blood mononuclear cells (PBMCs) were freshly isolated from healthy donors (thanks to the courtesy of the Centro Trasfusionale, Spedali Civili, Brescia) by Ficoll gradient (Histopaque-1077, Sigma, Milan, Italy). PBMC were washed and resuspended in complete medium. $CD14^+$ monocytes were isolated from PBMC by positive selection using Magnetic Blood Dendritic Cell Isolation kit I from Miltenyi Biotec by labeling with Abs against $CD14^+$. Cells were incubated at 4 °C for 10 min, washed twice in MACS buffer (PBS 1×, 0.5% BSA, 2 mM EDTA) and isolated by an autoMACS separator (Miltenyi Biotech, Bergisch Gladbach, Germany), according to the manufacturer's protocol. $CD14^+$ monocytes (purity > 98%) were washed and resuspended in RPMI 1640 (Sigma) containing penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and 10% heat inactivated FCS (Euroclone, Celbio, Milan, Italy), then seeded in 48-well plates (Falcon, BD Biosciences) and supplemented with 500 U/ml of rh-IL-4 and 800 U/ml of GM-CSF (R&D Systems, Minneapolis, MN, USA). After 3 days the medium was changed with complete medium containing cytokines. After 6 days of culture, the cells were harvested, pooled together and counted. On the 6th day immature DCs were stimulated by using 1 μg/ml of LPS from *Escherichia coli* 026:B6 (Sigma) or pidotimod (1 μg/ml). In some experiments pidotimod (1 μg/ml) was also used in combination with LPS (10 ng/ml) and a combination of LPS (10 ng/ml) and IFN-γ (100 ng/ml) was used as positive control. The supernatants were collected after 24 h and kept frozen at −80 °C for cytokine quantification assays.

The absence of endotoxin contamination (< 0.25 endotoxin unit/500 μg of pidotimod stock solution) in the pidotimod preparation was assessed by Limulus amoebocyte assay, Associates of Cape Cod Inc, Falmouth, MA).

2.2. Immunophenotyping analysis of MDDCs

Differentiation of monocytes ($CD14^{bright}$/HLA-DR^{low}/$CD86^-$/$CD83^-$) into immature MDDCs ($CD14^{low}$/HLA-DR^{++}/$CD86^{low}$/$CD83^{low}$) and then into mature MDDCs ($CD14^-$/HLA-DR^{++}/$CD86^{bright}$/$CD83^{high}$) was evaluated by FACScalibur flow cytometer using Cell Quest software (BD Biosciences). The cells were collected, washed in PBS and stained at 4 °C for 30 min with anti-human fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated CD14, HLA-DR, CD83, CD86 monoclonal antibodies and isotype controls (BD Biosciences, Palo Alto, CA).

2.3. Measurement of cytokines

Culture supernatants of iMDDCs were assayed for the presence of monocyte chemoattractant protein (MCP)-1, tumor necrosis factor (TNF)-α and interleukin (IL)-12p70 using ELISA kits purchased from Endogen (Boston, MA) according to the manufacturer's instructions.

2.4. Proliferation of allogeneic $CD4^+$ naïve T cells co-cultured with MDDCs

DCs have the ability to induce proliferation and differentiation of primary allogeneic naive T cells towards Th1 phenotype. PBMC were obtained from umbilical cord blood samples by Ficoll gradient as described above and naïve $CD4^+$ T cells were obtained from them by negative selection using $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech). Naïve $CD4^+$ T cells were cultured with different quantities of differentially matured MDDCs in the presence and absence of pidotimod in flat 96-well plates (BD Biosciences). The co-cultures of mature DCs and $CD4^+$ T cells were maintained for 6–10 days and cellular proliferation and intracellular cytokines production were analyzed. To assess proliferation of $CD4^+$ naïve T cells they were resuspended in PBS/BSA 0.1% and stained for 10 min at 37 °C with 10 μM of carboxyfluorescein diacetate succinimidyl ester (CFSE) (Molecular Probes Eugene, OR) according to the manufacturer's instructions. The cells were then washed twice with RPMI/20% FCS to remove the excess of CFSE. The proliferative activity of $CD4^+$ was established after 5 days of co-culture with unstimulated or pidotimod-treated DCs by FACScalibur flow cytometer. The proliferation of allogeneic $CD4^+$ naïve T cells co-cultured with MDDCs was evaluated on the basis of fluorescence loss which decreases proportionally to the number of cellular divisions.

2.5. Measurement of intracellular cytokines

The acquisition of cell functional polarization of $CD4^+$ T cells was analyzed through measurement of intracellular cytokines by FACScalibur flow cytometer. MDDCs were incubated with pidotimod for 24 h and the $CD4^+$ T cells were co-cultured with unstimulated or pidotimod-treated mature DCs. After 5 days of co-culture the $CD4^+$ T cells were re-stimulated for 9 h with PMA (10 ng/ml)/ionomycin (1 μM), in the presence of monensin (1 μM) (Sigma) for the last 6 h. After stimulation cells were washed in PBS/1% FCS and resuspended in Cytofix/Cytoperm buffer (BD Biosciences) according to the manufacturer's instructions for 20 min at 4 °C in dark. At the end of incubation, cells were washed with Perm/Wash buffer twice and then stained with human mAb FITC-conjugated anti-IFN-γ (BD Biosciences) for 30 min at 4 °C. After incubation the cells were washed with Perm/Wash Buffer and analyzed by FACScalibur flow cytometer.

2.6. Antigens, peptides

Ovalbumin (OVA) antigen (purity > 98%), purchased from Sigma, was used for *in vitro* and *in vivo* studies. Peptides representing the dominant epitope (Ova 257–264, SIINFEKL), subdominant epitope (Ova 55–62, KVVRFDKL) and cryptic epitope (Ova 11–18, CFDVFKEL) of OVA antigen, recognized by the major histocompatibility complex (MHC) class I (H-$2k^b$), were purchased from Primm (Milan, Italy).

2.7. Immunization protocols

Female C57BL/6 (H-2^b) mice (6 to 8 weeks old) were purchased from Harlan–Winkelmann GmbH (Borchen, Germany) and treated in

accordance with local and European Community guidelines. A group of six mice was immunized intranasally for three times at days 0, 14 and 21 with 50 μg of OVA alone and OVA co-administered with 100 μg of pidotimod. Negative controls received phosphate-buffered saline (PBS). Serum samples were collected from blood of the tail vein 1 day before each immunization and 1 week after the last immunization, when mice were sacrificed by inhalation of CO_2. Sera were stored at −20 °C prior to determination of specific anti-OVA antibodies. Bronchoalveolar lavage fluid samples were obtained by flushing the organs with PBS supplemented with 10 mM phenylmethylsulfonyl fluoride (PMSF).The lavages were then centrifuged (5 min at 13,000×g) to remove debris.

