ORIGINAL ARTICLES

UDC: 612.017
DOI: 10.2298/VSP1104301D

The influence of CD40 ligation and interferon-γ on functional properties of human monocyte-derived dendritic cells activated with polyinosinic-polycytidylic acid

Uticaj povezivanja CD40 molekula i interferona-γ na funkcionalna svojstva dendritičnih ćelija monocitnog porekla aktivisanih poliinosinsko-policitidilinskom kiselinom

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Abstract

Background/Aim. Ligation of a Toll-like receptor (TLR) by specific TLR agonists is a powerful tool for maturation induction of monocyte-derived dendritic cells (MoDCs). Studies so far have shown that the treatment of dendritic cells (DCs) with a TLR3 ligand, polyinosinic-polycytidylic acid [Poly(I:C)], may be an appropriate activation agent for obtaining mature MoDCs, competent to prime effective immune responses. However, little is known about how subsequent interaction of MoDCs with T cell-derived stimuli, such as CD40 or interferon-γ (IFN-γ), modulates MoDC functions. Therefore, this problem was the main objective of this study. **Methods.** Immature MoDCs were prepared by cultivation of monocytes from peripheral blood mononuclear cells with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 for 5 days. After that, maturation was induced by the treatment of these cells with Poly(I:C) for 2 days. At day 6, immature MoDCs and Poly(I:C)-activated MoDCs were incubated either with CD40 ligand (L)-transfected J558 cells or IFN-γ for additional 24 hours. Cytokine production was measured by ELISA and FlowCytomix Human T helper Th1/Th2 11plex. Allostimulatory capability of MoDCs was tested using an allogeneic mixed leukocyte reaction (MLR) assay. **Results.** Immature MoDCs showed a moderate potential for stimulation of proliferation of $CD4^+$ T cells, which was enhanced by the treatment with Poly(I:C). Ligation of CD40 or treatment with IFN-γ of immature or Poly(I:C)-treated MoDCs significantly up-regulated their allostimulatory activity. MoDCs matured in the presence of Poly(I:C) up-regulated the production of IL-12 and IL-10, which was followed by increased levels of IFN-γ and decreased levels of IL-5 in co-cultures with allogeneic $CD4^+$ T cells. Ligation of CD40 on immature MoDCs up-regulated the production of IL-12 and IL-23 which was accompanied by increased secretion of IFN-γ in co-culture. Stimulation of CD40 on Poly(I:C)-treated MoDCs significantly enhanced the production of IL-12, IL-23 and IL-10. However, such treated MoDCs decreased the production of IFN-γ and IL-10 and up-regulated the secretion of IL-17. Immature MoDCs treated with IFN-γ up-regulated IL-12, but lowered the production of IL-5 and IL-17 by $CD4^+$ T cells. Treatment of Poly(I:C)-activated MoDCs with IFN-γ down-regulated the production of IL-12 and up-regulated IL-10 by these cells and increased/decreased the levels of IL-10/IFN-γ, respectively, in co-culture with $CD4^+$ T cells. **Conclusion.** Treatment with Poly(I:C) or ligation of CD40 on immature MoDCs induces maturation of these cells into a phenotype that supports Th1 response. Activation of CD40 on Poly(I:C)-treated MoDCs shifts the immune response towards Th17. Treatment of immature MoDCs with IFN-γ down-regulated Th2 and Th17 responses. However, addition of IFN-γ to Poly(I:C)-activated MoDCs down-regulated Th1 response and promote T regulatory mechanisms. Each of these results may have functional and therapeutic implications.

Key words:
dendritic cells; CD40 ligand; interferon-gamma; poly I-C.

Apstrakt

Uvod/Cilj. Poliinosinsko-policitidilinska kiselina [*Polyinosinic-polycytidylic acid* – Poli (I:C)] stimuliše funkcionalno i fenotipsko sazrevanje dendritičnih ćelija (DC). Međutim, malo je podataka o modulaciji funkcije DC tokom interakcije sa T-limfocitima posredovanoj receptorom CD40 i interferonom-γ (IFN-γ), što je bio cilj ovog istraživanja.

