UNIVERZITET U BEOGRADU
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MODULACIJA FUNKCIJE HUMANIH DENDRITSKIH ĆELIJA KOMBINOVANOM PRIMENOM AGONISTA ENDOZOMNIH TOLL-SLIČNIH RECEPTORA, DEKTIN-1 RECEPTORA I PROINFLAMATORNIH CITOKINA

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MODULATION OF THE FUNCTION OF HUMAN MONOCYTE DERIVED DENDRITIC CELLS BY COMBINED USE OF THE ENDOSOMAL TOLL-LIKE RECEPTORS, DECTIN-1 RECEPTOR AGONISTS AND PROINFLAMMATORY CYTOKINES

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Modulacija funkcije humanih dendritskih ćelija kombinovanom primenom agonista endozomnih toll-sličnih receptora, dektin-1 receptora i proinflamatornih citokina

Rezime

Uvod/cilj. Dendritske ćelije (DĆ), najpotentnije antigen-prezentujuće ćelije (APĆ), integrišu signale koje primaju sa različitih receptora u jedinstveni odgovor. Ključni značaj u ostvarivanju funkcija DĆ imaju receptori za prepoznavanje konzervisanih struktura, tzv. molekularnih obrazaca patogena (engl. Pattern Recognition Receptor, PRR). Aktivacija pojedinačnih PRR, posebno Toll-sličnih receptora (engl. Toll-like receptor, TLR) ili lektinskih receptora C-tipa poput dektina-1, dovodi do sazrevanja DĆ, dok je za razvoj efikasnog imunskog odgovora neophodna kooperacija više receptora.

Agonisti PRR, posebno TLR agonisti, imaju veliku primenu u modulaciji funkcija DĆ u eksperimentalnim i kliničkim ispitivanjima u oblasti imunoterapije tumora. U fokusu aktuelnih istraživanja pripreme anti-tumorskih vakcina je primena Poly (I:C), sintetskog analoga dvolančane RNK (TLR3 agonist). Kultivacija MoDĆ u prisustvu Poly (I:C) dovodi do njihovog fenotipskog i funkcionalnog sazrevanja, pa su tako pripremljene ćelije efikasne u pokretanju imunskog odgovora. TLR3 zajedno sa TLR7-9 predstavlja subfamiliju TLR specifičnih za nukleinske kiseline lokalizovanih na membrani endozoma. Uprkos ohrabrujućim rezultatima, biološki potencijal DĆ za *in vivo* stimulaciju anti-tumorskog odgovora još uvek nije maksimalno iskorišćen. U te svrhe se kombinovanom primenom većeg broja TLR agonista ispituje potencijal MoDĆ da integrišu signale sa različitih receptora. Stoga je jedan od ciljeva naše studije bio da se ispita efekat kombinovane primene Poly (I:C) i loksoribina, selektivnog TLR7 agoniste na funkcionalne i fenotipske karakteristike MoDĆ.

Dektin-1 receptor je, pored TLR, jedini receptor urođenog imuniteta čija aktivacija samostalno indukuje signalnu kaskadu koja dovodi do sazrevanja MoDĆ sa sposobnošću indukcije Th1 i Th17 odgovora. Imajući navedno u vidu, sledeći cilj ovog istraživanja je bio da se ispita efekat kombinovane primene Poly (I:C) i kurdlana, agonista dektin-1 receptora, na funkcionalne i fenotipske karakteristike MoDĆ.

Za modulaciju sazrevanja i funkcija DĆ, pored stimulacije PRR, značajnu ulogu imaju i citokini prirodnog imuniteta koji se produkuju prilikom infekcije. Najnovija istraživanja ukazuju na ulogu TNF-α, jednog od najznačajnijih proinflamatornih citokina, u sazrevanju DĆ u ranim fazama infekcije kao i razvoju antigen-specifičnog odgovora. Naredni cilj našeg istraživanja bio je ispitivanje dozno- i vremenski-zavisnog efekta kombinovane primene TNF-α i Poly (I:C) na funkcionalne i fenotipske karakteristike MoDĆ.

Poslednji deo istraživanja se odnosio na ispitivanje uticaja signala stečenog imuniteta koje DĆ dobijaju tokom interakcije sa T-limfocitima, uključujući signalizaciju preko CD40 receptora i receptora za IFN-γ na njihove karakteristike.

Metode. Nezrele MoDĆ, dobijene kultivacijom humanih monocita, su stimulisane samim Poly (I:C) ili njegovom kombinacijom sa loksoribinom, kurdlanom ili TNF-α tokom 48h. Da bi ispitali uticaj signala stečenog imuniteta, MoDĆ stimulisane samim Poly (I:C) ili u kombinaciji sa TNF-α dalje su kultivisane u prisustvu ćelija J558 transfektovanim ligandom za CD40, solubilnog CD40L ili IFN-γ. U cilju ispitivanja uticaja kinetike aktivacije na kapacitet MoDĆ da polarizuju imunski odgovor, MoDĆ su stimulisane kombinacijama različitih koncentracija Poly (I:C) i TNF-α tokom 24h i 48h. Protočnom citofluorimetrijom su analizirane fenotipske karakteristike MoDĆ. Alostimulatorna sposobnost MoDĆ je određena testom mešane leukocitne reakcije. Produkcija citokina je određena ELISA metodom i testom za detekciju citokina pomoću imunofluorescentnih kuglica.

Rezultati. U preliminarnim eksperimentima nezrele MoDĆ su stimulisane različitim koncentracijama Poly (I:C) (5 μg/ml, 10 μg/ml, 25 μg/ml i 50 μg/ml), loksoribina (34 μg/ml i 85 μg/ml) i kurdlana (10 μg/ml , 50 μg/ml, 100 μg/ml i 200 μg/ml). Na osnovu fenotipskih i funkcionalnih karakteristika MoDĆ procenjeno je da je optimalna koncentracija za aktivaciju MoDĆ za Poly (I:C) 25 μg/ml, za loksoribin 85 μg/ml i za kurdlan 100 μg/ml, dok je koncentracija Poly (I:C) od 10 μg/ml i loksoribina od 34 μg/ml suboptimalna. Optimalne i suboptimalne koncentracije agonista su dalje korišćene za stimulaciju nezrelih MoDĆ u ovom istraživanju.

Stimulacija MoDĆ optimalnom koncentracijom Poly (I:C) dovela je do povećanja ekspresije HLA-DR, CD86, CD40, CD54, CD83 i CCR7 molekula, povećanja produkcije IL-12, umerene produkcije IL-23 i niske produkcije IL-10. Ovako stimulisane MoDĆ dovode do povećane produkcije IFN-γ i umerene produkcije IL-17 tokom kokultivacije sa $CD4^+$ T limfocitima.

MoDĆ kultivisane u prisustvu suboptimalnih koncentracija agonista TLR3 i TLR7 povećano su eksprimirale HLA-DR, CD86, CD83, CD54 i CD40 molekule i produkovale su veće nivoe IL-27, IL-23 i IL-10, u poređenju sa ćelijama tretiranim pojedinačnim agonistima. Ovako pripremljene MoDĆ su dovele do povećanja produkcije IFN-γ i IL-17 u kokulturi sa alogenim $CD4^+$ T limfocitima. Tretman MoDĆ suboptimalnom koncentracijom Poly (I:C) i optimalnom koncentracijom loksoribina u poređenju sa efektima pojedinačnih agonista, doveo je do povećanja ekspresija CD86, smanjenja HLA-DR, povećanja produkcije IL-12 i IL-23 i smanjenja produkcije IL-10 i IL-

27. MoDĆ diferencirane na ovaj način u kokulturi sa $CD4^+$ T ćelijama su indukovale povećanje produkcije IFN-γ i smanjenje produkcije IL-4 i IL-10. Stimulacija MoDĆ optimalnim koncentracijama oba TLR agonista dovela je do smanjenja ekspresije HLA-DR, CD83 i CD40 molekula, kao i povećanja produkcije IL-12, IL-27 i IL-10, dok je nivo IL-23 bio značajno smanjen, u poređenju sa efektima pojedinačnih TLR agonista. MoDĆ stimulisane na ovaj način su u kokulturi sa $CD4^+$ T limfocitima dovele do povećanja produkcije IFN-γ i smanjenje produkcije IL-4, IL-10 i IL-17.

Stimulacija MoDĆ kurdlanom dovela je do povećanja produkcije IL-23 koji je tokom kokultivacije sa $CD4^+$ T limfocitima usmerio odgovor T limfocita u Th17 pravcu uz manje izražen Th1 odgovor. MoDĆ kultivisane u prisustvu Poly (I:C) i kurdlana su ispoljile fenotip zrelih ćelija i stimulisale su proliferaciju alogenih $CD4^+$ T limfocita. Kostimulacija TLR3 i dektina-1 na MoDĆ je dodatno povećala produkciju IL-12, IL-23 i IL-10 u poređenju sa efektima angažovanja pojedinačnih receptora. Ovi rezultati su u korelaciji sa indukcijom snažnijeg Th1 i Th17 imunskog odgovora, procenjivanog na osnovu produkcije IFN-γ od strane naivnih ($CD45RA^+$) $CD4^+$ T limfocita, odnosno IL-17 od strane memorijskih ($CD45RO^+$) $CD4^+$ T limfocita.

MoDĆ stimulisane optimalnom koncentracijom Poly (I:C) i TNF-α ispoljile su povećane nivoe CD80, CD86 i CD54 molekula i smanjile produkciju IL-12, što je uticalo na smanjenje proliferacije T limfocita i produkcije IFN-γ u kokulturi sa alogenim $CD4^+$ T ćelijama, u poređenju sa efektom MoDĆ tretiranih samim Poly (I:C). Dozno-zavisni efekti Poly (I:C) i TNF-α na funkciju MoDĆ su izraženiji nakon 24h kultivacije u poređenju sa 48h, što je procenjeno na osnovu smanjenja produkcije IL-12 i IL-23 od strane MoDĆ i smanjenog nivoa IFN-γ i IL-17 u kokulturi sa $CD4^+$ T ćelijama. Uticaj interakcije CD40 i CD40L je najizraženiji u slučaju produkcije IL-12 od strane MoDĆ tretiranih najvećom koncentracijom Poly (I:C) i TNF-α. Smanjenje produkcije IL-12 od strane MoDĆ stimulisanih ovom kombinacijom je dovelo do smanjenja produkcije IFN-γ u kokulturi sa $CD4^+$ T ćelijama. Povezivanje CD40 molekula na MoDĆ stimulisanim najvećom koncentracijom Poly (I:C) i TNF-α je dovelo do smanjenja produkcije IL-23 i povećanja IL-10. Stoga su ovako pripremljene MoDĆ u kokulturi sa $CD4^+$ T ćelijama dovele do smanjenja produkcije IL-17, a povećanja IL-10.

Povezivanje CD40 molekula ili tretiranje sa IFN-γ nezrelih i MoDĆ diferenciranih u prisustvu Poly (I:C) značajno je povećalo njihov alostimulatorni

potencijal. Povezivanje CD40 molekula na nezrelim MoDĆ dovelo je do povećanja produkcije IL-12 i IL-23, što je praćeno povećanjem produkcije IFN-γ tokom kokultivacije ovako stimulisanih ćelija i $CD4^+$ T limfocita. Međutim, povezivanje CD40 molekula na MoDĆ pretretiranim sa Poly (I:C) dovelo je do povećanja produkcije IL-12, IL-23 i IL-10. Ovako stimulisane ćelije u kokulturi sa $CD4^+$ T ćelijama dovele su do povećanja produkcije IL-17, a smanjenja produkcije IFN-γ i IL-10. Nezrele MoDĆ stimulisane sa IFN-γ produkovale su veći nivo IL-12, a u kokulturi sa $CD4^+$ T limfocitima su dovele do smanjenja produkcije IL-5 i IL-17. Suprotno tome, tretman sa IFN-γ je doveo do smanjenja produkcije IL-12, a povećanja produkcije IL-10 od strane MoDĆ prethodno stimulisanih sa Poly (I:C). MoDĆ pripremljene na ovaj način su tokom kokultivacije sa T limfocitima dovele do smanjenja IFN-γ a povećanja nivoa IL-10.

Zaključak. Poly (I:C) dovodi do sazrevanja MoDĆ i stimulacije Th1 i Th17 imunskog odgovora. Primena veće koncentracije Poly (I:C) u kombinaciji sa loksoribinom utiče na karakteristike MoDĆ za polarizaciju Th1 imunskog odgovora. Kostimulacija MoDĆ sa Poly (I:C) i kurdlanom potencira efekte pojedinačnih agonista pa tako stimulisane MoDĆ indukuju snažniji Th1 i Th17 odgovor, u poređenju sa efektima pojedinačnih agonista. Povećanjem koncentracije Poly (I:C) u kombinaciji sa TNF-α moduliše se Th polarizujući potencijal MoDĆ od Th17 ka imunoregulatornom. IFN-γ i povezivanje CD40 molekula su aktivatori sazrevanja MoDĆ koje stimulišu razvoj Th1 odgovora. Ligacija CD40 molekula na MoDĆ aktiviranim Poly (I:C) usmerava sposobnost ovih ćelija da stimulišu Th17, a inhibiraju Th1 odgovor. U istom modelu IFN-γ inhibira Th1 odgovor, a stimuliše imunoregulatorne mehanizme. Dobijeni rezultati pokazuju veliku plastičnost u funkcionalnom odgovoru MoDĆ. Značajna imunogena svojstva Poly (I:C) koja su do sada iskorišćena u pripremi MoDĆ kao tumorskih vakcina se mogu dodatno pospešiti primenom stimulatora dektinskih receptora i TNF-α. Ipak neki od povoljnih efekata mogu biti umanjeni ili usmereni u suprotnom pravcu dodavanjem solubilnog CD40L i IFN-γ. Ovi rezultati mogu pomoći u boljem razumevanju biološkog ponašanja MoDĆ nakon transfera *in vivo*.