In some experiments spleens and bone marrow from shinbone and thigh bone were collected and grown in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/ml of penicillin, 50 μg/ml of streptomycin, 5×10^{-5} M 2-mercaptoethanol and 1 mM L-glutamine (GIBCO BRL, Karlsruhe, Germany) and were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.8. Detection of anti-OVA antibodies

The amount of specific antibodies anti-OVA was determined by ELISA. Briefly, 96-well Immuno MaxiSorp assay plates (Nunc, Roskilde, Denmark) were coated overnight at 4 °C with 100 μl of OVA at 5 μg/ml in carbonate buffer (pH 9.6). After blockage of wells, the plates were washed and further incubated with 100 μl of serial two-fold dilutions of sera for 1 h at 37 °C. After four washes, anti-mouse biotinylated detection antibody (Sigma Chemie, Deisenhofen, Germany), was added in each well. The plates were further incubated for 1 h at 37 °C. After the plates were washed, peroxidase-conjugated streptavidin (PharMingen) was added and the plates were incubated at room temperature for 45 min. After four washes, the reactions were developed by using ABTS [2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M citrate-phosphate buffer (pH 4.35) containing 0.01% H_2O_2 and the absorbance was read at a wavelength of 405 nm. The results were expressed as mean ± standard errors of the means (SEMs) for each group.

2.9. Detection of Ig-producing cells by ELISPOT assay

The total number of antigen-specific IgG secreting cells was determined 2 months after the last immunization by enzyme-linked immunospot (ELISPOT) assay. Briefly, polyvinylidene difluoride (PVDF) plates (Millipore, Bedford, MA) were coated with either 100 μl/well of isotype-specific antibodies (Sigma, Germany) at a concentration of 5 μg/ml or OVA antigen at 5 μg/ml in carbonate buffer, pH 9.6) to determine total and antigen-specific antibodies respectively. Different concentrations of splenocytes and bone marrow cells were incubated in quadruplicate for 6 h. Then, the plates were washed, 100 μl of the corresponding biotinylated detection antibody (Sigma) was added and the plates were further incubated overnight at 4 °C. After several washes, the plates were incubated for 1 h with 100 μl/well of peroxidase-conjugated streptavidin (BD-Pharmingen, Germany). Spots were developed using 3-amino-9-ethylcarbazole (Sigma) in 0.1 M acetate-buffer (pH 5.0) and 0.05% H_2O_2 (30%). Spots were scanned with an Immuno-Spot series 3A Analyzer and were counted by using ImmunoSpot image analyzer software (version 3.2; Cellular Technology, Ltd.).

2.10. Detection of IFN-γ producing cells by ELISPOT assay

The number of IFN-γ secreting spleen and bone marrow cells was determined by ELISPOT assay, according to the manufacturer's instructions (Becton Dickinson). In brief, spleen cells were seeded at final concentrations of 5×10^5 cells or 1×10^6 for well and were incubated in quadruplicate in the absence or presence of different concentrations of the MHC class I restricted dominant, subdominant and cryptic OVA peptides (SIINFEKL, KVVRFDKL and CFDVFKEL respectively). After 16 h of culture, the cells were removed and the local production of IFN-γ was detected by using anti-IFN-γ biotinylated antibody, which was developed by addition of peroxidase-conjugated streptavidin and substrates, as described above.

2.11. Determination of lymphocyte-mediated cytotoxicity in vivo

In order to determine the lymphocyte-mediated cytotoxicity, suspensions of splenocytes from naïve C57BL/6 mice were split into two equal portions. One portion was labeled with high concentration 1 μM of carboxyfluorescein-succinyl-ester (CFSE; Molecular Probes) and the other with a low concentration (0.1 μM) of CFSE. Half of each cell population was then stained with 10 μM of CMTMR (Molecular Probes). The splenocytes were further pulsed for 1 h at 37 °C with the dominant, subdominant and cryptic OVA peptide at a concentration of 15 μg/ml. 2×10^7 cells were then transferred by intravenous injection into the immunized mice and after 16 h the splenocytes were analyzed by flow cytometry with a FACScalibur instrument and BD Cell Quest Pro software. The percentage of OVA-specific lysis was determined by the loss of the peptide-pulsed CFSE (high) population and compared to the control CFSE (low) population [23].The following formula was used to calculate the percentage of specific lysis: 100 − {[(percent CFSE (high) in immunized mice/percent CFSE (low) in immunized mice)/(percent CFSE (high) in control mice/percent CFSE (low) in control mice)]×100} as previously described [24].

2.12. Statistical analysis

The statistical significance of the differences between the two groups examined was determined by the means and standard deviation using Student's *t*-test and between three or more groups by the one-way analysis of variance (ANOVA). Differences were considered significant at $p<0.05$.

3. Results

3.1. Pidotimod induces maturation of MDDCs

In order to investigate the effect of pidotimod on the maturation of MDDCs, human $CD14^+$ monocytes were initially cultured with GM-CSF and IL-4 for 6 days, to induce their differentiation into typical immature MDDCs (iMDDCs). At this time iMDDCs were characterized by a down-regulation of CD14 molecule expression and up-regulation of HLA-DR and CD86 antigens ($CD14^-$/HLA-DR^{++}/$CD86^+$) and showed the typical macroscopic aspect of immature myeloid dendritic cells (Fig. 1A). To investigate the direct effect of pidotimod on the maturation of MDDCs, iMDDCs were cultured for 24 h in both the presence and absence of 1 μg/ml pidotimod. LPS, a well-known factor that initiates terminal DCs maturation by stimulation of TLR4, was used at a concentration of 1 μg/ml as positive control of the reaction. DCs were then analyzed by flow cytometry for their phenotype. Analyzed by light microscopy human iMDDCs cultured in the presence of pidotimod had a phenotype belonging to that of mature DCs and superimposable to that induced in iMDDCs by LPS treatment. In fact, flow cytometric analysis revealed that pidotimod treatment for 24 h increased the expression of HLA-DR, CD83 and CD86 of 20%, 15% and 10% respectively compared to untreated iMDDC (Fig. 1B). These results suggested that pidotimod, similarly to LPS, has the ability to induce phenotypic maturation of iMDDCs by enhancing the expression levels of surface markers, which are fundamental for crosstalking with cells of adaptative immunity.