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Metode. Nezrele DC dobijene su kultivacijom monocita (Mo) iz periferne krvi u prisustvu faktora stimulacije granulocitno-makrofagnih kolonija (*Granulocyte-Macrophage Colony-Stimulating Factor* – GM-CSF) i interleukina (IL)-4 tokom pet dana. Sazrevanje je indukovano dvodnevnom inkubacijom MoDC sa Poli(I:C). Poslednja 24 časa, nezrele i zrele MoDC kultivisane su sa ćelijama J558 koje su transfektovane ligandom CD40 ili u prisustvu IFN-γ. Produkcija citokina određivana je ELISA metodom, a alostimulatorna sposobnost u mešanoj leukocitnoj kulturi. **Rezultati.** Stimulacija nezrelih MoDC sa Poli(I:C) povećala je sekreciju IL-12, njihovu alostimulatornu sposobnost i produkciju IFN-γ u kokulturi sa $CD4^+$ T limfocitima. Slični rezultati dobijeni su povezivanjem CD40 molekula ili tretiranjem nezrelih MoDC sa IFN-γ. Međutim, stimulacija CD40 molekula na MoDC koje su aktivisane sa Poli(I:C) povećala je produkciju IL-12, IL-23 i IL-10 što je pospešilo produkciju IL-17, a snizilo produkciju IFN-γ i IL-10 u MoDC/$CD4^+$ kokulturi. Suprotno tome, IFN-γ snizio je produkciju IL-12, a povećao produkciju IL-10 od strane MoDC aktivisanih sa Poli(I:C), što je bilo povezano sa sniženjem IFN-γ, a porastom nivoa IL-10 u ćelijskoj kokulturi. **Zaključak.** Poli(I:C), IFN-γ i povezivanje CD40 molekula su aktivatori sazrevanja MoDC i stimulatori Th1 imunog odgovora. Ligacija CD40 molekula na MoDC aktivisanim sa Poli(I:C) usmerava u pravcu Th17, a inhibira Th1 imuni odgovor. U istom modelu IFN-γ inhibira Th1 odgovor, a stimuliše imunoregulatorne mehanizme. Svaki od dobijenih rezultata može imati specifične funkcijske ili terapeutske implikacije.

Ključne reči:
ćelije, dendritične; CD40 ligand; interferon-gama; poli I-C.

Introduction

Dendritic cells (DCs) are bone marrow-derived cells that function as antigen-presenting cells (APCs). Immature DCs in the periphery capture and process antigens and have a low T cell stimulatory capability. These potent APCs express a wide variety of pattern recognition receptors (PRRs) by which they recognize a conserved groups of molecules, collectively known as molecular patterns (MPs). Activation of PRRs triggers signaling pathways resulting in phenotypic changes and functional maturation of DCs. An important group of PRRs are Toll-like receptors (TLRs) which are crucial proteins that link innate and adaptive immunity [1].

Upon encounter inflammatory cytokines, bacterial or viral products, DCs enter a crossroad where their fate, migratory type or cytokine-producing type is determined. At this stage DCs express costimulatory molecules, migrate to lymphoid organs and secrete cytokines to initiate immune responses [2, 3]. Inflammatory and innate cytokines create the environment in which antigen-specific adaptive T cells expand and differentiate into different effector $CD4^+$ T cells such as T helper (Th1, Th2, Th17) and various subsets of T cells with regulatory activities (Tregs) [4].

Dendritic cells are also important in antitumor immunity and DC-based cancer vaccines have given the encouraging results [5]. Human monocyte-derived DCs (MoDCs) are currently the major source of DCs used in clinical vaccination protocols for the treatment of cancer [6]. MoDCs can be easily prepared by plastic adherence of monocytes from peripheral blood mononuclear cells (PBMCs) and subsequent incubation of the cells for several days in granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4 containing medium [7]. *In vivo*, human DCs have been shown to be more efficient than immature DCs in inducing specific antitumor antigen proliferative and cytotoxic T cell responses [8,9]. Therefore, an important goal in immunotherapy is to identify an optimal protocol for DC maturation. *In vitro* generated mature DCs should produce IL-12 after migration to the lymph nodes and upon subsequent contact with T cell in order to stimulate Th1 immune response and thus maximize clinical efficacy [10].