Ključne reči: CD40, $CD4^+$ T-ćelijski odgovor, Dektin-1 receptor, humane dendritske ćelije monocitnog porekla, imunoterapija tumora, IFN-γ, TNF-α, Toll-sličan receptor 3, Toll-sličan receptor 7

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Modulation of the function of human monocyte derived dendritic cells by combined use of the endosomal Toll-like receptors, dectin-1 receptor agonists and proinflammatory cytokines

Abstract

Background/aim. Dendritic cells (DCs) are the most potent antigen presenting cells (APCs) which receive and integrate multiple signals to initiate and direct a response appropriate to extracellular milleu. These APCs perform these functions mostly due to the expression of a wide variety of pattern recognition receptors (PRRs). PRRs discriminate self-tissues from infectious non-self tissues through molecular pattern (MPs) recognition. Although triggering of a single pattern recognition receptor (PRR), especially Toll-like receptors (TLRs) or C-type lectins, results in phenotypic changes in DCs, for functional maturation cooperativity between multiple PRRs is needed in order to achieve an effective immune response.

Immune modulating strategies based on use of TLR-specific agonists are in the focus of current investigations in the field of tumor immunotherapy. There is currently much interest in the use of TLR3 agonist, Polyinosinic-polycytidylic acid (Poly (I:C)), in vaccine developement. Poly (I:C), a synthetic analogue of double stranded (ds)RNA, may be an appropriate activation agent for obtaining mature MoDCs competent to prime effective immune responses. TLR3 together with TLR7-9 represents a group of TLRs localized within an endosome specialised for recognition of nucleic acids. Cooperation of different TLR signals in the induction of immune responses is an emerging field in innate immune research. Therefore, we wanted to evaluate whether combined effect of Poly (I:C) and loxoribine, a selective TLR7 agonist, could be more advantageous over the use of single agonists for the maturation of MoDCs.

Recent studies have shown that the ligation of Dectin-1, C-type lectin receptor, on MoDCs elicits their maturation. Dectin-1, a DC-associated C-type lectin, is the first of many PRRs which mediate their own signaling and induces the maturation of DCs capable of eliciting the generation of different T helper (Th) effectors. The next aim of this work was to study the response of MoDCs to the combined effect of Poly (I:C) and curdlan, selective Dectin-1 agonists.

Tumor necrosis factor (TNF)-α is important for early DC maturation and as a bridge between initiation of the inflammatory cascade and generation of the antigen-specific response. To gain insight into this scientific problem we investigated the

kinetics of maturation and the length of exposure of MoDCs to a pathogen (mimicked by Poly (I:C) in our study) in an inflamed tissue (mimicked by TNF-α) on phenotypic and functional characteristics of MoDCs.

Finally, 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 last objective of this study.

Methods. Immature MoDCs (iMoDCs), generated from human monocytes, were treated with Poly (I:C) alone or in combination with loxoribine, curdlan or TNF-α for 48h. To investigate the influence of T-cell derived stimuli, MoDCs cultivated for 24h with Poly (I:C) alone or in combination with TNF-α were incubated either with CD40 ligand (L)-transfected J558 cells, soluble CD40L or IFN-γ for additional 24h. To examine the influence of kinetics of activation on the Th polarizing capability of MoDCs, we stimulated MoDCs with different doses of Poly (I:C) in combination with TNF-α for 24h and 48h. Phenotypic characteristics of MoDCs were determined by flow cytometry. Allostimulatory capability of MoDCs was tested using a mixed leukocyte reaction assay. Cytokine production was measured by ELISA and FlowCytomix.

Results. In preliminary experiments iMoDCs were treated with Poly (I:C) (5 μg/ml, 10 μg/ml, 25 μg/ml and 50 μg/ml), loxoribine (34 μg/ml and 85 μg/ml) or curdlan (10 μg/ml , 50 μg/ml, 100 μg/ml and 200 μg/ml). Based on phenotypic characteristics and functional capabilities of MoDCs, the concentrations of Poly (I:C) (25 μg/ml), loxoribine (85 μg/ml) and curdlan (100 μg/ml) were found to be optimal for activation of MoDCs, while the concentrations of Poly (I:C) (10 μg/ml) and loxoribine (34 μg/ml) were found to be suboptimal. For stimulation of iMoDCs we used these concentrations of the agonists.

Optimal concentration of Poly (I:C) stimulated the maturation of MoDCs as judged by the up-regulation of HLA-DR, CD86, CD40, CD54, CD83 and CCR7 expression. Poly (I:C)-treated MoDCs were potent producers of interleukin (IL)-12, moderate producers of IL-23 and weak producers of IL-10, which was followed by high production of IFN-γ and moderate production of IL-17 by allogeneic $CD4^+$ T cells.

The combined treatment of MoDCs with suboptimal concentrations of TLR3 and TLR7 agonists resulted in slight potentiation of HLA-DR, CD86, CD83, CD54 and CD40 molecules and stimulation of IL-27, IL-23 and IL-10 secretion, compared to effects of single agonists. This was followed by up-regulated secretion of IFN-γ and IL-17 in the

co-culture with allogeneic $CD4^+$ T cells. When the suboptimal concentration of Poly (I:C) was combined with the optimal concentration of loxoribine, MoDCs down-regulated HLA-DR and up-regulated CD86 expression, enhanced the production of IL-12 and IL-23 and down-regulated the levels of IL-10 and IL-27, compared to the effects of single agonists. MoDCs pretreated in this way stimulated the production of IFN-γ and lowered the levels of IL-4 and IL-10 by $CD4^+$ T cells. The treatment of MoDCs with optimal concentrations of both TLR agonists was followed by down-regulation of HLA-DR, CD83 and CD40 expression and augmented the production of IL-12, IL-27 and IL-10, whereas the level of IL-23 was significantly lower, compared to relevant controls. These MoDCs promoted the production of IFN-γ and inhibited the production of IL-4, IL-10 and IL-17 in co-culture, compared to the effect of corresponding controls.

The combination of Poly (I:C) and curdlan induced phenotypic maturation of MoDCs with the capability to stimulate alloreactive response. Such treated MoDCs up-regulated the production IL-12, IL-23 and IL-10, compared to the effect of Poly (I:C), alone. The opposite effect was observed for IFN-γ production. When combined, these agonists primed MoDCs to further increase the production of IFN-γ by $CD4^+$ T cells in co-culture, especially those of naïve ($CD45RA^+$) phenotype, and IL-17 by memory ($CD45RO^+$) $CD4^+$ T cells.

MoDCs stimulated with optimal concentration of Poly (I:C) and TNF-α up-regulated the expression of CD80, CD86 and CD54 molecules and lowered the production of IL-12 which was followed with inhibition of cellular proliferation and IFN-γ production in co-culture with allogeneic $CD4^+$ T cells, compared to the effect of Poly (I:C)-treated MoDCs. Dose- and time- dependent effect of Poly (I:C) and TNF-α on the functions of MoDCs were more pronounced after 24h than 48h of stimulation, as judged by down-regulation of IL-12 and IL-23 production by this cells and reduced levels of IFN-γ and IL-17 in co-cultures with $CD4^+$ T cells. The influence of CD40:CD40L interaction was most prominent in the case of production of IL-12 when MoDCs were pretreated with the highest dose of Poly (I:C) and TNF-α. This effect was reflected on reduced production of IFN-γ by allogeneic $CD4^+$ T cells co-cultured with MoDCs treated with this combination. The addition of CD40L lessen the levels of IL-23 and increased the production of IL-10 in cultures of MoDCs stimulated with the highest dose of Poly

(I:C) and TNF-α which was reflected on significant reduction of the levels of IL-17 and up-regulation of IL-10 produced by allogeneic $CD4^+$ T cells.

Ligation of CD40 or treatment with IFN-γ of iMoDCs or Poly (I:C)-treated MoDCs significantly up-regulated their allostimulatory activity. Ligation of CD40 on iMoDCs 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. iMoDCs treated with IFN-γ up-regulated IL-12 production, 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.

Conclusions. Higher concentrations of Poly (I:C) combined with loxoribine divert Th polarizing capabilities of MoDCs from Th1 and Th17 towards Th1. Co-stimulation of MoDCs with Poly (I:C) and curdlan induces superior Th1 and Th17 immune responses, compared to effects of single agonists. Higher concentrations of Poly (I:C) combined with TNF-α redirect Th polarizing capabilities of MoDCs from Th17-promoting to immunoregulatory influence. Ligation of CD40 on iMoDCs induces their maturation into a phenotype that supports Th1 response, while on Poly (I:C)-treated MoDCs the immune response shifts towards Th17. Treatment of iMoDCs with IFN-γ down-regulated Th2 and Th17 responses, while adding IFN-γ to Poly (I:C)-activated MoDCs down-regulated Th1 response and promoted T-regulatory mechanisms. Each of these findings may have therapeutic implications for the use of MoDCs in immunotherapy.

Key words: CD40, Dectin-1 receptor, human monocyte-derived dendritic cells, IFN-γ, TNF-α, T helper immune response, Toll-like receptor 3, Toll-like receptor 7, tumor immunotherapy

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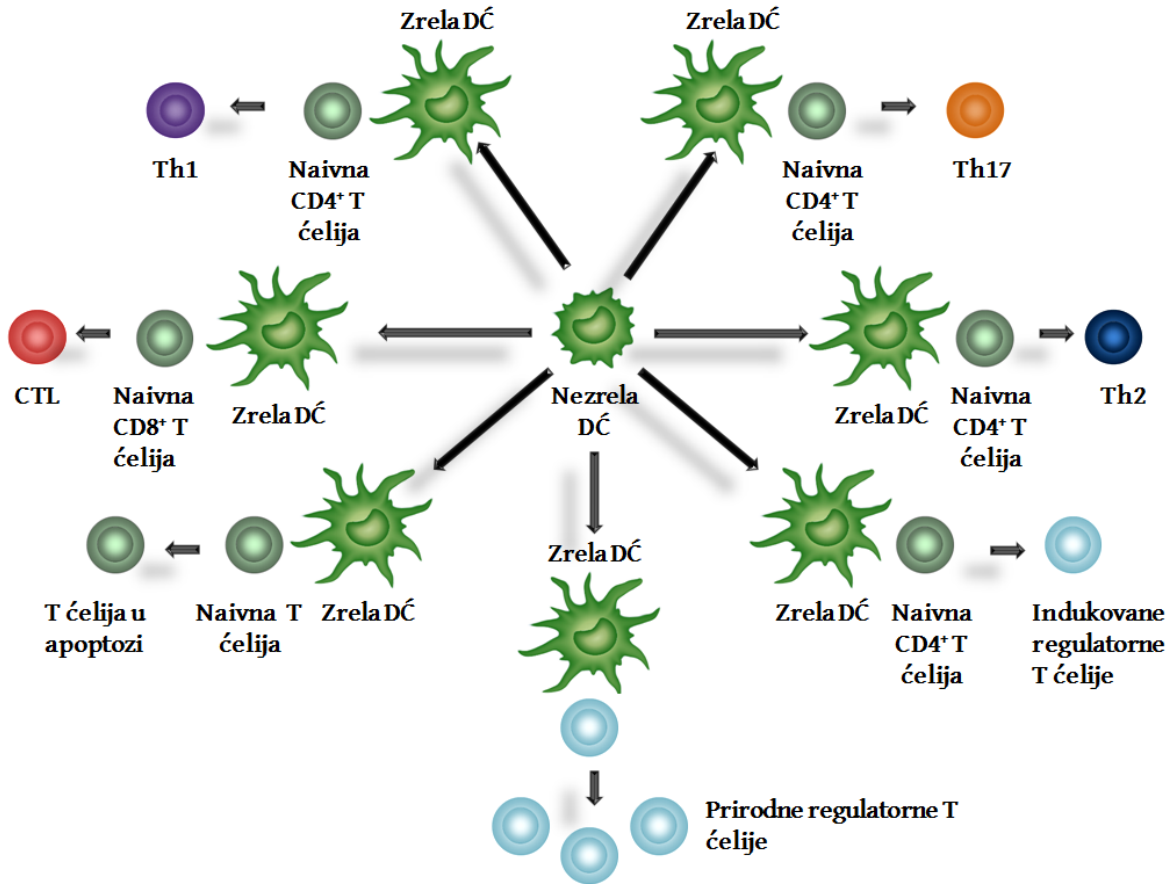
SADRŽAJ

1. UVOD

Imunski sistem je evoluirao sa ciljem da održi homeostazu u organizmu štiteći ga od širokog spektra patogena spoljašnje sredine. Komponente ovog jedinstvenog sistema odbrane, urođeni i stečeni imunitet, udružuju svoje efektorske mehanizme u cilju što efikasnije eliminacije patogena. Urođeni imunski sistem predstavlja prvu liniju odbrane i ima ključnu ulogu u kontroli infekcije tokom vremenskog perioda neophodnog za pokretanje efektorskih mehanizama stečenog imuniteta (Janeway i Medzhitov, 2002). Stečeni imunitet je evoluirao kasnije u odnosu na urođeni i zasniva se na specifičnom prepoznavanju antigena od strane receptora na T limfocitima i antitela. Pretpostavka o prefinjenoj ulozi urođene imunosti kao sistema koji precizno detektuje tip patogena koji narušava homeostazu organizma (Janeway, 1992) potvrđena je nakon identifikacije receptora koji prepoznaju evolutivno konzervirane strukture patogena (tzv. molekularne obrasce karakteristične za patogene (engl. Pathogen-Associated Molecular Patterns, PAMP) (Medzhitov i sar., 1997; Medzhitov i Janeway, 1997). Dendritske ćelije imaju ključnu ulogu u aktivaciji, regulaciji i povezivanju ove dve grane imunskog sistema zahvaljujući svojim jedinstvenim morfološkim, fenotipskim i funkcionalnim karakteristikama (Banchereau i Steinman, 1998).