3.2. Cytokine production by MDDCs in response to pidotimod treatment

DCs play an important role in T cell-mediated immune response via secretion of different cytokine profiles. In particular we focused on MCP-1 and TNF-α, since they represent important local inflammation

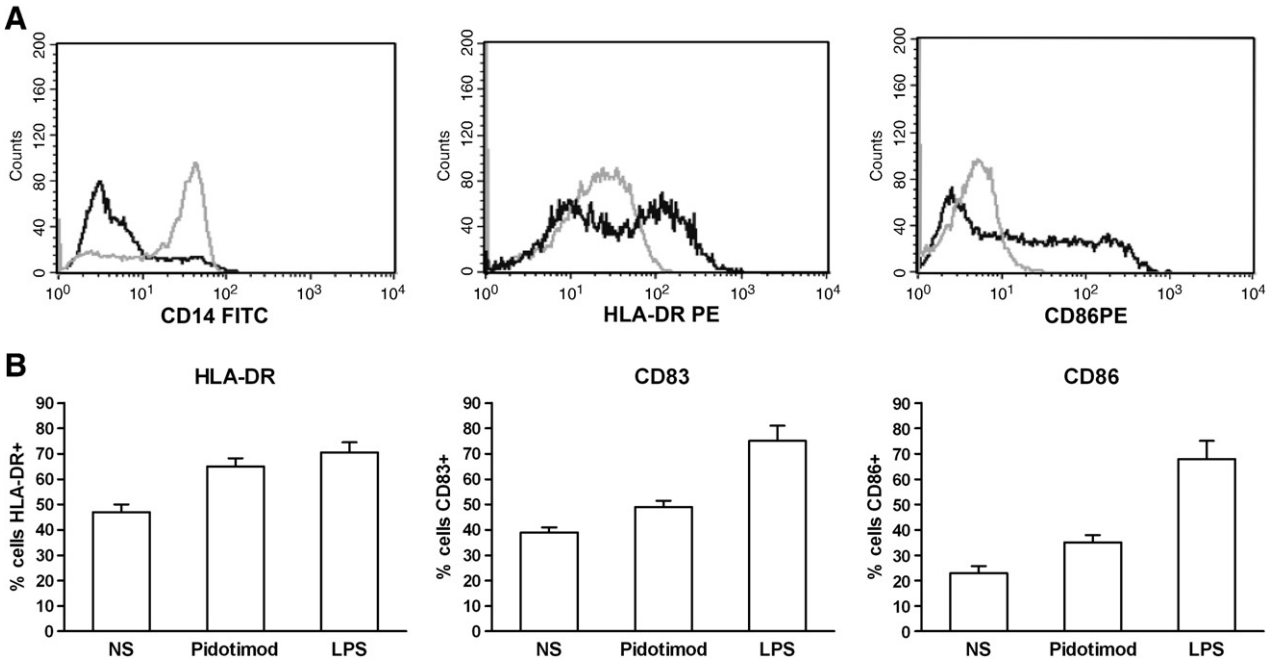

Fig. 1. (A) Monocytes (grey line) stimulated with GM-CSF and IL-4 for 6 days acquire the phenotype of iMDDC (black line). Cells were stained with mAbs anti CD14, HLA-DR and CD86 to determine the phenotype of DCs by flow cytometry. The histogram shows the down-regulation of CD14 and up-regulation of HLA-DR and CD86. (B) After stimulation of human $CD14^+$ monocytes with GM-CSF and IL-4 for 6 days, the iMDDC were incubated in μg/ml LPS. After 24 h cells were collected and stained with mAbs to HLA-DR, CD83 and CD86. Results represent the mean ± SD of triplicate samples and are expressed as percentage of cells expressing respectively HLA-DR, CD83 and CD86. Data are representative of six independent experiments with similar results.

mediators. In fact, these molecules are able to activate the vascular endothelium and increase its permeability, increase the recruitment of inflammatory cells and the synthesis of other cytokines. Experiments were performed to assess the capability of pidotimod to modulate the production of these pro-inflammatory molecules by iMDDC. iMDDCs, generated as described before, were cultured for 24 h in both the presence and absence of pidotimod (1 μg/ml) and supernatant tested for the presence of MCP-1 and TNF-α by quantitative ELISA. As shown in Fig. 2, pidotimod-treated dendritic cells produced significantly larger amounts of MCP-1 and TNF-α than untreated cells, the increase was approximately of 180% and 450% for MCP-1 and TNF-α respectively ($p<0.01$). These results indicate that pidotimod exerts a potent activity on DC function by inducing them to release high amounts of pro-inflammatory molecules MCP-1 and TNF- α cytokines.

3.3. Pidotimod-treated DCs induce proliferation and polarization of co-cultured $CD4^+$ naïve T cells towards Th1 phenotype

DCs have the unique ability to induce a primary immune response through proliferation, activation and polarization of naive T cells. Therefore, we evaluated the ability of pidotimod to trigger MDDCs to stimulate a primary allogeneic naive T cell response. Naive $CD4^+$ T cells, previously stained with CFSE, were co-cultured for 5 days with DCs treated and not treated with pidotimod for 24 h. After 5 days of co-culture, cells were collected and washed, and proliferation of $CD4^+$ naive T lymphocytes was determined by flow cytometry. As shown in Fig. 3 (A–C–D) $CD4^+$ naive T cells co-cultured for 5 days with pidotimod-treated DCs, displayed a proliferative activity significantly stronger than cells co-cultured with untreated DCs (>10%, $p<0.01$). Moreover, when $CD4^+$ naive T cells were co-cultured for 5 days with pidotimod-treated DCs and expanded for further 8 days in culture medium containing IL-2 (25 U/ml), they maintained a stronger proliferative activity if compared to cells co-cultured with mock-treated DCs (data not shown).