Ligation of different TLRs by specific TLR agonists is a powerful tool for induction of DC maturation both *in vitro* and *in vivo*. Polyinosinic-polycytidylic acid – Poly(I:C), a synthetic analogue of dsRNA and a TLR3 agonist, triggers the maturation of MoDCs into a phenotype that strongly supports the Th1 responses [11]. Poly(I:C)-treated DCs show a mature phenotype with high expression of costimulatory molecules and a maturation marker, CD83 [12]. Therefore, Poly(I:C) may be an appropriate maturation agent for obtaining stable homogenous mature DCs that are potentially competent to prime effective immune responses *in vivo*. This is supported by the experiments showing that such treated DCs retain the ability to secrete bioactive IL-12 in lymph nodes which is initiated during the *ex vivo* maturation step [10].

CD40 is a cell surface receptor that belongs to the tumor necrosis factor-R (TNF-R) family. Ligation of CD40 on DCs plays an important role in an enhanced survival of these cells, secretion of cytokines and enzymes as well as in enhanced tumoricidal activity and NO synthesis. CD40 ligand (CD40L), is mainly expressed on activated $CD4^+$ T cells. CD40:CD40L interaction has shown the complexity and importance in T cell-dependent humoral immune responses, in acquired cellular immune responses as well as in innate immunity. DC:T-cell interaction via CD40:CD40L upregulates the expression of costimulatory and adhesion molecules on DCs and triggers DCs to secrete IL-12 [13, l4]. These data suggest that ligation of CD40 on DCs may be an additional way to enhance IL-12 production and Th1 immune response. So far, therapies targeting CD40 have been designed to trigger CD40 signaling and thus boost the immune response against tumor. It should be noted that biologically relevant production of IL-12 by DCs is not induced by CD40 engagement alone but requires a second signal [15] which can be provided by other stimuli such as IFN-γ, a key Th1 cytokine.

Interferon-γ is one of the most powerful DC potentiating agent. This cytokine, which is produced by natural killer (NK) and by Th1 cells [16, 17] promotes specific cytotoxic im-

munity by up-regulation of costimulatory and adhesion molecules, chemokines, antigen processing and presentation. IFNγ is a necessary costimulus for IL-12 production in MoDCs [15] and this amplification may be important in stabilization of the Th1 response.

Dendritic cells constatly receive multiple signals and need to integrate them to give a response appropriate to extracellular milieu. The involved factors (TLR3 ligand, CD40L, IFN-γ) may be of crucial importance for modulation of *ex vivo* generated DCs. However, little is known whether their combination may act sinergistically or antagonistically on DC functions and this scientific problem was the principle aim of this study.

Methods

Medium and reagents

Human MoDCs were cultured in RPMI 1640 medium (ICN, Costa Mesa, CA, USA) supplemented with 2 mM L-glutamine, 20 μg/mL gentamicin, 50 μM 2-mercaptoethanol (2-ME) and 10% heat inactivated fetal calf serum (FCS). Recombinant human IL-4 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Recombinant human GM-CSF (Leucomax, spec. activity 4.44×10^6 UI) was obtained from Schering-Plough (Basel, Switzerland). Final concentrations of Poly(I:C) (Sigma-Aldrich, Munich, Germany) and IFN-γ (R&D Systems, Minneapolis, USA) were 25 μg/mL and 5 ng/mL, respectively. The number of CD40L-expressing J558 cells was 1.8 x 10^6/mL.

Cell preparation and MoDC cultures

MoDCs were generated from PBMCs. Briefly, PBMCs from buffy coats of six healthy volunteers were isolated by density centrifugation on Lymphoprep gradient (Nycomed, Oslo, Norway), resuspended in 5 ml of 10% FCS with 2-ME in RPMI medium and allowed to adhere to plastic flasks. After 2 h at 37°C, non-adherent cells were removed and adherent cells were cultured in 5 ml of control medium containing GM-CSF (100 ng/mL) and IL-4 (20 ng/mL). At day 3, 2.5 mL of medium was removed and replaced by the same volume of fresh medium containing GM-CSF and IL-4. After 6 days MoDCs were replated (5 x 10^5 cells/mL) in medium with a GM-CSF/IL-4 and Poly(I:C). At day 7, half of each of these cultures were incubated with J558 cells or with IFN-γ for additional 24 hours. After 8 days, cell-free supernatants were collected and stored at − 20°C for the subsequent determination of cytokine levels.