1.1. Karakteristike dendritskih ćelija

Dendritske ćelije (DĆ) predstavljaju sistem antigen-prezentujućih ćelija (APĆ) koje efikasno stimulišu komponente urođenog i stečenog imuniteta integrišući njihove efektorske mehanizme u jedinstveni imunski odgovor. Jedan od podtipova DĆ, Langerhansove ćelije (LĆ), kao zasebna populacija ćelija opisane su još 1868. godine (Langerhans, 1868). Nova vrsta ćelija, identifikovana u slezini miša, je na osnovu jedinstvenih morfoloških karakteristika, izraženih dužih i kraćih citoplazmatskih nastavaka, nazvana dendritskim ćelijama 1973. godine (Steinman i Cohn, 1973). Potencijal ovih ćelija da stimulišu T-ćelijski imunski odgovor pokazan je pet godina kasnije u mešanoj leukocitnoj kulturi (Steinman i Witmer, 1978) i potvrđen u brojnim studijama koje su usledile.



Slika 1. Funkcije dendritskih ćelija u ćelijskom imunskom odgovoru

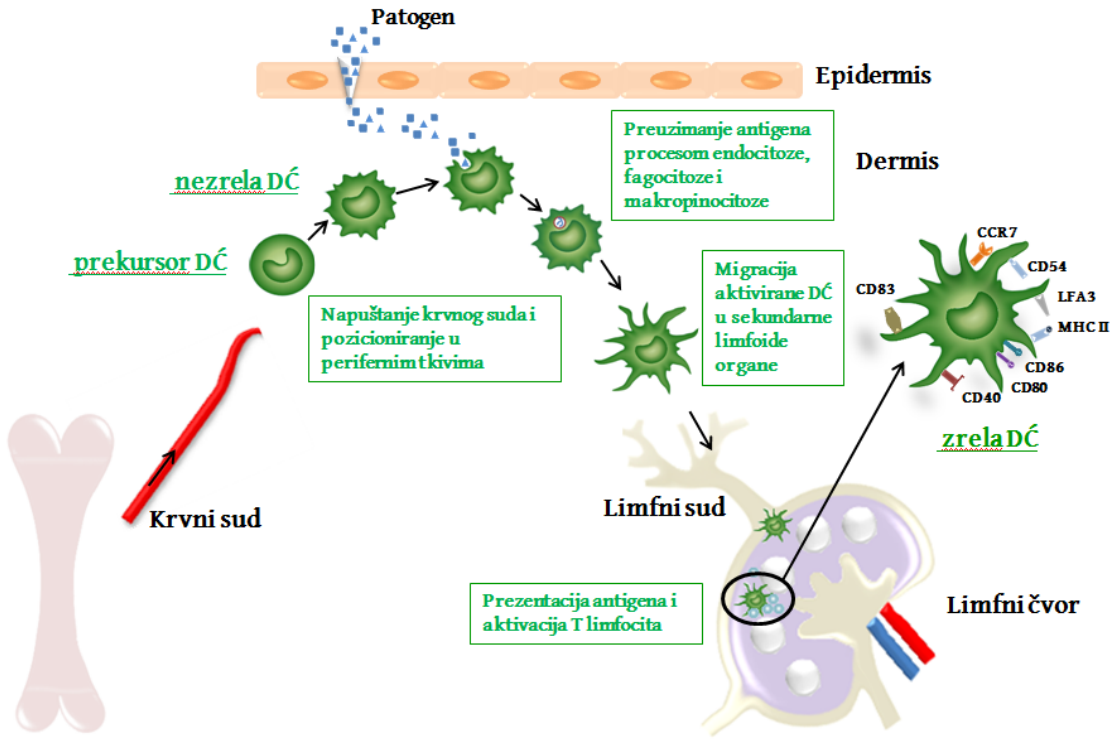
U poređenju sa drugim APĆ, makrofagama i B limfocitima, ove ćelije se izdvajaju kao najznačajnije APĆ zbog svoje jedinstvene sposobnosti da aktiviraju naivne $CD4^+$ i $CD8^+$ T limfocite i obezbede signale neophodne za njihovu potpunu aktivaciju (Banchereau i Steinman, 1998; Steinman i Hemmi, 2006) *(Slika 1).* Pored ove ključne uloge u ćelijskom imunskom odgovoru, DĆ su važne za pokretanje humoralne imunosti aktiviranjem B limfocita (Jego i sar., 2005; Qi i sar., 2006), dok u okviru urođene imunosti aktiviraju ćelije prirodne ubice (engl. natural killer, NK) (Lucas i sar., 2007) i NKT ćelije (Fujii i sar., 2002). Takođe, DĆ imaju centralnu ulogu u uspostavljanju imunološke tolerancije u timusu i perifernim organima (Steinman i sar., 2003).

Za obavljanje pomenutih brojnih, često i međusobno suprotstavljenih, funkcija neophodna je uska specijalizacija subpopulacija u okviru ovog heterogenog sistema APĆ.

DĆ se morfološki karakterišu prisustvom brojnih dendritskih nastavaka pomoću kojih stupaju u blizak kontakt sa ostalim ćelijama imunskog sistema. U zavisnosti od svog porekla, stepena zrelosti i funkcionalnog statusa, DĆ se mogu fenotipski karakterisati različitim nivoom ekspresije molekula glavnog kompleksa histokompatibilnosti klase I i II (engl. major histocompatibility complex, MHC); CD1(a-e) molekula koji prezentuju lipidne i glikolipidne antigene endogenog i egzogenog porekla; kostimulatornih molekula: B7 familije, B7.1 (CD80) i B7.2 (CD86), i CD40 molekula; adhezivnih molekula koji pripadaju klasi integrina ili receptora za integrine: molekula CD11 familije (CD11a, CD11b i CD11c), CD54 (intraćelijski adhezivni molekul-1, engl. intracellular adhesion molecule, ICAM-1), familije antigena asociranih sa funkcijom leukocita (engl. leukocyte function-associated antigen, LFA) i markera zrelih DĆ, CD83 molekula (Banchereau i sar., 2000; Liu i sar., 2001; Wu i Dakic, 2004; Steinman i Hemmi, 2006).

Prema tradicionalnom stanovištu, DĆ su strateški pozicionirane tako da se nalaze na mestima kontakta sa spoljašnjom sredinom, uključujući kožu, creva i pluća (Nelson i Loffert, 1994; Nestle i sar., 1993; Sertl i sar., 1986), gde imaju ulogu nadzornika koji detektuje "opasne" signale, pa regrutuje i aktivira ostale ćelije imunskog sistema (Fernandez i sar., 1999; Foti i sar., 1999; Rescigno i sar., 1998). Fenotipski i funkcionalno nezrele DĆ se odlikuju izraženim svojstvima internalizacije i proteolitičke obrade antigena (Banchereau i Steinman, 1998). Nezrele DĆ ispoljavaju nizak nivo MHC molekula klase II kao i kostimulatornih i adhezivih molekula CD40, CD54, CD80 i CD86 (Sallusto i Lanzavecchia, 1994). DĆ mogu biti aktivirane brojnim signalima, među koje spadaju patogeni, apoptotične ćelije, produkti inflamacije, kao i ostale ćelije imunskog sistema. DĆ se odlikuju izuzetnom plastičnošću i sposobnošću prilagođavanja svog odgovora u zavisnosti od tipa stimulusa kome su izložene. Na primer, ove ćelije su sposobne da naprave razliku između pristustva infekcije i inflamacije. *In vitro* studije su pokazale da su proinflamatorni medijatori manje efikasni od patogena u pogledu aktivacije DĆ. DĆ izložene dejstvu proinflamatornih medijatora su efikasne u stimulisanju proliferacije T limfocita, ali ne i stvaranju efektorskih $CD4^+$ T limfocita. Sa druge strane, izlaganje DĆ patogenima dovodi do kompletne aktivacije DĆ koje pokreću

odgovarajući $CD4^+$ T-ćelijski odgovor (Sporri i Reis e Sousa, 2005). Dakle, priroda signala koje DĆ detektuju u svojoj mikrosredini utiče na karakteristike stečenog imunskog odgovora koji će one pokrenuti. Nakon aktivacije, DĆ menjaju morfologiju koja se ogleda u reorganizaciji citoskeleta, gubitku adhezivnih struktura i povećanju pokretljivosti ćelija. Aktivirane DĆ ispoljavaju hemokinski receptor 7 (engl. chemokine receptor 7, CCR7) zahvaljujući kome migriraju u sekundarne limfne organe i na taj način povezuju mesta inicijacije imunskog odgovora sa perifernim inficiranim tkivima. Naime, blizak kontakt između aktiviranih DĆ i T limfocita je prvi korak u efikasnom pokretanju imunskog odgovora. Migracija je praćena sazrevanjem koje se ogleda u smanjenju kapaciteta obrade antigena i povećanju sposobnosti njihove prezentacije, povećanju ekspresije kostimulatornih molekula i produkcijom citokina (Banchereau i Steinman, 1998). Fenomen sazrevanja DĆ je pokazan u *in vitro* i *in vivo* eksperimentima. Najpre je u *in vitro* studijama pokazano da LĆ nakon 2-3 dana kultivacije efikasnije stimulišu proliferaciju T limfocita u odnosu na proliferativni odgovor indukovan sveže izolovanim LĆ koje su, sa druge strane, efikasnije u prezentaciji peptidnih antigena (Schuler i Steinman, 1985; Romani i sar., 1989). Sekvencijalno smenjivanje funkcija DĆ tokom njihove migracije potvrđeno je i *in vivo*. Nakon stimulacije lipopolisaharidom (engl. lipopolysaccharide, LPS) ili ekstratkom *Toxoplasma gondii*, dolazi do redistribucije DĆ slezine iz marginalne zone i crvene pulpe, gde obrađuju antigene, u belu pulpu, u kojoj su lokalizovane T ćelije kojima prezentuju antigene (De Smedt i sar., 1996; Reis e Sousa i sar., 1997). Razvojni put, tzv. "paradigma Langerhansovih ćelija", predstavlja prototip životnog ciklusa DĆ (Villadangos i Heath, 2005) *(Slika 2).*



Slika 2. Aktivacija i sazrevanje dendritskih ćelija

DĆ imaju sposobnost da detektuju patogene zahvaljujući receptorima za prepoznavanje molekularnih obrazaca patogena (engl. pattern recognition receptors, PRR) (Akira, 2009). Određeni tip patogena se odlikuje jedinstvenom kombinacijom PAMP. PRR je velika familija receptora među kojima su do sad najbolje okarakterisane subfamilije Toll-sličnih receptora (engl. Toll like receptors, TLR) i lektinskih receptora C-tipa (engl. C-type lectin receptors, CLR).

1.1.1. Toll-slični receptori

TLR su najveća familija receptora urođene imunosti koja je do sada identifikovana. Njihovo otkriće je vezano za eksperimente koji su pokazali da su mutanti za Toll protein *Drosophila melanogaster* vrlo podložni gljivičnim infekcijama. Tokom embriogeneze vinske mušice Toll protein je neophodan za uspostavljanje dorzo-ventralne ose, dok kod odraslih mušica ima ulogu u razviću imunosti na gljivice. Homologni sistem receptora je na osnovu homologije sa Toll proteinom identifikovan i kod sisara (Hoffmann i sar., 1996; Lemaitre i sar., 1996; Medzhitov i sar., 1997). Otkriće TLR je bilo od suštinskog značaja, slično kao i

otkriće receptora na limfocitima, za razumevanje kompleksnosti urođenog imunskog odgovora i mehanizama kojima se uspostavlja balans između zaštitnog imunskog odgovora i imunopatologije.

TLR su transmembranski proteini tipa I izgrađeni od ekstraćelijskog domena koji prepoznaje ligande i sadrži ponovke bogate leucinom, transmembranskog regiona i intraćelijskog domena homologog receptoru za IL-1 koji je neophodan za prenos signala u ćeliju (engl. Toll-interleukin 1 receptor domain, TIR) (Medzhitov i sar., 1997). Do sada je identifikovano 10 humanih i 12 TLR u mišjem sistemu. TLR1-9 su konzervisane strukture u oba sistema. TLR10 je funkcionalan kod ljudi, dok je kod miševa njegova funkcija poremećena insercijom endogenog retrovirusa. Ligand za TLR10 još uvek nije poznat. TLR 11-13 nisu prisutni u humanom genomu (Kawai i Akira, 2010). Funkcionalne analize pojedinačnih TLR receptora su pokazale da svaki član prepoznaje različite molekulske obrasce mikroorganizama i to u okviru plazma membrane, endozoma, lizozoma i endolizozoma (Akira i sar., 2006). Receptori su pozicionirani tako da se osigura kolokalizacija sa odgovarajućim ligandima, ali i da se spreči interakcija sa „sopstvenim“ molekulima i održi imunološka tolerancija. Sve više podataka ide u prilog tome da se u određenim patološkim stanjima za TLR vezuju i endogeni molekuli što može biti povezano sa razvojem autoimunskih bolesti (Kawai i Akira, 2010).