In order to investigate if pidotimod-treated iMDDCs induce polarization of co-cultured $CD4^+$ naïve T cells towards a Th1 phenotype, the secretion of the IFN-γ Th1 cytokine was evaluated. Naive $CD4^+$ T cells were co-cultured for 8 days with pidotimod-treated or mock-treated iMDDCs and then stimulated for 9 h with PMA and ionomycin, in the presence of monensin for the last 6 h of

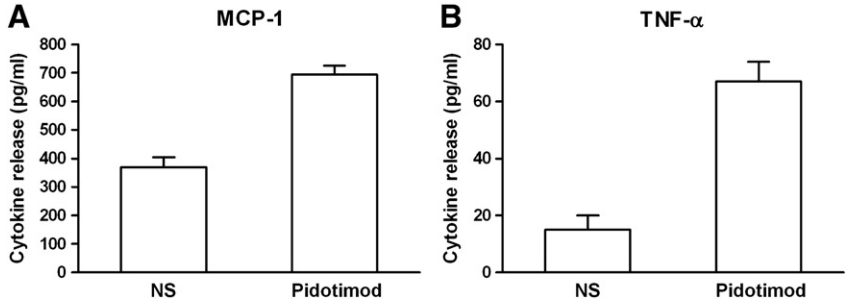

Fig. 2. (A–B) After 6 days of stimulation with GM-CSF and IL-4 the immature DCs were cultured in the presence and absence of pidotimod for 24 h. After the treatment with pidotimod the supernatant from cell cultures was collected and the secreted pro-inflammatory cytokines were quantified by ELISA. The histograms show the MCP-1 (A) and TNF-α (B) production levels after pidotimod stimulation ($p<0.01$). Results represent the mean ± SD of triplicate samples. Data are representative of six independent experiments with similar results.

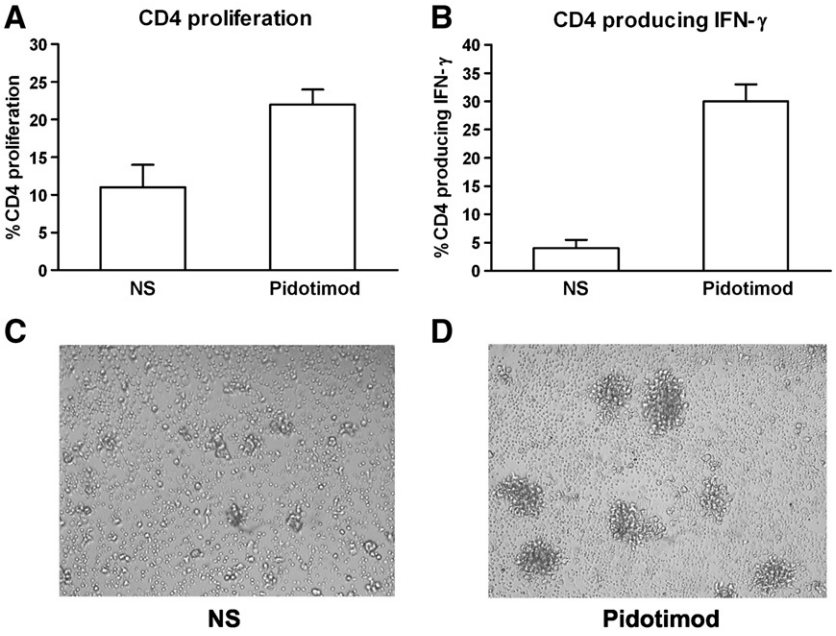

Fig. 3. (A) MDDCs were incubated with pidotimod for 24 h and they were then co-cultured with $CD4^+$naive T cells previously stained with CFSE. After 5 days of co-culture, the proliferation of $CD4^+$ naive T lymphocytes induced by unstimulated mature DCs or pidotimod-treated DCs was evaluated. The proliferation of $CD4^+$ naive T lymphocytes was analyzed by flow cytometry. The data show the percentage of proliferating $CD4^+$ cells co-cultured with unstimulated mature DCs and pidotimod-treated DCs ($p<0.01$). Results represent the mean ± SD of triplicate samples. Data are representative of six independent experiments with similar results. (B) Naive $CD4^+$ T cells co-cultured for 8 days with pidotimod-treated DCs differentiate towards a Th1 phenotype and secrete IFN-γ. After 6, 9 and 12 h of PMA/ionomycin stimulation, in the presence of monensin, $CD4^+$ T cells were collected, washed, fixed and permeabilized at 4 °C for 20 min. After permeabilization the cells were then stained with a FITC-conjugated monoclonal antibody to evaluate and detect the presence of intracellular IFN-γ by flow cytometry. Here we show the percentage of IFN-γ producing $CD4^+$ cells co-cultured with unstimulated mature DCs and pidotimod-treated DCs ($p<0.01$). Results represent the mean ± SD of triplicate samples. Data are representative of six independent experiments with similar results. (C–D) Images of $CD4^+$ T cells after 5 days of co-culture with unstimulated MDDCs and pidotimod-treated DCs. The presence of cellular rosette in T cells co-cultured with pidotimod-treated DCs provides evidence for activation of naive T cells. The images are representative of at least three different experiments.

culture. The cells were then analyzed for intracellular expression of IFN-γ by flow cytometry.

We found that naive T cells co-cultured with pidotimod-treated iMDDCs secreted substantially higher amounts of the Th1 cytokine IFN-γ (Fig. 3B) ($p<0.01$) than cells co-cultured with mock-treated iMDDCs. This finding provides evidence that pidotimod drives DCs to stimulate T cell differentiation towards a Th1 phenotype.

Because the level of IL-12 production by myeloid DCs during activation of naïve Th cells is a major factor driving the development of Th1 cells, we also evaluated the effects of pidotimod on bioactive IL-12p70 production by iMDDCs. We tested the supernatants of iMDDCs for the presence of IL-12 by quantitative ELISA after 24 h of culture with pidotimod (1 μg/ml) either as a unique stimulus or in combination with the classical maturation stimulus LPS (10 ng/ml). In fact, it is known that it is especially important at the onset of a primary immune response the need for a second signal to induce IL-12 production [25,26]. As positive control we cultured iMDDCs in the presence of LPS (10 ng/ml) and IFN-γ (100 ng/ml) and, as expected, we observed a consistent secretion of IL-12p70. Surprisingly, DCs didn't show enhanced IL-12p70 production when treated with pidotimod alone or in combination with LPS (data not shown). This suggests that the ability of pidotimod to induce the development of Th1-inducing DCs is due to the existence of an alternative Th1-driving mechanism.

3.4. Intranasal immunization with OVA co-administered with pidotimod results in the elicitation of strong antigen-specific antibody responses in serum

The upper airway mucosa of humans contains a dense network of dendritic APCs both in the epithelium and in the lamina propria [27]. These mucosal DCs express an immature phenotype with low levels of co-stimulatory molecules and are functionally characterized by high antigen-capturing and low antigen-presenting capacity [28].