Allogeneic T-cell activation

The ability of T cells to proliferate was tested in an allogeneic mixed leukocyte reaction (MLR). $CD4^+$ T cells were used as responders in MLR, after their isolation from PBMCs using immunomagnetic sorting with $CD4^+$ isolation kits (MACS technology, Myltenyi Biotec, Bergish Gladbach, Germany) following instructions of the manufacturer. After loading the cell suspension onto a column placed in the magnetic field of a MACS Separator, unlabeled cells run through and this cell fraction consists mainly of the $CD4^+$ T-cell subset as determined by flow cytometry using an anti-CD4 FITC (Serotec, Oxford, UK).

Purified $CD4^+$ T cells (1×10^5 cells/well) were cultivated for 5 days with different numbers of allogeneic MoDCs in complete RPMI medium with 10% FCS in 96-well round-bottomed cell culture plates. Different DC: T cells ratios were used. To assess cell proliferation, cells were pulsed with [^{3}H]-thymidine for the last 18 h (1 μCi/well, Amersham, Books, UK). Labeled cells were harvested onto glass fiber filters and the incorporation of the radionuclide into DNA was further measured by β-scintillation counting (LKB-1219 Rackbeta, Finland). Results were expressed as count per minute (cpm) ± SD of triplicates.

Cytokine assays

After 8 days MoDCs were treated with PMA (20 ng/mL) and ionomycin (500 ng/mL) for 8 hours to stimulate excretion of the synthesized cytokines. A similar procedure was used for stimulation of MoDC/$CD4^+$ T cell coculture after a 5 day incubation period. Cells were harvested, centrifugated and cell-free supernatants were collected and stored at − 20°C for the subsequent determination of cytokine levels. The levels of IL-12p70, IL-23, IL-17 and IL-10 were measured by sandwich ELISA assays from R&D Systems (Minneapolis, USA), following the manufacturer's instructions. The leveles of IFN-γ and IL-5 cytokines were evaluated using FlowCytomix Human Th1/Th2 11plex Kit from Bender MedSystems (Vienna, Austria).

Statistical analysis

Data were analyzed for significant differences using Student`s paired *t*-test ($p < 0.05$ was considered statistically significant).

Results

Effects of CD40 ligation and IFN-γ treatment on the cytokine production by MoDCs

Immature MoDCs were generated by incubating monocytes with GM-CSF and IL-4 for 5 days. After that, maturation was induced by the treatment of these cells with Poly(I:C) for 2 days. At day 6 immature MoDCs and MoDCs induced to mature with Poly(I:C) were incubated either with CD40L-transfected J558 cells or IFN-γ for additional 24 hours. The levels of IL-12, IL-23 and IL-10 were detected in culture supernantans.

The results presented in Figure 1 show that immature MoDCs produced a very small quantity of all three cytokines. Poly(I:C) treatment significantly enhanced the production of IL-12 and IL-10, whereas the production of IL-23 was not significantly changed. Ligation of CD40 on immature DCs was followed by up-regulation of IL-12 and IL-23. However, such treatment of Poly(I:C)-stimulated MoDCs resulted in about 2-fold, 20-fold and 3-fold increase in the production of IL-12, IL-23 and IL-10, respectively. The addition of IFN-γ to immature MoDCs exerted similar stimulatory effect on IL-12 production, as Poly(I:C) did. No significant effect was observed regarding IL-23 and

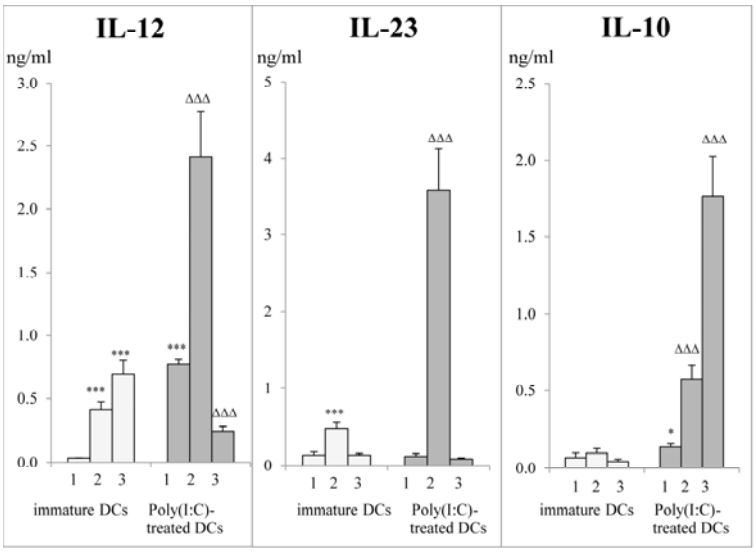

Fig. 1 - Cytokine production by immature human monocyte-derived dendritic cells (MoDCs) and polynosinic-polycytidylic acid – Poly(I:C)-treated MoDCs activated by CD40 ligation and interferon (IFN)-γ