TLR su klasifikovani u dve grupe u zavisnosti od lokalizacije i odgovarajućih liganada. U prvu grupu su svrstani TLR1, TLR2, TLR4, TLR5, TLR6 i TLR11 koji su ispoljeni na plazma membrani i prepoznaju komponente membrana patogena, uključujući lipide, lipoproteine i proteine.

Prvi sisarski homolog Toll proteina, TLR4, identifikovan je 1998. godine kao receptor za LPS kodiran Lps lokusom (Poltorak i sar., 1998). LPS je komponenta membrane Gram-negativnih bakterija i uzročnik je septičkog šoka. TLR4 i mijeloidni faktor diferencijacije 2 (engl. myeloid differentiation factor 2, MD2) formiraju kompleks za koji se vezuje LPS (Park i sar., 2009; Akashi-Takamura i Miyake, 2008). TLR2 prepoznaje širok spektar PAPM poreklom od bakterija, virusa, mikoplazme i gljivica (Akira i sar., 2006). Ligandi za TLR2 su lipoproteini bakterija, peptidoglikan i lipotejhnoična kiselina Gram-pozitivnih bakterija,

lipoarabinomanan mikoplazme, zimozan gljivica i hemaglutinin virusa iz Paramyxovirus familije. TLR2 formira heterodimere sa TLR1 ili sa TLR6. TLR1/TLR2 kompleks prepoznaje triacil lipopeptide Gram-negativnih bakterija i mikoplazme, a TLR2/TLR6 kompleks vezuje diacil lipopeptide Gram-pozitivnih bakterija i mikoplazme (Jin i sar., 2007; Kawai i Akira, 2010). TLR5 je najviše ispoljen na DĆ *lamina propria* u tankom crevu gde prepoznaje flagelin, protein bakterijskih flagela (Akira i sar., 2006). TLR11 je prisutan kod miševa u bubrezima i bešici gde prepoznaje uropatogene bakterije. Njegov homolog u humanom sistemu je TLR5 (Zhang i sar., 2004; Yarovinsky i sar., 2005).

Drugu grupu ovih receptora čine TLR3, TLR7, TLR8 i TLR9 koji se isključivo nalaze u intraćelijskim vezikulama, uključujući endoplazmatski retikulum (ER), endozome, lizozome i endolizozome gde prepoznaju nukleinske kiseline (Akira i sar., 2006; Kawai i Akira, 2010). TLR3 prepoznaje dvolančanu RNK poreklom od virusa i njen sintetski analog, poliinozinsku:policitidilinsku kiselinu (engl. polyinosinic:polycytidylic acid, Poly (I:C)) (Akira i sar., 2006). TLR3 prepoznaje i genomsku RNK reovirusa, dvolančanu RNK koja nastaje tokom replikacije virusa koji imaju jednolančanu RNK i pojedine male interferirajuće RNK (Kawai i Akira, 2010). TLR7 je najpre okarakterisan kao receptor koji prepoznaje derivate imidazokvinolina (imikvimod i rezikvimod) i analoge guanina (loksoribin). Pored ovih analoga purina, mišji TLR7/8 i humani TLR7 vezuju virusnu jednolančanu RNK i male interferirajuće RNK (Hornung i sar., 2005; Akira i sar., 2006). Humani TLR8 prepoznaje jednolančanu RNK i jedinjenje rezikvimod (Akira i sar., 2006). Ligandi za TLR9 su nemetilovana DNK sa CpG motivima poreklom od bakterija i virusa (Haas i sar., 2008) i nerastvorljivi kristal hemozin koji predstavlja nusprodukt digestije hemoglobina od strane parazita *Plasmodium falciparum* (Coban i sar., 2010). TLR3, TLR7 i TLR9 se nalaze u ER i nakon stimulacije se transportuju u endolizozome. Sprečavanje acidifikacije endolizozoma dovodi do izostanka efekata karakterističnih za stimulaciju ovih receptora što ukazuje na to da se u endolizozomima odvija interakcija liganada sa ovim receptorima (Kim i sar., 2008). Translokacija receptora se nalazi pod kontrolom proteina ER, UNC93B1. UNC93B1 se specifično vezuje za transmembranske regione TLR3, TLR7 i TLR9 u ER (Brinkmann i sar., 2007). Kod miševa koji su mutanti za ovaj protein javljaju se

poremećaji u produkciji citokina i ekspresiji kostimulatornih molekula, kao i povećana podložnost infekcijama od strane bakterija i virusa čiji su PAMP ligandi za TLR3, TLR7 i TLR9 (Tabeta i sar., 2006).

1.1.2. Lektinski receptori C-tipa

Lektinski receptori C-tipa (engl. C-type lectin receptor, CLR) prepoznaju antigene koji su po strukturi ugljeni hidrati, prisutni kod širokog spektra patogena (Figdor i sar., 2002; Geijtenbeek i sar., 2004). Ekspresija CLR je specifična za određene subpopulacije DĆ, pa je tako BDCA-2 karakterističan za plazmacitoidne DĆ (Dzionek i sar., 2001), langerin (CD207) za LĆ (Valladeau i sar., 2000), a DC-SIGN (engl. DC-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing non-integrin, CD209) je specifičan za intersticijalne DĆ (Geijtenbeek i sar., 2000a; Geijtenbeek i sar., 2000b). Poznato je da CLR imaju brojne funkcije. DC-SIGN stupa u interakcije sa: CD11b/CD18 i CEACAM 1 (engl. carcinoembryonic antigen (CEA)-related cellular adhesion molecule 1) ispoljenim na neutrofilima i time posreduje u aktivaciji DĆ od strane neutrofila (van Gisbergen i sar., 2005); ICAM-2 ispoljenim na ćelijama endotela čime se omogućava migracija DĆ iz krvnih i limfnih sudova u periferna tkiva i ICAM-3 koji je ispoljen na T limfocitima čime se olakšava njihova aktivacija od strane DĆ (Geijtenbeek i sar., 2000a). Neki od CLR, poput DC-SIGN i dektina-1, u citoplazmatskom domenu poseduju imunoreceptor tirozinske aktivacione sekvence (engl. immunoreceptor tyrosine-based activation motif, ITAM) putem kojih prenose aktivacione signale DĆ (Geijtenbeek i sar., 2000a; Ariizumi i sar., 2000; Brown, 2006). Sa druge strane, DCIR (engl. dendritic cell immunoreceptor) i MICL (engl. myeloid inhibitory C-type lectin receptor) u citoplazmatskom domenu imaju imunoreceptor tirozinske inhibitorne sekvence (engl. immunoreceptor tyrosine-based inhibitory motif, ITIM) čija funkcija nije u potpunosti ispitana (Bates i sar., 1999; Marshall i sar., 2004).

1.1.2.1. Imunomodulacija fenotipa i funkcija dendritskih ćelija aktivacijom dektin-1 receptora

Imunomodulatori su jedinjenja koja pri interakciji sa imunskim sistemom menjaju karakteristike imunskog odgovora pospešujući ili smanjujući neka njegova

svojstva. Primena imunomodulatora ima za cilj manipulisanje imunskim sistemom u cilju njegove kontrole, posebno razvoja efikasnijeg imunskog odgovora, uključujući i pospešivanje anti-tumorskog imunskog odgovora (Tzianabos, 2000).

Polisaharidi su jedna od klasa imunomodulatora a među njima su najznačajniji β glukani. β-glukani su polimeri glukoze koji se sastoje od β(1→3) skeleta za koji su vezane β-D-glukoporanozil jedinice sa β(1→4) ili β(1→6) bočnim lancima varijabilne dužine. β-glukani su komponente ćelijskog zida gljivica, kvasaca i bakterija, a sadrže ih i ovas i ječam. Ova jedinjenja se ne nalaze kod životinja i predstavljaju molekulske obrasce koji pokreću mehanizme urođene i adaptivne imunosti (Brown i Gordon, 2001; Brown i Gordon, 2005). Efekti koje β-glukani imaju na komponente imunskog sistema zavise od njihove molekulske mase, konformacije, dužine bočnih lanaca, stepena grananja i rastvorljivosti (Zekovic i sar., 2005). β-glukani velike molekulske mase (>100 kDa), poput zimozana, pokreću fagocitne i antimikrobne aktivnosti makrofaga kao i produkciju proinflamatornih citokina i hemokina (Fadok i sar., 2000; Brown, 2006; Czop, 1986). β-glukani srednje i male molekulske mase (5-10 kDa) sa niskim stepenom grananja su biološki neaktivni (Zekovic i sar., 2005; Miyazaki i sar., 1979). β-glukani rastvorljivi u vodi su snažniji imunomodulatori od nerastvorljivih (Xiao i sar., 2004). Konformacija je još jedan od ključnih faktora koji utiču na biološku aktivnost β- glukana. Naime, β-glukani mogu imati tri konformacije: trostruki heliks, jednostruki heliks ili nasumičnu spiralu. U literaturi postoje kontradiktorni rezultati u pogledu biološke aktivnosti β-glukana zavisno od konformacije. Prema jednoj grupi autora β-glukani su najaktivniji kada imaju konformaciju jednostrukog heliksa (Aketagawa i sar., 1993) dok je prema drugima biološki najaktivnija konformacija trostrukog heliksa (Falch i sar., 2000; Ohno i sar., 1987). Ova jedinjenja su u *in vivo* uslovima snažni stimulatori imunskog odgovora usmerenog protiv razvoja tumora, kao i infekcija izazvanih gljivicama, virusima i bakterijama (Brown i Gordon, 2003). Pored imunomodulatorne uloge β-glukani kao molekulski obrasci predstavljaju ligande za brojne receptore ćelija imunskog sistema. Laktozilceramid je glikofosfolipid na plazma membrani neutrofila i endotelnih ćelija koji prepoznaje β(1→3) glukane iz gljivica (Zimmerman i sar., 1998). Komplement receptor 3 (engl. complement receptor 3, CR3), heterodimer koji se

sastoji od CD11b i CD18, prepoznaje β-glukane i ispoljen je na NK ćelijama, neutrofilima i monocitima (Ross, 2000). Receptori čistači (engl. scavenger receptors) koji su ispoljeni na mijeloidnim i endotelnim ćelijama takođe interaguju sa β-glukanima (Rice i sar., 2002).

Dektin-1, receptor CLR familije, je nedavno identifikovan kao glavni receptor koji posreduje u efektima β-glukana (Brown i Gordon, 2001). Najpre je identifikovan kao receptor na DĆ koji prepoznaje tada još uvek neidentifikovan ligand na T ćelijama (Ariizumi i sar., 2000) i prvobitno je nazvan lektin-1 tipa C na DĆ. Kasnije je utvrđeno da je ispoljen i na drugim tipovima ćelija kao što su neutrofili, makrofage, monociti i podtipu T ćelija (Taylor i sar., 2002). Kod ljudi ispoljen je i na B ćelijama i eozinofilima (Willment i sar., 2005). Dektin-1 je transmembranski receptor sa ekstraćelijskim domenom koji prepoznaje ugljene hidrate (engl. carbohydrate recognition domain, CRD) i intraćelijskim domenom koji sadrži ITAM preko koje se odvija signalizacija. Dektin-1 prepoznaje β(1→3) i β(1→6) glukane (Brown i Gordon, 2001). Takođe prepoznaje i zimozan koji pored β-glukana sadrži hitin, manan, proteine i lipide (Brown, 2006). Dektin-1 receptor je jedini, pored TLR, koji pokreće signalnu kaskadu. Signalna komponenta dektin-1 receptora je ITAM sekvenca koja je takođe prisutna kod receptora na B i T limfocitima kao i Fcγ receptoru. Veliki broj studija je proučavao efekat zimozana koji pored dektin-1 receptora aktivira i TLR2. Ispitivanja na makrofagama i DĆ korišćenjem zimozana su pokazala da dektin-1 zajedno sa TLR2 dovodi do produkcije TNF-α, IL-2, IL-10 (Brown i Gordon, 2003; Gantner i sar., 2003), indukcije respiratornog praska (Gantner i sar., 2003; Underhill i sar., 2005), kao i fagocitoze (Herre i sar., 2004; Underhill i sar., 2005). Ispitivanja na mišjim i humanim DĆ su pokazala da stimulacija dektin-1 receptora dovodi do fenotipskog sazrevanja ovih ćelija i polarizacije imunskog odgovora u Th1 i Th17 pravcu (LeibundGut-Landmann i sar., 2007; Skrzypek i sar., 2009; Gringhuis i sar., 2009; Ferwerda i sar., 2008).