Evidence strongly suggests that APCs, especially mucosal DCs, play a key role in induction of protective immune responses via processing and presenting peptides in an immunogenic form to the adaptive immune system [29].

Once demonstrated that pidotimod has the ability of inducing *in vitro* phenotypic maturation of iMDDCs, our aim was to evaluate if, during intranasal immunization, use of pidotimod co-administered with OVA could result in a stronger stimulation of antibody response. C57BL/6 ($H\text{-}2^b$) mice were intranasally immunized with PBS, OVA alone (50 μg/dose) and OVA co-administered with pidotimod (100 μg/dose) on days 0, 14 and 21. As shown in Fig. 4A, animals immunized with OVA alone didn't significantly increase the OVA-specific antibody titer. In contrast, when pidotimod was co-administered with OVA, a significant rise in OVA-specific antibody titer was observed just after a single boost. At the end of immunization protocol, the OVA-specific antibody titer was approximately 35-fold higher in animals vaccinated with OVA in the presence of pidotimod than in animals receiving OVA alone (Fig. 4A). After intranasal immunization, we had no evidence of behavioural changes in the pidotimod-treated mice when compared to the PBS or OVA-treated animals.

3.5. Intranasal immunization with pidotimod results in increased differentiation of splenocytes and bone marrow cells into OVA-specific effector cells

After 1 and 8 weeks from the last intranasal immunization, splenocytes and bone marrow cells were stimulated for 16 h with peptides representing OVA MHC class I restricted epitopes. Then, the number of IFN-γ-producing cells was evaluated by ELISPOT. Intranasal immunization with OVA alone didn't result in any increase in IFN-γ-producing cells (Fig. 4C–D). In contrast, co-administration of the OVA protein with pidotimod triggered splenocytes and bone marrow cells to produce IFN-γ (Fig. 4B).This result suggests that intranasal

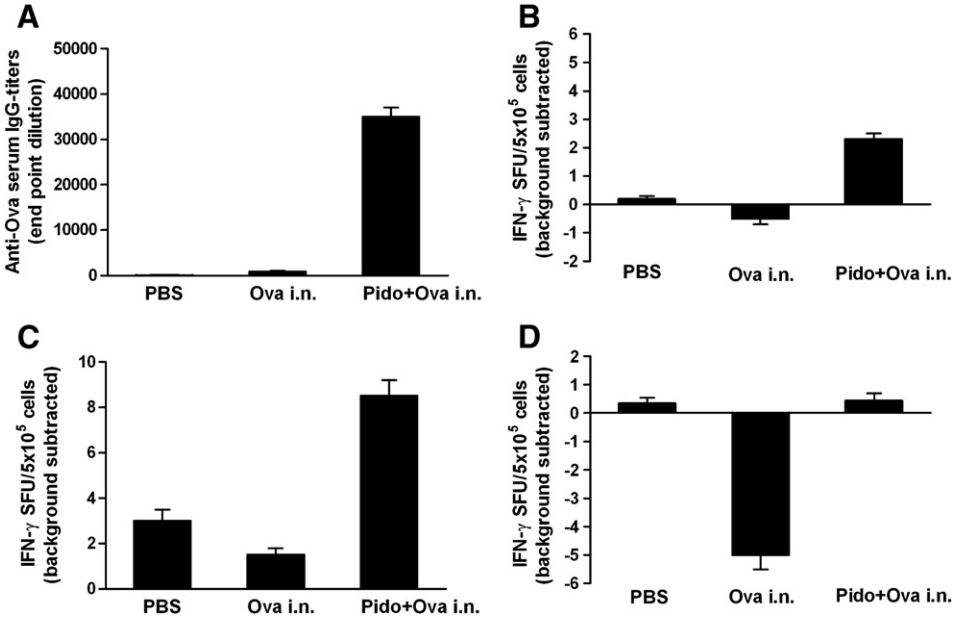

Fig. 4. (A) Analysis of OVA-specific serum antibodies in C57BL/6 mice after intranasal immunization with PBS, OVA alone and OVA co-administered with pidotimod. On day 28, OVA-specific IgG antibody titer was determined by end-point dilution ELISA. Each bar represents the group mean end-point titre. The SEM is indicated by vertical line. Differences were statistically significant at $p<0.05$. (B–C–D) Percentage of splenocytes (C–D) and bone marrow (B) cells producing IFN-γ after 8 weeks from the last intranasal immunization. The cells were stimulated for 16 h with the dominant (C) and subdominant (D) epitopes of OVA antigen recognized by MHC class I. The number of IFN-γ-secreting cells was determined by ELISPOT. Results are presented as IFN-γ spot forming unit (SFU)/10^5 cells. The values reported are those obtained from stimulated cells subtracted of background from non-stimulated cells. The SEM of four values is indicated by vertical lines. Differences were statistically significant at $p<0.05$.

injection of a model antigen with pidotimod results in an OVA-specific Th1 immune response.

3.6. Intranasal immunization with pidotimod results in the production of OVA-specific IgG from splenocytes and bone marrow cells

It is known that after initial contact with an antigen, plasmablasts generate a short humoral immune response. However, most antibodies secreting cells (ASCs), generated by a second contact with the antigen, leave the secondary lymphoid organs to move towards bone marrow, mucosal-associated tissues and chronically inflamed tissues [30]. These ASCs with a plasma cell mature phenotype have a potential lifespan of more than 1 year. Because the different IgG subclasses have a half-life of 3 weeks, the maintenance of antibody titres over long period of time from bone marrow antibody secreting cells is very important [31]. Therefore, we examined the ability of bone marrow cells of secreting