Supernatants of immature and Poly(I:C)-treated MoDCs challenged with CD40L-transfected J558 cells or IFN-γ were collected and processed to determination of cytokine levels using sandwich ELISA assays.
Data represent mean values of six different experiments ± standard deviations (six donors).
Treatment: 1- control; 2- +CD40L; 3- +IFN-γ
***p* < 0.05, ****p* < 0.005 compared with immature MoDCs**
$^{\Delta\Delta\Delta}p$ < 0.005 compared with Poly(I:C)-treated MoDCs

IL-10 production. However, the addition of IFN-γ to the cultures of Poly(I:C)-treated MoDCs down-regulated the production of IL-12 and subsequently up-regulated (7-fold increase) the production of IL-10.

Effects of CD40 ligation and IFN-γ treatment on the allostimulatory activity of MoDCs

The influence of CD40 ligation and IFN-γ on the allostimulatory potential of immature and Poly(I:C)-treated MoDCs was examined in a MLR, where allogeneic $CD4^+$ T-cells were used as responders. The results are presented in Figure 2.

Immature MoDCs showed a moderate potential for stimulation of $CD4^+$ T-cell proliferation, which progressively decreased with lowering the number of DCs as stimulators. MoDCs matured in the presence of Poly(I:C) enhanced the allostimulatory activity of MoDCs at the highest (1 : 10) DC:$CD4^+$ T-cells ratio. Ligation of CD40 on immature MoDCs or IFN-γ treatment of these cells was followed by significant up-regulation in their allostimulatory activity. Such treatment of Poly(I:C)-activated MoDCs additionally enhanced the proliferation of allogeneic $CD4^+$ T-cells.

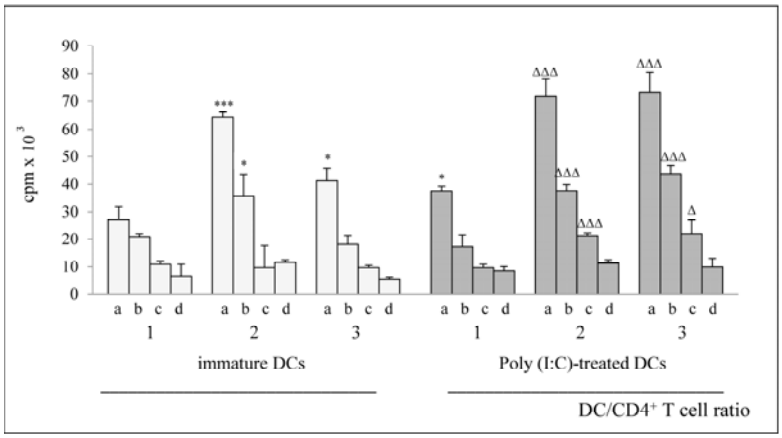

Fig. 2 - Allostimulatory activity of immature human monocyte-derived dendritic cells (MoDCs) and polynosinic-polycytidylic acid – Poly(I:C)-treated MoDCs stimulated with CD40L-transfected J558 cells and interferon (IFN)-γ

The ability of $CD4^+$ T cells to proliferate was tested in allogeneic mixed leukocyte reaction. Different rations of MoDC/$CD4^+$ T cells were used (a- 1:10; b- 1:20; c- 1:40; d- 1:80). After five days of culture cells were pulsed with $[H]^3$-thymidine (1 μCi/well) for the last 18 h. Incorporation of the radionuclide into DNA was measured by β-scintillation counting.
Data represent the mean value of triplicates ± standard deviations.
Treatment: 1- control; 2- +CD40L; 3- +IFN-γ.
***p* < 0.05, ****p* < 0.005 compared with control immature MoDCs**
$^{\Delta}p$ < 0.05, $^{\Delta\Delta\Delta}p$ < 0.005 compared with control Poly(I:C)-treated MoDCs

Effects of CD40 ligation and IFN-γ treatment on the Th polarization capability of MoDCs

The effect of MoDCs on the polarization of Th immune responses was measured by production of cytokines in DC/CD4$^+$ T-cell co-cultures.