1.2. Poreklo dendritskih ćelija

U cilju održavanja homeostaze populacija DĆ se mora kontinuirano osvežavati novim DĆ koje se razvijaju iz prekursora. Poput ostalih ćelija

hematopoetskog porekla, DĆ nastaju diferencijacijom matičnih ćelija kostne srži koje integrišu signale iz svoje mikrosredine i usmeravaju svoj dalji razvoj ka mijeloidnoj ili limfoidnoj liniji (Manz i sar., 2001; Kondo i sar., 2003). Shodno tome, a u skladu sa markerima ispoljenim na površini DĆ, poreklo DĆ može biti mijeloidno odnosno limfoidno (Shortman i Naik, 2007).

Najpre se smatralo da su sve DĆ mijeloidnog porekla. Najranija istraživanja su pokazala da se u prisustvu faktora stimulacije rasta kolonija granulocita i makrofaga (engl. granulocyte-macrophage colony-stimulating factor, GM-CSF) mijeloidni prekursori iz kostne srži miša razvijaju u makrofage, granulocite i DĆ (Inaba i sar., 1993). Takođe, humane $CD34^+$ ćelije kultivisane u prisustvu GM-CSF i TNF-α su se diferencirale u $CD1a^-$ monocite, prekursore granulocita i DĆ, odnosno u makrofage, u prisustvu faktora stimulacije rasta kolonija makrofaga (engl. macrophage colony-stimulating factor, M-CSF) (Reid i sar., 1992; Szabolcs i sar., 1996; Caux i sar., 1996). Direktna potvrda mijeloidnog porekla DĆ usledila je nakon detektovanog obnavljanja populacija DĆ u slezini i timusu miševa posle transplantacije zajedničkih mijeloidnih progenitora (ZMP) iz kostne srži u radioaktivno ozračene primaoce (Traver i sar., 2000; Wu i sar., 2001; Manz i sar., 2001).

Ispitivanjem membranskih markera na DĆ timusa, slezine i limfnih čvorova pokazano je da su neki od njih (CD8α, CD4, CD2, BP1 i CD25) karakteristični za ćelije limfoidnog porekla (Vremec i sar., 1992). Iz ove studije je proistekla pretpostavka o limfoidnom poreklu DĆ koja je potvrđena u eksperimentima sa transferom $CD4^{low}$ timusnih limfoidnih prekursora u timus ozračenih primalaca od kojih su nastali T limfociti i $CD8^+$ timusne DĆ (Wu i sar., 1995; Ardavin i sar., 1993). Ovi prekursori su imali potencijal diferenciranja i u $CD8^+$ i $CD8^-$ DĆ slezine (Wu i sar., 2001; Martin i sar., 2000). Takođe je pokazano da zajednički limfoidni progenitori (ZLP) u mišjem sistemu imaju sposobnost da se diferenciraju u DĆ i *in vivo* i *in vitr*o (Wu i sar., 2001; Manz i sar., 2001; Izon i sar., 2001).

Kada je pokazano da i mijeloidni i limfoidni progenitori mogu da se razviju u $CD8\alpha^+$ i $CD11b^+$ DĆ (Wu i sar., 2001), kao i u plazmacitoidne DĆ (Karsunky i sar., 2005) postavilo se pitanje biološkog značaja višestrukih razvojnih puteva DĆ (Schmid i sar., 2010). Smatra se da je značaj multilinijskog razvojnog programa DĆ osiguravanje efikasne selekcije funkcionalnih B i T limfocita koji nisu autoreaktivni (Rolink i sar., 2001; Goldrath i Bevan, 1999). U prilog ovoj hipotezi ide podatak da su u timusu najzastupljenije DĆ limfoidnog porekla, dok u svim ostalim organima dominiraju DĆ mijeloidnog porekla (Ardavin i sar., 1993; Wu i sar., 1996; Merad i Manz, 2009).

DĆ se mogu razviti direktno, od ZMP i ZLP, ili indirektno, od delimično ili potpuno diferenciranih ćelija mijeloidne i limfoidne linije. ZLP i ZMP od kojih će nastati DĆ eksprimiraju Flt-3 molekul (engl. FMS-related tyrosine kinase 3, Flt) (Shortman i Naik, 2007) koji je receptor za Flt-3 ligand, najznačajniji faktor za diferencijaciju DĆ *in vivo* (Waskow i sar., 2008). Flt-3 molekul je tirozin kinaza kontinuirano eksprimirana od stadijuma progenitora do potpuno diferenciranih DĆ (Dong i sar., 2002) i predstavlja obeležje ovog razvojnog puta DĆ. DĆ se indirektno mogu razviti od monocita, granulocitno-monocitnih prekursora u okviru mijeloidnog puta, odnosno od timusnih limfoidnih prekursora u okviru limfoidnog puta (D'Amico i Wu, 2003; Traver i sar., 2000; Manz i sar., 2001; Wu i sar., 2001; Karsunky i sar., 2005).

Monociti imaju značajnu ulogu kao prekursori DĆ (Cella i sar., 1997). Ove ćelije imaju sposobnost da se diferenciraju u različite subpopulacije fagocita u zavisnosti od lokalnih faktora kojima su izložene u tkivu u kome se nalaze. Monociti eksprimiraju CD11b, CD11c, CD13, CD14 i CD33 (Liu i sar., 2001), manozni receptor kao i CD1a, b, c i d. Monociti se u prisustvu M-CSF diferenciraju u makrofage, dok se pri kultivaciji sa GM-CSF i IL-4 diferenciraju u nezrele DĆ. Ove nezrele DĆ poreklom od monocita (MoDĆ) su nalik intersticijalnim DĆ i mogu se aktivirati i sazreti pod uticajem proinflamatornih citokina, produkata patogena ili signalima poreklom od T ćelija (CD40L) (Cella i sar., 1996). Dodavanje TGF-β u kulturu monocita u prisustvu GM-CSF i IL-4 usmerava diferencijaciju monocita ka LĆ (Geissmann i sar., 1998). Mogućnost diferenciranja monocita u DĆ je potvrđena i

in vivo i praćena je migracijom ovih DĆ u T ćelijske oblasti regionalnih limfnih čvorova (Randolph i sar., 1999).

1.3. Klasifikacija dendritskih ćelija

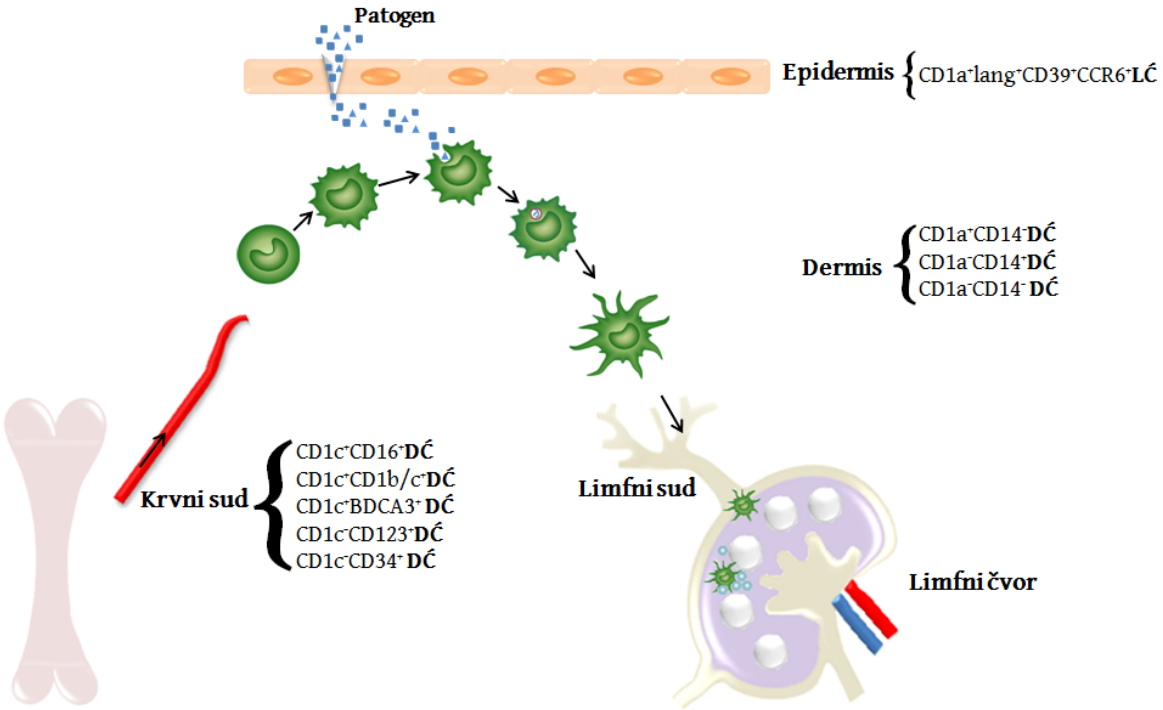
DĆ su heterogena populacija ćelija čije su razlike u fenotipskim i funkcionalnim svojstvima rezultat brojnih faktora kao što su stadijumi ontogeneze, putevi migracije, tkivna distribucija i uticaj faktora mikrosredine (Shortman i Naik, 2007). Potreba za podelom DĆ na podtipove se javila sedamdesetih godina prošlog veka kad je ustanovljeno da se LĆ razlikuju od DĆ slezine kod miša. U to vreme se ovaj fenomen pripisivao različitim stupnjevima u razvoju DĆ i smatralo se da su DĆ slezine kasniji stupanj razvoja LĆ. Konačan dokaz o postojanju različitih podvrsta DĆ usledio je identifikacijom DĆ slezine miša koje su se međusobno razlikovale po ekspresiji CD8 molekula ($CD8\alpha^+$ i $CD8\alpha^-$ DĆ) (Vremec i sar., 1992). Paralelno sa studijama na mišjem sistemu ispitivane su humane DĆ i izvršena je klasifikacija na mijeloidne DĆ (mDĆ), limfoidne i plazmacitoidne DĆ (pDĆ) (Grouard i sar., 1997). Ovakva podela je primenjena i na DĆ u mišjem sistemu (Asselin-Paturel i sar., 2001; O'Keeffe i sar., 2002). Aktuelna klasifikacija zanemaruje ontogenetsko poreklo DĆ usled izrazite plastičnosti fenotipskih i funkcionalnih karakteristika ovih ćelija i prema njoj DĆ se dele na konvencionalne DĆ (kDĆ) i pDĆ (Shortman i Naik, 2007).

1.3.1. Konvencionalne dendritske ćelije

Podela kDĆ na subpopulacije u mišjem sistemu se zasniva na ekspresiji CD4, CD8, CD205, CD11b i langerina. DĆ slezine se dele na tri podvrste uključujući $CD4^+CD8\alpha^-CD11b^+CD205^-$, $CD4^-CD8\alpha^+CD11b^-CD205^+$ i $CD4^-CD8\alpha^-CD11b^+CD205^-$ DĆ (Vremec i sar., 2000). Ove DĆ vode poreklo od prekursora koji su se razvili u slezini (Naik i sar., 2006; Diao i sar., 2006). DĆ slezine su fenotipski i funkcionalno nezrele (Wilson i O'Neill, 2003) i imaju ulogu u detektovanju patogena prisutnih u krvi (Lundie i sar., 2008; Sponaas i sar., 2006). $CD8\alpha^-$ DĆ su zastupljene u marginalnoj zoni slezine, subkapsularnim sinusima limfnih čvorova i subepitelnom regionu Pajerovih ploča, dok su $CD8\alpha^+$ DĆ prisutne u T-ćelijskim zonama limfoidnih organa

(Pulendran i sar., 1997; Shortman i Liu, 2002). CD8α$^-$ DĆ mogu da migriraju u T-ćelijske zone nakon stimulacije (De Smedt i sar., 1996). U timusu miša prisutne su dve subpopulacije DĆ koje su CD4$^-$CD11b$^-$CD205$^+$ i međusobno se razlikuju po nivou ekspresije CD8α molekula (Vremec i sar., 2000). U limfnim čvorovima su prisutne dve subpopulacije CD4$^-$CD11b$^+$CD205$^+$ koje se razlikuju po intenzitetu ekspresije CD8α molekula i prisustvu odnosno odsustvu ekspresije langerina (Henri i sar., 2001). U koži su identifikovane tri različite subpopulacije DĆ: LĆ, dermalne DĆ i langerin$^+$CD11b$^-$CD103$^+$ DĆ (Heath i Carbone, 2009).

U humanom sistemu najbolje su okarakterisane DĆ prisutne u koži i krvi *(Slika 3).*



Slika 3. Tipovi dendritskih ćelija u humanom sistemu

U epidermisu kože se nalaze LĆ, dok su u dermisu prisutne 3 podvrste tzv. intersticijalnih DĆ uključujući CD1a$^+$CD14$^-$, CD1a$^-$CD14$^+$ i CD1a$^-$CD14$^-$ DĆ (Nestle i sar., 1993; Angel i sar., 2006). LĆ eksprimiraju CD1a, langerin, E-kadherin,

Birbekove granule, CD39 i CCR6. CCR6 se specifično se ispoljava na LĆ i omogućava migraciju nezrelih LĆ u epidermis, vezujući se za MIP-3α, koji se isključivo ispoljava na epitelu (Romani i sar., 2003; Santegoets i sar., 2008). Dermalne $CD14^+$ DĆ ispoljavaju receptore iz CLR familije (DC-SIGN, DEC -205, LOX-1, CLEC-6, Dektin-1 i DCIR). $CD1a^+$ DĆ eksprimiraju manozni receptor, DC-SIGN, CD36, faktor XIIIa i CCR5. LĆ i DĆ koje eksprimiraju CD1a se međusobno razlikuju po fenotipskim i funkcionalnim svojstvima. $CD1a^+$ DĆ su aktivirane, migratorne DĆ koje eksprimiraju visoke nivoe kostimulatornih, adhezivnih molekula, hemokina i hemokinskih receptora (CCL22, MIP-1α, MIP-1β, CCR7 i CXCR4) i sekretuju proinflamatorne citokine u odsustvu stimulusa. Sa druge strane, $CD1a^+$ LĆ se odlikuju niskom ekspresijom kostimulatornih molekula i slabom produkcijom proinflamatornih citokina (Santegoets i sar., 2008). Prema novim nalazima, LĆ se smatraju transporterima antigena koje prenose iz kože u regionalne limfne čvorove i predaju rezidentnim DĆ koje imaju ulogu u stimulaciji T-ćelija (Allan i sar., 2006).