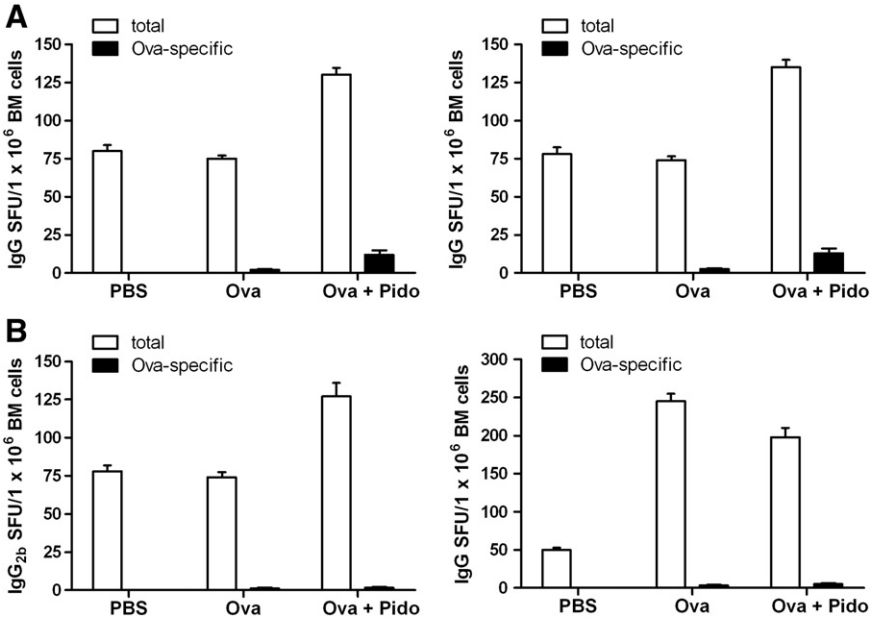

Fig. 5. (A) Determination of total and OVA-specific IgG (left) and IgG1 (right) in bone marrow antibody secreting cells present in immunized animals. Spleen cells from C57BL/6 mice immunized intranasally with PBS, OVA alone and OVA co-administered with pidotimod were cultured for 6 h and the number of IgG secreting cells was evaluated by ELISPOT. Results are presented as spot forming unit (SFU)/10^6 cells. The SEM of four values is indicated by vertical lines. Differences were statistically significant at $p<0.05$. (B) Determination of total and OVA-specific IgG2b by ELISPOT in bone marrow antibody secreting cells (left) and determination of total and OVA-specific IgG in spleen antibody secreting cells (right) present in immunized animals. Humoral immune response was stimulated in C57BL/6 mice after intranasal immunization with PBS, OVA alone and OVA co-administered with pidotimod. Results are presented as spot forming unit (SFU)/10^6 cells. The SEM of four values is indicated by vertical lines. Differences were statistically significant at $p<0.05$.

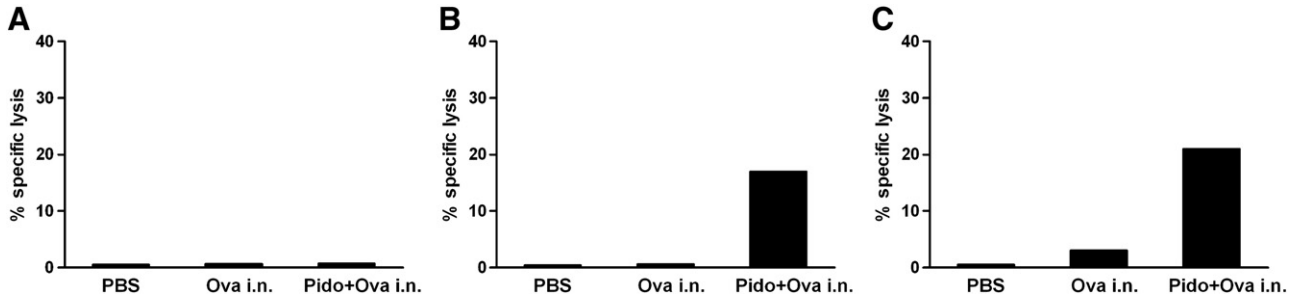

Fig. 6. (A–B–C) Analysis of cell-mediated cytotoxic activity induced *in vivo* in mice immunized with OVA + pidotimod. Splenocytes of C57BL/6 naïve mice, previously labeled with 0.1 μM and 1 μM CFSE and incubated with and without subdominant (A), dominant (B) and cryptic (C) OVA peptides, were then transferred by intravenous injection into mice after 21 days from immunization. After 16 h spleens were harvested and CFSE (high) and CFSE (low) cells were detected by flow cytometry. Results are expressed as percentage of lysed cells pulsed with peptides.

total and OVA-specific IgG after 6 h of stimulation with OVA by ELISPOT assay. This short incubation period is not enough for the differentiation of memory B cells into ASCs, thus, only actively secreting mature plasma cells can be determined [32]. We observed a significant increase in the number of bone marrow cells secreting total IgG, IgG1, IgG2b and OVA-specific IgG and IgG1, in mice intranasally immunized with OVA in combination with pidotimod if compared to mice immunized with OVA alone (Fig. 5A–B). In contrast, we did not find any statistically significant difference in the number of bone marrow cells secreting OVA-specific IgG2b in immunized mice with OVA alone or in combination with pidotimod. Moreover, we didn't observe any increase in the number of splenocytes secreting total and OVA-specific IgG in C57BL/6 (H-2^b) mice intranasally immunized with OVA co-administered with pidotimod.

3.7. Pidotimod generates in vivo an OVA-specific cytotoxic response

In order to evaluate the ability of pidotimod to induce specific cytotoxic responses against OVA, splenocytes from naïve mice were collected after 21 days from the last intranasal immunization, stained with CFSE and treated for 1 h with dominant, subdominant and cryptic OVA peptides. Cells were then inoculated in C57BL/6 mice previously immunized intranasally with OVA and pidotimod. As shown in Fig. 6, the ability of splenocytes of intranasally immunized mice to induce a specific cytotoxic response against cells expressing dominant and cryptic OVA peptides was higher in mice immunized with OVA in combination with pidotimod (Fig. 6B–C) than in mice immunized with OVA alone. On the other hand no response was seen by inoculating splenocytes treated with subdominant peptide into immunized mice with OVA alone or OVA plus pidotimod.

4. Discussion

Activation and maturation of DCs have a pivotal impact on immune response. The majority of adjuvants in experimental use today provide signals which the innate immune system vigorously reacts to, resulting in a generalized potentiation of the immune system so that the Ag coadministrated with the adjuvant can be more effectively taken up, processed and presented by the activated DCs. Molecules with immunomodulatory activity and adjuvant properties at the mucosal site are able to enhance humoral and cellular immune responses against co-administered antigens at both mucosal and systemic levels [14–16].

In this study we analyzed for the first time the effect of pidotimod on DC maturation. Data obtained demonstrate that pidotimod induces a phenotypic DC maturation, being able to decrease CD14 expression and up-regulate the expression of co-stimulatory molecules, such as HLA-DR, CD83 and CD86, which are very important as co-receptors for DC/T cell crosstalk. Maturation of DCs leads to the production of pro-inflammatory cytokines [3] which can play an important role in T cell-mediated immune responses.