As shown in Figure 3 treatment of MoDCs with Poly(I:C) up-regulated the production of Th1 cytokine (IFN-γ), and down-regulated the production of Th2 cytokine (IL-5), while the levels of IL-17 and IL-10 were not changed. Treatment of immature MoDCs with CD40L-transfected cells exerted similar effect on cytokine production as Poly(I:C) did, except that the production of IL-5 was not significantly changed. In contrast, ligation of CD40 on Poly(I:C)-treated DCs enhanced Th17 response and down-regulated Th1 response and production of IL-10. The addition of IFN-γ to immature MoDCs showed no significant effect on the production of IFN-γ, but lowered the production of IL-17 and IL-5. IFN-γ treatment of MoDCs, matured in the presence of Poly(I:C), significantly enhanced the levels of IL-10 and decreased production of IFN-γ, whereas the secretion of IL-17 and IL-5 was not significantly modulated.

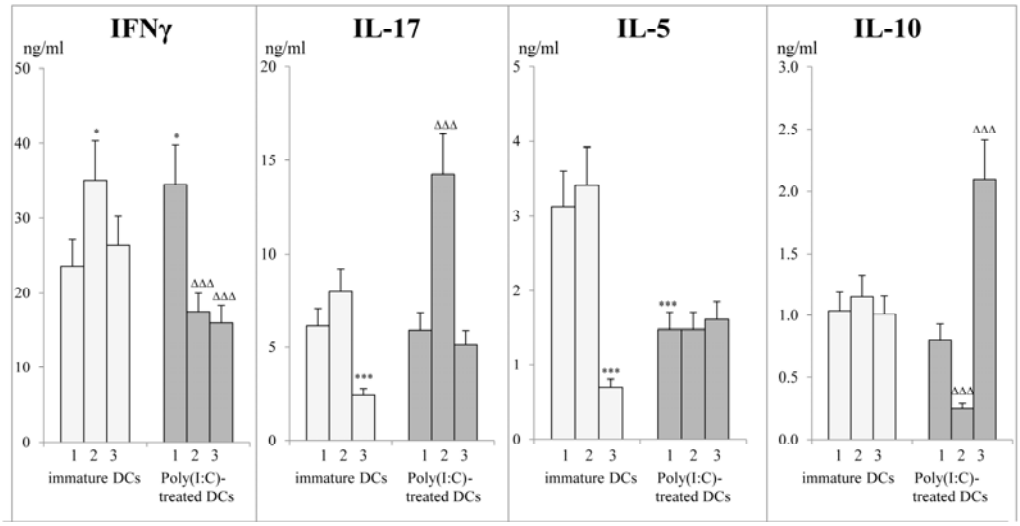

Fig. 3 - Polarization of Th immune response by immature human monocyte-derived dendritic cells (MoDCs) and polynosinic-polycytidylic acid – Poly(I:C)-treated MoDCs activated with CD40L-transfected cells and interferon (IFN)-γ
Production of cytokines in MoDC/CD4$^+$ T-cell cocultures was measured using sandwich ELISA assays and FlowCytomix Human Th1/Th2 11plex. Data represent mean values of three experiments ± standard deviations (three donors). Similar differences between groups were obtained with three other donors. However, the levels of all cytokines in these cultures were significantly lower (data not shown).
Treatment: 1- control; 2- +CD40L; 3- +IFN-γ.
***$p < 0.05$, *** $p < 0.005$ compared with control immature MoDCs**
$^{\Delta\Delta\Delta}p < 0.005$ compared with control Poly(I:C)-treated MoDCs

Discussion

Dendritic cells are professional APCs with an unique ability to prime naive T cells upon antigen presentation, regulate the type of T cell–mediated immune response, but also to induce immunological tolerance [18]. In our study we generated DCs *in vitro* from peripheral blood monocytes with GM-CSF and IL-4. It has been confirmed, similarly as in our previous study [19] and numerous other publications [10, 12, 20, 21] that such MoDCs are immature, triggered moderate allogeneic T cell response in MLR and produced low levels of IL-12 and IL-23, dominant Th1 and Th17 polarizing cytokines, respectively. Such characteristics are in accordance with the knowledge that the capacity of immature DCs to stimulate the immune response is rather weak and thus limits their clinical efficacy, especially as tumor vaccines [22].