U perifernoj krvi ljudi prisutno je 5 podtipova DĆ. U okviru $CD11c^+$ subpopulacije DĆ podela je izvršena na osnovu ekspresije CD16, CD1b/c i BDCA-3 (engl. blood dendritic cell antigen, BDCA). Najbrojnije su $CD1c^+CD16^+$ DĆ koje imaju nisku ekspresiju CD14 i CD33, HLA-DR i ne ispoljavaju kutani limfocitni antigen (engl. cutaneous lymphocyte antigen, CLA). $CD1c^+CD16^+$ DĆ ispoljavaju visoke nivoe kostimulatornih molekula, CD86 i CD40. $CD11c^+CD1b/c^+$ DĆ eksprimiraju CD33, HLA-DR i vrlo niske nivoe CD14. Najmanje brojna subpopulacija $CD11c^+$ DĆ u krvi su $BDCA\text{-}3^+$ DĆ koje ispoljavaju niske nivoe CD11c i ne eksprimiraju CD14. $BDCA\text{-}3^+$ DĆ imaju najnižu ekspresiju CD86 i najveću ekspresiju CD40 i DEC-205 u poređenju sa ostalim podtipovima DĆ u krvi. $CD11c^-$ DĆ se prema ekspresiji CD123 i CD34 mogu podeliti na dve subpopulacije, $CD123^+$ DĆ i $CD34^+$ DĆ. $CD11c^-CD123^+$ DĆ imaju slabu ekspresiju CD86 molekula i umerenu ekspresiju CD86, dok $CD11c^-CD34^+$ DĆ ne eksprimiraju CD86 i umereno ispoljavaju HLA-DR (MacDonald i sar., 2002).

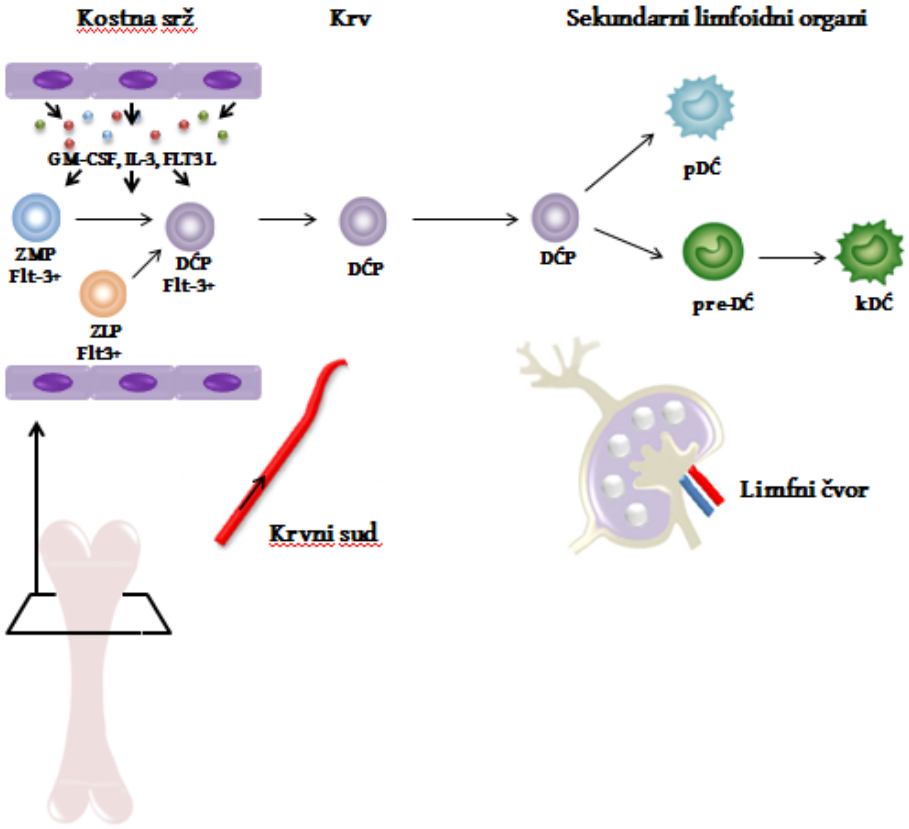
1.3.2. Plazmacitoidne dendritske ćelije

Lennert i Remmele su 1958. godine opisali ćelije prisutne u T-ćelijskoj zoni limfoidnog tkiva ljudi koje po morfologiji podsećaju na plazma ćelije, ali ne ispoljavaju markere B ćelija i plazma ćelija. Ove ćelije su nazvane plazmacitoidne T ćelije. Dvadeset godina kasnije pokazano je da nakon izlaganja leukocita periferne krvi virusima ili virusom inficiranim ćelijama jedan mali broj ćelija produkuje interferone tipa I (IFN-α i IFN-β) koji stimulišu citotoksične funkcije NK ćelija (Trinchieri, 1978). Ove IFN tip I- produkujuće ćelije nisu imale karakteristike T limfocita, B limfocita, NK ćelija niti monocita, a ispoljavale su MHC molekule klase II i niskoafinitetne Fcγ receptore (Trinchieri, 1978; Abb i sar., 1983; Ronnblom i sar., 1983; Perussia i sar., 1985). Deset godina kasnije u perifernoj krvi ljudi identifikovane su $CD11c^-$ nezrele DĆ sa niskom ekspresijom MHC molekula klase II i slabom sposobnošću stimulacije proliferacije T limfocita (O'Doherty i sar., 1994). Nakon kultivacije u kondicioniranom medijumu ove ćelije su povećale ekspresiju MHC molekula klase II. Potom su iz tonzila izolovane $CD11c^-CD4^+CD45RA^+$ ćelije sa gotovo identičnim karakteristikama kao i $CD11c^-$ ćelije periferne krvi. Ove ćelije su opstajale u kulturi samo u prisustvu IL-3 i CD40L pod čijim uticajem su se diferencirale u ćelije sa morfologijom DĆ (Grouard i sar., 1997). Uticaj IL-3 na preživljavanje ovih ćelija se bazirao na visokom nivou ekspresije α-lanca IL-3 receptora (IL-3Rα, CD123). Krajem XX veka je definitivno potvrđeno da se radi o posebnom podtipu DĆ koje su prisutne u perifernoj krvi i sekundarnim limfoidnim organima i nakon stimulacije virusima produkuju IFN tipa I (Siegal i sar., 1999; Cella i sar., 1999a).

U krvi, kostnoj srži i sekundarnim limfoidnim organima miša nalaze se $CD11c^+CD45RA^+CD19^-Gr1^+$ pDĆ (Nikolic i sar., 2002). Humane pDĆ su fenotipski okarakterisane kao $CD4^+CD45RA^+CD123^+ILT3^+ILT1^-CD11c^-$ ćelije. Niskoafinitetni Fcγ receptor identifikovan u ranijim studijama je FcγRIIa (CD32) koji moduliše produkciju IFN tipa I od strane pDĆ (Bave i sar., 2003). pDĆ prisutne u krvi i kostnoj srži ispoljavaju i dva specifična markera, BDCA-2 i BDCA-4. BDCA-2 je

transmembranski glikoprotein koji pripada CLR familiji receptora sa svojstvima internalizacije antigena u cilju prezentacije T limfocitima. Primena antitela specifičnih za BDCA-2 inhibira produkciju IFN tipa I od strane pDĆ (Dzionek i sar., 2001). BDCA-4 je identičan neuropilinu-1, neuronalnom receptoru za klasu 3 semaforinske subfamilije i koreceptor je za vaskularni endotelni faktor rasta prisutan na endotelnim i tumorskim ćelijama. Primena antitela specifičnih za BDCA-4 nema uticaj na funkciju pDĆ i mogu se koristiti za izolaciju ovih ćelija pozitivnom selekcijom (Dzionek i sar., 2002).

Poreklo pDĆ je predmet brojnih debata i istraživanja. Za diferencijaciju pDĆ od hematopoetskih matičnih ćelija i kod čoveka i kod miša je ključan Flt3-ligand (Blom i sar., 2000; Karsunky i sar., 2003), a za njihovu migraciju iz kostne srži faktor stimulacije rasta kolonija granulocita (engl. Granuclocyte colony-stimulating factor, G-CSF) (Pulendran i sar., 2000; Arpinati i sar., 2000). Prisustvo transkripta za pre-T-ćelijski receptor α (pre-Tα), λ5 i spi-B je išlo u prilog hipotezi o limfoidnom poreklu pDĆ (Rissoan i sar., 2002; Corcoran i sar., 2003) dok nije pokazano da se $Flt3^+$ ćelije mogu diferencirati i u mDĆ i u pDĆ (Karsunky i sar., 2003; D'Amico i Wu, 2003). Intrigantni su rezultati istraživanja sprovedenih od strane dve istraživačke grupe koji su ukazali na moguće postojanje zajedničkog prekursora pDĆ i kDĆ u mišjem sistemu. U kostnoj srži miša identifikovane su ćelije koje su okarakterisane kao $CD11c^-$ MHC $II^-Lin^-Flt3^+$M-$CSFR^+$c-Kit^{int} koje se nakon tranfera u slezinu i limfni čvor miša diferenciraju u $CD8^+$ DĆ, $CD8^-$ DĆ i pDĆ. Ove ćelije se *in vitro* diferenciraju u kDĆ i pDĆ u prisustvu Flt-3L. Razviću u kDĆ, za razliku od pDĆ, prethodi stupanj $CD11c^-$MHC II^+ pre-DĆ (Onai i sar., 2007; Naik i sar., 2007) *(Slika 4).*



Slika 4. Hipotetički model diferencijacije DĆ od jedinstvenog DĆ prekursora

pDĆ u krvi ispoljavaju i L-selektin (Cella i sar., 1999a) čija se ekspresija smanjuje u limfoidnim organima u kojima su pDĆ prisutne u blizini visokih endotelnih venula (Grouard i sar., 1997; Facchetti i sar., 2003). Aktivacija pDĆ je praćena morfološkim promenama i povećanjem ekspresije MHC molekula klase I i II, kostimulatornih molekula (CD40, CD80 i CD86) kao i produkcijom IFN tipa I. Uprkos opisanim fenotipskim i funkcionalnim promenama pDĆ su manje efikasne APĆ od kDĆ (Asselin-Paturel i sar., 2001).

1.4. *In vitro* priprema dendritskih ćelija

DĆ čine svega 0.1-1% ukupnog broja ćelija u organizmu i odlikuju se širokom tkivnom distribucijom i heterogenošću. Značajan napredak u istraživanju DĆ je usledio tek dve decenije od njihove identifikacije, kada su razvijene metode za njihovu *in vitro* pripremu (Inaba i sar., 1992; Talmor i sar., 1998; Lutz i sar., 1999). Kako je priprema DĆ iz kostne srži miša bila najefikasniji i najzastupljeniji metod

većina studija je izvođena upravo na ovom tipu DĆ. U početku je korišćen koktel antitela (antitela specifična za CD4, CD8, B220) u cilju eliminacije limfocita i GM-CSF kao jedini faktor za stvaranje DĆ od progenitora. Nakon nedelju dana kultivacije dobijao se dovoljan broj DĆ, ali je postojala kontaminacija sa granulocitima i makrofagima (Inaba i sar., 1992). Kasnije su modifikovani protokoli produženjem vremena kultivacije, smanjenjem koncentracije GM-CSF i uvođenjem IL-4 (Lutz i sar., 1999; Garrigan i sar., 1996). DĆ dobijene kultivisanjem progenitora iz kostne srži miša u prisustvu GM-CSF i IL-4 su fenotipski i funkcionalno nalik MoDĆ, ali razlike ipak postoje. Sallusto i Lanzavecchia su prvi kultivisali humane monocite iz periferne krvi u prisustvu IL-4 i GM-CSF u cilju dobijanja MoDĆ (Sallusto i Lanzavecchia, 1994) *(Slika 5).*