Release of MCP-1 and TNF-α, makes possible for DCs to activate and increase the permeability of vascular endothelium, the recruitment of inflammatory cells, the synthesis of other cytokines and finally to regulate the function of different immune cells [33]. Moreover, MCP-1 has been reported to promote T helper (Th) cell development [34] and memory T cells chemoattraction [35]. Our results show that pidotimod induced DCs to release large amounts of MCP-1 and TNF-α cytokines, confirming its capability of promoting not only a phenotypic but also a functional DC maturation.

Fully competent DCs can activate naïve T lymphocytes to induce adaptative immunity, [36–38]. We investigated if pidotimod-treated DCs could induce proliferation and polarization of co-cultured $CD4^+$ naïve T cells towards a Th1 phenotype.

Our results show that pidotimod-treated DCs were able to induce a significant increase in proliferation of $CD4^+$ naive T cells if compared to untreated DCs. Moreover, naive T cells stimulated by pidotimod-treated DCs differentiate towards helper T cell type 1 (Th1) response as judged by elevated IFN-γ level even if iMDDCs did not show enhanced IL-12p70 production. The level of IL-12 production by myeloid DCs during activation of naïve Th cells is a major factor driving the development of Th1 cells, so Th1-promoting capacity by pidotimod-treated DCs suggests the existence of an alternative Th1-driving mechanism as already demonstrated for other immunomodulant molecules [39–41]. According to Viola et al. the effect of DCs on differentiation of naive T cells towards a Th1 response may be due to their higher expression level of co-stimulatory molecules when treated with pidotimod, which facilitates T cell activation by lowering the activation threshold [42]. Cell receptors and/or intracellular pathways engaged by pidotimod to activate DCs are not known yet. We can speculate, on the basis of its chemical structure, that pidotimod's action may be exerted through the interaction with some Pattern Recognition receptors (as TLR) and this might be a matter for further investigation.

The mucosa contains cells of the innate immune system, which through a variety of mechanisms significantly contribute to host defense against pathogens [43] and to initiating adaptative mucosal immune responses. Mucosal DCs play a key role in regulating effector immune responses. They prime T and B cell responses, resulting in optimal protection of the host at the mucosal surfaces, providing antigenic and co-stimulatory signal to lymphocytes and upregulating chemokines and homing receptors. Thus, drugs capable of activating and inducing DC maturation — and therefore acting as adjuvants at the mucosal level — may critically favour the induction of protective mucosal cellular responses. Because of its *in vitro* capability of inducing phenotypic and functional maturation of DCs, we decided to perform *in vivo* experiments with the aim to evaluate if pidotimod could promote strong and specific immune responses when co-administered intranasally with antigens and trying to understand if it can become a possible candidate for improving effective mucosal immune responses. We evaluated in particular whether intranasal co-administration of a model antigen (OVA) with pidotimod in C57BL/6

mice resulted in a potent enhancement of humoral and cellular immune responses. The results obtained *in vivo* demonstrate that intranasal administration of OVA with pidotimod resulted in the stimulation of a strong and specific antibody response and in the generation of antigen-specific Th-1 effector cells, as well as a potent cytotoxic T-lymphocyte response.

Mucosal vaccination is the most effective strategy to achieve an efficient systemic and local immune response at the portal of entry, which is the first line of defense. However, mucosally administered antigens are usually poorly immunogenic and the co-administration with adjuvants, which increase the local and systemic immune responses, is needed to elicit effective mucosal and systemic immune responses [14–16]. Pidotimod capability of improving antigen presentation *in vivo* to cells of adaptive immunity after intranasal delivery might make this molecule an attractive adjuvant for the development of mucosal vaccine formulations. The antigen-specific immune response induced by pidotimod might also have a significant role in the control of infection processes and explain most of the clinical observations that attest for the activity of pidotimod in preventing and/or controlling upper and lower respiratory tract infections in both children and adults.

In conclusion, this study provides new insights into the actions of pidotimod as an immunomodulatory agent and points for the first time on DCs as a major potential target for pidotimod to exert its immunomodulatory functions. The cellular target and mechanism of action makes possible to use pidotimod as adjuvant molecule for the development of new strategies to promote and direct mucosal immune responses.