Poly(I:C) is a synthetic analog of double-stranded RNA that binds to TLR3, a PRR highly expressed in immature MoDCs [23]. This TLR3 agonist behaves like MP and upon binding to TLR3 signals the presence of infectious agent, followed by activation of DCs and induction of protection to viral cytopathic effects [11]. Activation of DCs leads to induction of inflammatory cytokines and activation of IFN-β promoter, NF-κB and MAP kinases through engagement of the TRIF adaptor protein that cause DCs to mature [24, 25]. It is known that Poly(I:C) induces phenotypic maturation of MoDCs by up-regulation of co-stimulatory molecules (CD80, CD86 and CD40), and maturation marker, CD83 [10, 12]. This could be a dominant mechanism of increased allostimulatory activity of Poly(I:C)-treated MoDCs in our experiments. Poly(I:C) is also a very potent stimulator of IL-12 production and subsequent activator of the Th1 immune response both *in vitro* and *in vivo*, the properties desirable for induction of anti-tumor immunity [26]. This is confirmed in our present study, too. Therefore, Poly(I:C) may be an appropriate maturation agent for obtaining stable homogenous mature DCs that are potentially competent to prime effective immune responses *in vivo*. This is also supported by the experiments showing that Poly(I:C)-treated DCs retain the ability to secrete bioactive IL-12 in lymph

nodes which is initiated during the *ex vivo* maturation step [10]. We also showed that Poly(I:C)-treated MoDCs down-regulated the Th2 immune response, whereas the Th17 immune response was not significantly changed. Down-regulation of Th2 immune response is in agreement with the current concept of reciprocal regulation of Th1/Th2 balance [27].

Ligation of CD40 on DCs plays an important role in maturation and functional modulation of these cells [28, 29]. We used a CD40L-transfected cell line to simulate CD40:CD40L bidirectional crosstalk between DCs and T cells that provides reciprocal regulation of both lymphocytes and DCs [28, 29]. We showed that ligation of CD40 on immature or Poly(I:C)-treated MoDCs significantly up-regulated their allostimulatory activity, most probably as a consequence of increased expression of adhesion and co-stimulatory molecules, such as ICAM-1, HLA-DQ, CD80 and CD86 [30]. It has already been shown that the engagement of CD40 on immature MoDCs as a single signal induces high levels of Th1 polarizing cytokine IL-12 [15] and subsequent production of IFN-γ. We confirmed such results in our study. Moreover, we demonstrated that ligation of CD40 on immature MoDCs was followed by an increased production of IL-23 and IL-17, a phenomenon that has not been described so far. The production of IL-17 was additionally enhanced following ligation by CD40 on Poly(I:C)-treated MoDCs. IL-17 is a signature cytokine of the Th17 subset of $CD4^+$ T cells, whose expansion and maturation is promoted by IL-23 [31]. The Th17 immune response was potentiated after ligation of CD40 on Poly(I:C)-treated MoDCs, but this was followed by down-regulation of Th1 immune response.

Th1 cells were considered as the most important CD+ T cell subset for generating antitumor immunity because of their potential to enhance cytotoxic function of $CD8^+$ cells by producing IFN-γ, as a key activating factor. Recent publications shed new light on potential benefits of Th17 cells in rejection of tumors [32, 33]. Athough first it was considered that the effects of Th17 cells were dependent on IFN-γ and independent of IL-17 and IL-23, due to conversion of Th17 to Th1 [32], the protective function of Th17 cells, with maintained cytokine expression profile, against tumors have been confirmed [33]. The properties of Th17 cells, such as the ability to enhance inflammatory responses and to increase antigen presentation by DCs, promotion of leukocyte homing to tumors, facilitation of $CD8^+$ T cell priming and effector differentiation offer new possibilities for developing the Th17 cell-based therapy for tumors. Regarding the results of our study which are consistent with new insights of tumor immunotherapy, Poly(I:C) together with CD40 ligation generates desirable $CD4^+$ T cell subsets with suitable cytokine mileu for the treatment of tumors. However, such hypothesis needs further testings *in vivo* because we showed that signaling through CD40 on Poly(I:C)-treated MoDCs decreased the Th1 immune response. At the moment it is not known whether such balance between Th1 and Th17 immune response is optimal for antitumor immune response or not. It is known that the Th1 type of immune response could be harmful if exaggerated [34] and thus CD40 signaling could be protective and immunomodulatory.