Slika 5. In vitro priprema DĆ

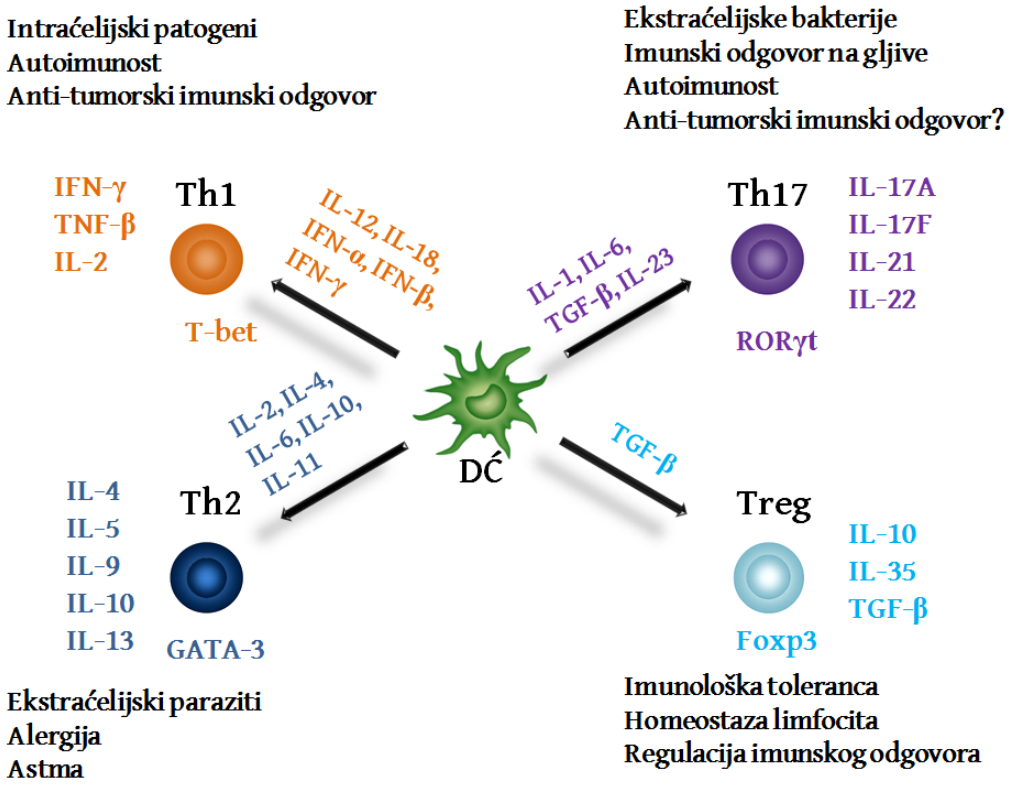
Postoji još jedan način za pripremu DĆ iz krvi i to je kultivacija preko noći sortiranih mononuklearnih ćelija u RPMI 1640 medijumu sa AB serumom kojom se dobijaju $CD11c^+$ DĆ. Ove ćelije eksprimiraju CD86 i u manjoj meri HLA-DR. Razlika između $CD11c^+$ DĆ i MoDĆ je u tome što $CD11c^+$ DĆ ne eksprimiraju DC-SIGN, manje su efikasne u prezentaciji antigena i indukuju jači Th1 odgovor (Osugi i sar., 2002). Za pripremu DĆ korišćeni su i $CD34^+$ hematopoetske progenitorske ćelije iz kostne srži i periferne krvi (Bernhard i sar., 1995) i $CD14^+$ monociti dobijeni metodom imunomagnetnog sortiranja mononukleara periferne krvi (Babatz i sar., 2003). Postoji još jedan tip kratkotrajnih kultura u kojima DĆ dobijaju kultivacijom humanih $CD14^+$ monocita tokom prvih 24h u prisustvu GM-CSF i IL-4 i tokom

sledećih 24h u prisustvu proinflamatornih medijatora (TNF-α, IL-1β, IL-6, PGE2) (Dauer i sar., 2003).

1.5. Uloga dendritskih ćelija u polarizaciji imunskog odgovora

DĆ oblikuju $CD4^+$ T-ćelijski odgovor u skladu sa prirodom patogena koji su prepoznali a koji ugrožava homeostazu organizma (Lafaille, 1998). Stimulusi koji aktiviraju DĆ i dovode do njihovog sazrevanja povećavaju efikasnost obrade antigena i produžavaju ispoljavanje kompleksa antigen-MHC molekul klase II na površini DĆ (Cella i sar., 1997).

Kada dođe do interakcije T-ćelijskog receptora sa antigenom u sklopu MHC molekula II klase na DĆ i kada T-ćelije uspostave stabilan kontakt sa DĆ dolazi do formiranja imunološke sinapse (Al-Alwan i sar., 2001). Prepoznavanje antigena je praćeno aktivacijom intraćelijskih signalnih molekula uključujući protein kinazu C (PKC), jone kalcijuma i nuklearni faktor κB (engl. nuclear factor kappa-B, NF-κB) (Constant i Bottomly, 1997; Noble i sar., 2000; Rogers i Croft, 2000). Kostimulatori ispoljeni na DĆ (CD40, CD80, CD86, PD-L1 (engl. programmed death-1 ligand), PD-L2 i ICOS-L (engl. inducible costimulator ligand) se vezuju za odgovarajuće molekule na T ćelijama (CD40L, CD28/CTLA-4, PD-1 i ICOS) (Van Gool i sar., 1996; Greenwald i sar., 2005). Za odgovarajuću diferencijaciju aktiviranih naivnih T ćelija neophodni su citokini. Optimalno vreme za sekreciju citokina je nakon uspostavljanja kontakta DĆ i T ćelija i uspostavljanja interakcije CD40:CD40L. Dakle, fenotip polarizovanih T-ćelija koje se diferenciraju od naivnih $CD4^+$ T limfocita je rezultat kompleksnih interakcija sa DĆ uz učešće citokina, kostimulatornih molekula i polarizujućih faktora. Ovi faktori aktiviraju intraćelijske signalne puteve i ekspresiju gena specifičnih za određeni podtip $CD4^+$ T ćelija. Aktivacija je praćena klonalnom ekspanzijom antigen-specifičnih efektorskih ćelija koje se mogu diferencirati u Th1, Th2, Th17 ili Treg fenotip *(Slika 6).*



Slika 6. Uticaj DĆ na polarizaciju Th ćelijskog odgovora

Razvoj Th1 imunskog odgovora stimulisan je u prisustvu IL-12, IL-18, IFN-α i IFN-β. Ove citokine oslobađaju makrofage i DĆ nakon aktivacije intraćelijskim patogenima (Farrar i sar., 2002). IL-12 stimuliše produkciju IFN-γ od strane APĆ uspostavljajući pozitivnu povratnu spregu koja dovodi do dodatne produkcije IL-12. Sa druge strane, IFN-γ deluje inhibitorno na razvoj Th2 ćelija (Murphy i sar., 2000). IFN-γ u naivnim T ćelijama dovodi do aktivacije transkripcionog faktora STAT-1 (engl. signal transducer and activator of transcription 1) koji potom pokreće aktivaciju transkripcionog faktora T-bet. T-bet dovodi do remodelovanja genskog lokusa za IFN-γ, produkcije IFN-γ i ekspresije receptora za IL-12 (Mullen i sar., 2001). IL-12 se potom vezuje za svoj receptor ispoljen na Th1 ćelijama i signalna kaskada pokrenuta ovim receptorom aktivira transkripcione faktore STAT-3, STAT-4 i NF-κB čime se stabilizuje produkcija citokina koji su karakteristični za Th1 fenotip (Afkarian i sar., 2002). Th1 ćelije su efikasne u zaštiti od intraćelijskih patogena kao i eliminaciji tumorskih ćelija (Kidd, 2003).

Th2 ćelije se razvijaju pod uticajem IL-2 IL-4, IL-6, IL-10 i IL-11 (Swain i sar., 1990; Curti i sar., 2001; Laouini i sar., 2003; Cote-Sierra i sar., 2004; Krishnamoorthy i sar., 2007). DĆ ne produkuju IL-4 i smatra se da ovaj citokin produkuju NKT ćelije, eozinofili ili mast ćelije (Wang i sar., 2006). IL-4 aktivira transkripcioni faktor STAT-6 u naivnim T ćelijama što je praćeno aktivacijom transkripcionog faktora GATA-3 (Kaplan i sar., 1996; Ouyang i sar., 1998). GATA-3 i T-bet deluju antagonistički. Kada su prisutne visoke koncentracije IFN-γ, IL-12 i T-bet, inhibira se GATA-3, a visok nivo produkcije GATA-3 i IL-4 je praćen supresijom T-bet (Mullen i sar., 2001; Ouyang i sar., 1998). GATA-3 je odgovoran za produkciju citokina karakterističnih za Th2 fenotip, IL-4, IL-5, IL-9, IL-10 i IL-13 (Farrar i sar., 2002). c-MAF je još jedan transkripcioni faktor specifičan za Th2 ćelije odgovoran je za regulaciju sinteze IL-4 (Ho i sar., 1996). Th2 ćelije produkuju sve veće količine IL-4 i uspostavlja se pozitivna povratna sprega kojom se održava Th2 fenotip (Kidd, 2003).

Tokom ranog Th2 imunskog odgovora produkuje se IL-6 koji povećava produkciju IL-4 i inhibira fosforilaciju STAT-1 (Dodge i sar., 2003; Detournay i sar., 2005). Sa druge strane, IL-11 deluje direktno na T ćelije stimulišući sintezu IL-4 i IL-5 a istovremeno inhibira produkciju IFN-γ. Takođe, IL-11 suprimira sekreciju IL-12 od strane makrofaga (Curti i sar., 2001). IL-10 smanjuje ekspresiju IL-12Rβ2 receptora (Romano i sar., 2005) čime smanjuje usmeravanje ka Th1. Za razliku od Th1 ćelija koje posreduju u ćelijskom, Th2 ćelije posreduju u humoralnom imunskom odgovoru. Tako je IL-4 neophodan za produkciju IgE, IL-5 za aktivaciju eozinofila, dok IL-4 i IL-13 stimulišu proliferaciju i degranulaciju mast ćelija.

Th17 ćelije eksprimiraju transkripcioni faktor RORγt i produkuju IL-17A, IL-17F, IL-21 i IL-22. Osnovna uloga Th17 ćelija je zaštita od ekstraćelijskih patogena, uključujući i gljivice. IL-17A je uključen i u regrutovanje, aktivaciju i migraciju neutrofila (Dubin i Kolls, 2008). Kod miša Th17 ćelije se razvijaju od naivnih T ćelija pod uticajem IL-6 i TGF-β dok su IL-21 i IL-23 neophodni za održavanje Th17 fenotipa (Mangan i sar., 2006; Veldhoen i sar., 2006; Huber i sar., 2008). Za diferencijaciju Th17 ćelija kod ljudi neophodni su IL-1, IL-6 i TGF-β (Benwell i Lee, 2010). Specifično je da se Th17 ćelije ljudi odlikuju izaženom plastičnošću. Naime,

ustanovljeno je postojanje Th1/Th17 ćelija koje sekretuju kako IL-17 tako i IFN-γ ispoljavaju istovremeno IL-23R, IL-12Rβ2 i transkripcione faktore RORC i T-bet (Annunziato i sar., 2007). Takođe su kod ljudi i miša identifikovane Th2/Th17 ćelije koje koeksprimiraju transkripcione faktore GATA3 i RORγt i pored Th2 citokina produkuju IL-17 i IL-22, a imaju ulogu razvoju astme (Wang i sar., 2010).

Funkcije efektorskih Th1, Th2 i Th17 ćelija se nalaze pod kontrolom $CD4^+CD25^+$ T regulatornih ćelija (Treg). Treg ćelije su neophodne za uspostavljanje imunološke tolerance i regulaciju imunskog odgovora. Ove ćelije inhibiraju proliferaciju i produkciju citokina od strane $CD4^+$ i $CD8^+$ T limfocita, produkciju imunoglobulina od strane B limfocita, citotoksičnu aktivnost NK ćelija kao i sazrevanje DĆ. Za diferencijaciju ovih ćelija ključni transkripcioni faktor je Foxp3 (engl. Forkhead box P3, FoxP3) (Sakaguchi, 2005; Akbar i sar., 2007). Kao i u slučaju Th17 ćelija, za razvoj Treg neophodan je TGF-β. Kada su DĆ aktivirane mikroorganizmima koji dovode do produkcije proinflamatornih citokina IL-1 i IL-6, prisustvo TGF-β ih usmerava ka Th17 fenotipu. Sa druge strane, izostanak proinflamatornih citokina dovodi do razvoja Treg ćelija. Regulatorne T ćelije sekretuju IL-10, IL-35 TGF-β i imaju imunosupresorsku ulogu (O'Garra i sar., 2004; Vignali i sar., 2008; Collison i sar., 2010).

1.6. Struktura TNF-α, receptori za TNF-α i biološki efekti TNF-α

Sposobnost imunskog sistema da razvije anti-tumorski imunski odgovor uočena je pre više od 100 godina zahvaljujući hirurgu William Coley-u koji je uočio regresiju tumora nakon bakterijske infekcije pacijenta obolelog od sarkoma (Coley, 1906). U istraživanjima koja su usledila identifikovan je faktor koji ispoljava citotoksičan efekat na tumorske ćelije i dovodi do nekroze tumora kod animalnih modela kancera (Shear i sar., 1943; Shear, 1944; O'malley i sar., 1962). Izolovana su dva različita proteina sa sličnim sekvencama: faktor nekroze tumora alfa (engl. tumor necrosis factor, TNF-α) i limfotoksin beta (danas poznatiji pod imenom TNF-β) (Aggarwal i sar., 1985; Aggarwal i sar., 1984). Otkriće da se TNF-α i TNF-β vezuju za isti receptor (Aggarwal i sar., 1985) je bilo prva indikacija o postojanju

superfamilije za koju se danas zna da se sastoji od 19 liganada i 30 receptora uključenih u regulaciju funkcija imunskog sistema (Croft, 2009; Sabbagh i sar., 2007; Hehlgans i Pfeffer, 2005).