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References

[1] Borghi MO, Fain C, Barcellini W, Del Papa N, La Rosa L, Nicoletti F, Uslenghi C, Meroni PL. Ex vivo effect of pidotimod on peripheral blood mononuclear cell immune functions: study of an elderly population. Int J Immunotherapy 1994;10:35–9.
[2] Pugliese A, Uslenghi C, Torre D, D'Alonzo L, Corgnati F, Girardello R. Evaluation of the effect of pidotimod on the in vitro production of interferons. Int J Immunotherapy 1995;11:71–6.
[3] Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767–811.
[4] Sallusto F, Cella M, Danieli C, Lanzavecchia A. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 1995;182:389–400.
[5] Reis C, Sousa, Stahl PD, Austyn JM. Phagocytosis of antigens by Langerhans cells in vitro. J Exp Med 1993;178:509–19.
[6] Sheng KC, Pietersz GA, Wright MD, Apostolopoulos V. Dendritic cells: activation and maturation applications for cancer immunotherapy. Curr Med Chem 2005;12:1783–800.
[7] Miller RA. The ageing immune system: primer and prospectus. Science 1996;273:70–4.
[8] Yoshikawa TT. Epidemiology and unique aspects of ageing and infectious diseases. Clin Infect Dis 2000;30:931–3.
[9] Murasco DM, Bernstein ED, Gardner EM, Gross P, Munk G, Dran S, Abrutyn E. Role of humoral and cell-mediated immunity in protection from influenza disease after immunization of healthy elderly. Exp Gerontol 2002;37:427–39.
[10] Webster RG. Immunity to influenza in the elderly. Vaccine 2000;18:1686–9.
[11] Donnini A, Argentari K, Mancini R, Smorlesi A, Bartozzi B, Bernardini G, Provinciali M. Phenotype, antigen-presenting capacity, and migration of antigen-presenting cells in young and old age. Exp Gerontol 2002;37:1097–112.
[12] Gon Y, Hashimoto S, Hayashi S, Koura T, Matsumoto K, Horie T. Lower serum concentrations of cytokines in elderly patients with pneumonia and the impaired production of cytokines by peripheral blood monocytes in the elderly. Clin Exp Immunol 1996;106:458–60.
[13] De Martinis M, Modesti M, Ginaldi L. Phenotypic and functional changes of circulating monocytes and polymorphonuclear leukocytes from elderly persons. Immunol Cell Biol 2004;82:415–20.
[14] Rharbaoui F, Drabner B, Borsutzky S, Winckler U, Morr M, Ensoli B, Muhlradt PF, Guzman CA. The mycoplasma-derived lipopeptide MALP-2 is a potent mucosal adjuvant. Eur J Immunol 2002;32:2857–65.
[15] Becker PD, Fiorentini S, Link C, Tosti G, Ebensen T, Caruso A, Guzman CA. The HIV-1 matrix protein p17 can be efficiently delivered by intranasal route in mice using the TLR 2/6 agonist MALP-2 as mucosal adjuvant. Vaccine 2006;24:5269–76.
[16] Fiorentini S, Becker PD, Marini E, Marconi P, Avolio M, Tosti G, Link C, Manservigi R, Guzman CA, Caruso A. HIV-1 matrix protein p17 modulates in vivo preactivated murine T-cell response and enhances the induction of systemic and mucosal immunity against intranasally co-administered antigens. Viral Immunol 2006;19:77–88.
[17] Coppi G, Mailland F. Immunopharmacological studies on PGT/1A, a new immunostimulating drug. Pharmacol Res 1990;22:126–8.
[18] Migliorati G, D'Adamio L, Coppi G, Nicoletti I, Ricardi C. Pidotimod stimulates natural killer cell activity and inhibits thymocyte cell death. Immunopharmacol Immunotoxicol 1992;14:737–48.
[19] Marelli P, Lupetti A, Senesi S, Uslenghi C, Girardello R, Campa M. Potenziamento della resistenza alle infezioni virali e batteriche dopo somministrazione di Pidotimod nel topo. Drugs Exp Clin Res 1993;19:15–21.
[20] Careddu P, Biolchini A, Alfano S, Zavattini G. Pidotimod in the prophylaxis of recurrent acute tonsillitis in childhood. Adv Otorhinolaryngol 1992;47:328–31.
[21] Careddu P. Role of immunoactivation with pidotimod in recurrent respiratory infections in childhood. Arzneimittelforschung/Drug Res 1994;44:1506–11.
[22] Aivazis V, Hatzimichail A, Papachristou A, Valeri R, Iuga-Donca G. Clinical evaluation and changes of the respiratory epithelium function after administration of pidotimod in Greek children with recurrent respiratory tract infections. Minerva Pediatr 2002;54:315–9.
[23] Hermans IF, Silk JD, Yang J, Palmowski MJ, Gileadi U, McCarthy C, Salio M, Ronchese F, Cerundolo V. The VITAL assay: a versatile fluorometric technique for assessing CTL- and NKT-mediated cytotoxicity against multiple targets in vitro and in vivo. J Immunol Methods 2004;285:25–40.
[24] Becker PD, Nörder M, Guzmán CA, Grinstein S. Immune modulator adamantylamide dipeptide stimulates efficient major histocompatibility complex class I-restricted responses in mice. Clin Vaccine Immunol 2007 May;14(5):538–43.
[25] Snijders A, Kalinski P, Hilkens CM, Kapsenberg ML. High-level IL-12 production by human dendritic cells requires two signals. Int Immunol 1998 Nov;10(11):1593–8.
[26] Frasca L, Nasso M, Spensieri F, Fedele G, Palazzo R, Malavasi F, Ausiello CM. IFN-gamma arms human dendritic cells to perform multiple effector functions. J Immunol 2008 Feb 1;180(3):1471–81.
[27] Jahnsen FL, Gran E, Have R, Brandtzaeg P. Human nasal mucosa contains antigen-presenting cells of strikingly different functional phenotypes. Am J Respir Cell Mol Biol 2004;30:31–7.
[28] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392:245–52.
[29] Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol 2002;2:151–61.
[30] Manz RA, Radbruch A. Plasma cell for a lifetime? Eur J Immunol 2002;32:923–7.
[31] Moser K, Tokoyoda K, Radbruk A, MacLennan I, Manz RA. Stromal niches, plasma cell differentiation and survival. Curr Opin Immunol 2006;18:265–7.
[32] Masihi KN. Immunomodulators in infectious disease: panoply of possibilities. Int J Immunopharmacol 2000;22:1083–91.
[33] Daly C, Rollins BJ. Monocyte chemoattractant protein-1 (CCL2) in inflammatory disease and adaptive immunity: therapeutic opportunities and controversies. Microcirculation 2003;10:247–57 Review.
[34] Gu L, Rutledge B, Fiorillo J, Ernst C, Grewal I, Flavell R, Gladue R, Rollins B. In vivo properties of monocyte chemoattractant protein-1. J Leukoc Biol 1997;62:577–80.
[35] Carr MW, Roth SJ, Luther E, Rose SS, Springer TA. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. Proc Natl Acad Sci U S A 1994;91:3652–6.
[36] Trinchieri G. Interleukin-12: a proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. Annu Rev Immunol 1995;13:251–76.
[37] Kadowaki N, Antonenko S, Lau JY, Liu YJ. Natural interferon alpha/beta-producing cells link innate and adaptive immunity. J Exp Med 2000;192:219–26.
[38] De Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhsh M, Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. J Immunol 2002 Feb 15;168(4):1704–9.
[39] De Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA, Yazdanbakhsh M, Kapsenberg ML. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse Th cell-polarizing signals. J Immunol 2002 Feb 15;168(4):1704–9.
[40] Schijns VE, Haagmans BL, Wierda CM, Kruithof B, Heijnen IA, Alber G, Horzinek MC. Mice lacking IL-12 develop polarized Th1 cells during viral infection. J Immunol 1998;160:3958.
[41] Hoene V, Peiser M, Wanner R. Human monocyte-derived dendritic cells express TLR9 and react directly to the CpG-A oligonucleotide D19. J Leukoc Biol 2006 Dec;80(6):1328–36.
[42] Viola A, Lanzavecchia A. T cell activation determined by T cell receptor number and tunable thresholds. Science 1996;273:104–6.
[43] Yuan Q, Walker WA. Innate immunity of the gut: mucosal defense in health and disease. J Pediatr Gastroenterol Nutr 2004;38:463–73.