Interferon-γ is classified as type II IFN in accordance with its receptor specificity and sequence homology [35]. IFNs were initially described as agents that interfere with viral replication [36]. IFN-γ is produced by NK cells and possibly by APCs during the early course of infection, while T lymphocytes became a major source of this cytokine in the adaptive immune response [37]. Cytokine increases antigen processing, presentation and APC costimulatory molecules [35]. We showed in this work that the treatment of immature or Poly(I:C)-activated MoDCs with IFN-γ also enhanced the proliferative activity of allogeneic $CD4^+$ T cells. The allostimulatory potential of MoDCs decreased by lowering the DC:$CD4^+$ T cell ratio. At higher ratios MoDCs showed lesser proliferative capability. One explanation could be that stimulatory effects of costimulatory and adhesion molecules and suitable levels of IL-12 are abrogated by low numbers of producing cells.

A significant finding of this study was related to the dual role of IFN-γ on IL-12 production: stimulation by immature MoDCs; suppression by Poly(I:C)-treated MoDCs. The increased production of IL-12 was followed by increased IFN-γ production and down-regulation of IL-5 and IL-17 production by $CD4^+$ T cells in co-culture. Up-regulation of IL-12 by immature MoDCs is in agreement with previous results [17]. The produced IL-12 attracts and activates T cells and NK cells to produce IFN-γ [14] which, in return, stimulate further production of IL-12 by amplifying loop. Down-regulation of IL-5 production could be explained by reciprocal regulation of Th1 and Th2 immune response [27] and by direct inhibitory effect of IFN-γ on the growth of Th2 cells [35]. The reason why IFN-γ-treated MoDCs inhibited IL-17 production without significant changes of IL-23 production is not clear. Since IL-23 predominantly acts on already differentiated Th17 cell subset [31], it is possible that IFN-γ-treated MoDCs inhibited the differentiation of $Th17^+$ cells by modulating the production of Th17 differentiation cytokines such as TGF-β, IL-1β, IL-6 and IL-21 [38]. Therefore, this hypothesis should be tested in the next experiment.

The inhibition of IL-12 production and stimulation of IL-10 production by IFN-γ- and Poly(I:C)-treated MoDCs is an important finding which could be relevant for down-regulation of Th1 immune response and promotion of an IL-10-mediated immunoregulatory milieu. It is not completely clear whether IFN-γ primarily acts on down-regulation of IL-12 by Poly(I:C)-activated MoDCs or on up-regulation of IL-10 production. It is known that IL-10 is a potent anti-inflammatory and immunosuppresive cytokine that inhibits the production of IL-12 by MoDCs [39]. Therefore, IL-10 is a very important cytokine for self-limiting Th1 cell-mediated immunopathology in conditions of strong inflammatory stimuli [40, 41].

Recently it has been shown that IFN-γ, beside amplifying production of pro-inflammatory cytokines during activation of DCs, also triggers an immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) activity in DCs [42]. It is known that IDO^+ DCs exert immunoregulatory potential which is important for down-regulation of the immune response [43]. In addition, cytokine can also induce the development of adaptive

regulatory T cells [42]. Cumulatively, our results support the concept that IFN-γ, as a dominant Th1 effector cytokine, with the pro-inflammatory properties could be also an important down-regulator of strong immune response.

Conclusion

Treatment with Poly(I:C) or ligation of CD40 on immature MoDCs induced maturation of these cells into a phenotype that supports Th1 response. Activation of CD40 on Poly(I:C)-treated MoDCs shifted the immune response towards Th17. Treatment of immature MoDCs with IFN-γ down-regulated Th2 and Th17 responses. However, addition of IFN-γ to Poly(I:C)-treated MoDCs down-regulated Th1 response and promoted immunoregulatory mechanisms by induction of IL-10, thus limiting the exaggerated and potentially harmful immune response.

Acknowledgments

This work was supported by the grant (VMA/06-10/A.5) of the Military Medical Academy, Belgrade and the Ministry of Science and Technological Development of the Republic of Serbia (project number 175102).

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Received on April 28, 2010.
Revised on October 8, 2010.
Accepted on November 16, 2010.