TNF-α se primarno sintetiše kao transmembranski protein tipa II u vidu homotrimera (Kriegler i sar., 1988; Tang i sar., 1996). Od ove membranske forme proteolitičkom delovanjem metaloproteaze TNF-α konvertujućeg enzima (engl. TNF alpha coverting enzyme, TACE) oslobađa se citokin u vidu solubilnog heterotrimera (Black i sar., 1997). TNF-α se vezuje za dva različita tipa receptora: TNF receptor tipa 1 (TNFR1; p55; CD120a) i TNF receptor tipa 2 (engl. TNF receptor type, TNFR2; p75; CD120b) (Vandenabeele i sar., 1995). Ovi receptori se međusobno razlikuju po tipu ćelija na kojima se eksprimiraju, stabilnosti, afinitetu za vezivanje liganda, dužini poluživota kompleksa ligand-receptor, kao i signalnim putevima koje pokreću (Beutler i Cerami, 1989; McCoy i Tansey, 2008). TNFR1 je konstitutivno eksprimiran na svim ćelijama izuzev eritrocita, dok je ekspresija TNFR2 regulisana i ograničena na ćelije imunskog sistema, endotelne ćelije i pojedine populacije neurona. Smatra se da TNF-α većinu svoju funkcija ispoljava vezivanjem za TNFR1 za koji se vezuje solubilna forma TNF-α, dok se za TNFR2 vezuje membranska forma TNF-α (Grell i sar., 1995; Grell i sar., 1998). Ekstraćelijski domeni ovih receptora su podložni proteolitičkoj obradi čime se oslobađaju solubilni fragmenti receptora koji imaju neutrališuće efekte (Wallach i sar., 1991). TNFR2 se proteolitički obrađuje TACE enzimom (Solomon i sar., 1999), dok enzim odgovoran za proteolizu TNFR1 još uvek nije identifikovan. Intraćelijski domen TRAF1 sadrži tzv. domen smrti (engl. death domain, DD). U signalnoj kaskadi koju pokreće aktivacija TNFR1 učestvuju i članovi iz familije faktora asociranih sa TNF receptorom (engl. TNF receptor-associated factor, TRAF). Finalno dolazi do aktivacije NF-κB i produkcije proinflamatornih i antiapoptotskih proteina ili do apoptoze putem aktivacije kaspaza 3 i 8 (Tracey i sar., 2008). Signalizacija preko TNFR2 dovodi do produkcije proinflamatornih medijatora aktivacijom NF-κB (Yang i sar., 2002). Ovaj receptor ne poseduje DD tako da može biti uključen u apoptozu samo indirektno putem kooperacije sa TNFR1 (Tartaglia i sar., 1993) ili na nivou intraćelijskih signalnih puteva amplifikacijom signala poreklom od TNFR1 (Fotin-Mleczek i sar., 2002).

TNF-α je zajedno sa IL-1 ključni citokin u modelima za uspostavljanje hronične inflamacije u mišjem sistemu, poput reumatoidnog artritisa (Williams i sar., 2000). Sve je više dokaza koji idu u prilog tome da hronična inflamacija prethodi razvoju kancera i da, pored anti-tumorskih efekata, TNF-α ima i protumorska svojstva (Szlosarek i sar., 2006). Ovaj citokin, između ostalog, dovodi do apoptoze T limfocita koji se infiltriraju u tumore (Mocellin i sar., 2005) i doprinosi neoplastičnoj transformaciji stimulacijom produkcije reaktivnih kiseoničnih intermedijera i azot oksida (Szlosarek i sar., 2006).

Stimulacija DĆ sa TNF-α dovodi do njihovog fenotipskog sazrevanja koje se ogleda u povećanju ekspresije MHC molekula klase II i kostimulatornih molekula. Međutim, ovaj citokin je slab stimulus i DĆ koje se diferenciraju u njegovom prisustvu produkuju niske nivoe proinflamatornih citokina (Lutz i Schuler, 2002; Repnik i sar., 2008). Sa druge strane, TNF-α ispoljava svoju regulatornu ulogu uspostavljajući balans u produkciji proinflamatornih i anti-inflamatornih citokina od strane DĆ u prisustvu liganada TLR. Pokazano je da u mišjem sistemu, TNF-α pojačava produkciju anti-inflamatornog citokina IL-10 od strane DĆ poreklom iz kostne srži (Hirata i sar., 2011) dok u humanom sistemu dovodi do povećanja produkcije IL-12 od strane MoDĆ stimulisanih ligandom za TLR3 (Spisek i sar., 2001). Kako je balans u produkciji proinflamatornih i anti-inflamatornih citokina od ključnog značaja za uspostavljanje homeostaze i sprečavanja razvoja imunopatologija, ispitivanje imunoregulatorne uloge TNF-α je predmet aktuelnih istraživanja pogotovo zbog moguće primene u imunoterapiji.

1.7. Struktura, receptori i biološki efekti IFN-γ

Interferoni su otkriveni u eksperimentima sa horioalantoinskom membranom pileta kao faktori prisutni u fluidu nakon dodavanja virusa influence na osnovu svoje sposobnosti da spreče replikaciju virusa (Isaacs i Lindenmann, 1957). Najpre su klasifikovani prema tipu ćelija koji ih sekretuje, dok se prema aktuelnoj klasifikaciji dele na tip I i tip II. IFN tipa I su IFN-α, IFN-β i IFN-ω koji se sekretuju od strane leukocita i fibroblasta nakon virusne infekcije (Adolf, 1995). IFN-γ je INF tipa II koji se po strukturi i receptorima razlikuje od IFN tipa I.

Najpre se smatralo da IFN-γ produkuju isključivo Th1 ćelije, $CD8^+$ T limfociti i NK ćelije (Bach i sar., 1997). Kasnije je pokazano da ga sekretuju i B limfociti, NKT ćelije i APĆ (Gessani i Belardelli, 1998; Yoshimoto i sar., 1998; Carnaud i sar., 1999; Frucht i sar., 2001). Produkcija IFN-γ se nalazi pod kontrolom citokina IL-12 i IL-18 koje sekretuju APĆ (Otani i sar., 1999; Munder i sar., 2001). Negativni regulatori produkcije IFN-γ su IL-4, IL-10 i TGF-β (Schindler i sar., 2001; Hochrein i sar., 2001).

IFN-γ je biološki aktivan u formi homodimera (Boehm i sar., 1997). Receptor za IFN-γ (IFNGR) je heterotetramer koji se sastoji od dva IFNGR1 lanca, koji vezuju ligand, i dva IFNGR2 lanca, koji posreduju u prenosu signala. IFNGR1 i IFNGR2 su članovi klase II familije citokinskih receptora (Bazan, 1990; Thoreau i sar., 1991). Oba lanca se konstitutivno eksprimiraju sa tom razlikom što je IFNGR1 lanac prisutan u višku, dok je nivo ekspresije IFNGR2 lanca vrlo nizak. Ekspresija IFNGR2 se nalazi pod striktnom kontrolom i ova subjedinica IFNGR predstavlja ograničavajući faktor u prenosu signala poreklom od IFN-γ (Bach i sar., 1997; Bernabei i sar., 2001). Intraćelijski domen IFNGR1 sadrži sekvencu za koju se vezuje Jak1 (engl. janus tyrosine kinase 1) i STAT-1 koji fosforilišu receptor i neophodni su za dalji prenos signala i ispoljavanje bioloških efekata IFN-γ (Farrar MA, i sar., 1991; Kaplan i sar., 1996; Greenlund i sar., 1994). Intraćelijski domen IFNGR2 sadrži sekvencu za koju se vezuje Jak2 (Kotenko i sar., 1995).

IFN-γ je multifunkcionalan citokin čiji su plejotropni efekti rezultat modulacije funkcije velikog broja gena pod uticajem ovog citokina. Nakon vezivanja liganda za IFNGR, Jak1 i Jak2 fosforilišu STAT-1 što dovodi do formiranja STAT-1 homodimera, poznatog i kao faktor aktiviran gamom (engl. gamma activated factor, GAF). GAF se translocira u nukleus gde se vezuje za GAS sekvencu (engl. gamma activated site) i pokreće transkripciju nekoliko transkripcionih faktora (Boehm i sar., 1997). IFN-γ aktivira i fosfatidil-inozitol kinazu 3 (engl. phosphatidylinsositol-3-kinase, PI3K) što dalje dovodi do aktiviranja protein kinaze Cε, protein kinaze aktivirane mitogenom (engl. mitogen activated protein kinase MAPK) i aktivacije STAT-1 (Choudhury, 2004). Članovi familije faktora koje regulišu interferoni (engl. interferon regulation factors, IRF) IRF-1, IRF-2 i IRF-9, su takođe aktivirani sa IFN-γ (Mahboubi i Pober, 2002; Rouyez i sar., 2005). IFN-γ pokreće

brojne MAPK signalne puteve uključene u regulaciju proliferacije, diferencijacije i apoptoze ćelija. Kod makrofaga iz kostne srži IFN-γ aktivira p38 MAPK i pokreće ekspresiju gena uključenih u hemotaksu i inflamaciju uključujući CXCL10, TNF-α i iNOS. IFN-γ moduliše funkcije makrofaga selektivnom aktivacijom ERK (engl. extracellular signal-regulated kinases) i JNK (engl. c-Jun N-terminal kinases) kinaza. Naime, ERK signalni put posreduje u ekspresiji proinflamatornih gena, dok JNK signalni put posreduje u ekspresiji gena uključenih u prezentaciju antigena (Valledor i sar., 2008). Negativni regulatori Jak/STAT signalnog puta pokrenutog vezivanjem IFN-γ za svoj receptor su SOCS (engl. suppressor of cytokine signaling) proteini koji inhibiraju aktivnost Jak (Hilton i sar., 1998; Krebs i Hilton, 2001), kao i fosfataza SHP2 (engl. SH2 domain-containing tyrosine phosphatase) (David i sar., 1995). IFN-γ je uključen u pokretanje odgovora na virusnu infekciju povećanjem ekspresije brojnih molekula koji su uključeni u obradu i prezentaciju antigena, adhezivnih molekula i hemokina (Schroder i sar., 2004). Takođe, IFN-γ reguliše i inflamatorne procese, produkciju reaktivnih kiseoničnih intermedijera i azot oksida (Brewington i sar., 2001; Malu i sar., 2003; Prasanna i sar., 2007). NK ćelije produkuju IFN-γ u cilju uspostavljanja kontrole nad bakterijskim i virusnim infekcijama (Biron i sar., 1999). Za razvoj optimalnog anti-tumorskog imunskog odgovora neophodna je kooperacija u produkciji IFN-γ od strane NK ćelija, makrofaga i $CD4^+$ T ćelija (Li i sar., 2007). Pored navedenog, IFN-γ ima značajnu ulogu i u humoralnom imunskom odgovoru i razvoju autoimunosti (Saha i sar., 2010).

1.8. Značaj CD40:CD40L interakcije u ćelijskom imunskom odgovoru

CD40 molekul je transmembranski protein tipa I koji pripada superfamiliji TNF receptora (Stamenkovic i sar., 1989). CD40 je najpre opisan kao molekul ispoljen na B limfocitima čija aktivacija dovodi do proliferacije ovih ćelija (Ledbetter i sar., 1987). Istraživanja koja su usledila su pokazala da je CD40 molekul prisutan i na drugim ćelijama uključujući monocite, DĆ, endotelne ćelije i epitelne ćelije (Clark, 1990) (Hart i McKenzie, 1988; Romani i sar., 1989). Ligand za CD40 molekul (poznat kao CD40L, CD154, gp39, T-BAM ili TRAP) je integralni membranski

protein tipa II ispoljen na aktiviranim $CD4^+$ i $CD8^+$ T limfocitima, aktiviranim B limfocitima, trombocitima, glatkim mišićnim ćelijama i drugim tipovima ćelija (Banchereau i sar., 1994; Grewal i Flavell, 1996; Mach, i sar., 1997; Henn i sar., 1998). CD40L postoji i u solubilnoj formi i ima iste biološke funkcije kao i membranska forma (Ludewig i sar., 1996).

Nizak nivo CD40 je konstitutivno eksprimiran na nezrelim DĆ (Banchereau i sar., 1994), a nakon aktivacije ovih ćelija njegova ekspresija se povećava (Kawai i Akira, 2007). CD40L se ispoljava na T limfocitima nakon aktiviranja T ćelijskog receptora i koreceptora (CD80 i CD86) (Cella i sar., 1996; Koch i sar., 1996). Interakcija CD40:CD40L je bidirekciona pa sa jedne stane ima važnu ulogu u kompletiranju procesa sazrevanja DĆ, dok je sa druge strane neophodna za diferencijaciju i sticanje efektorskih funkcija T ćelija. Naime, aktivacija CD40 molekula na DĆ dovodi do povećanja ekspresije kostimulatornih i adhezivnih molekula i produkcije IL-12 (VanKooten i Banchereau, 2000; Walzer i sar., 2005). Krajnji ishod ove interakcije je pospešivanje antigen-prezentujućih svojstava DĆ, polarizacija T ćelijskog odgovora ka Th1 tipu i aktivacija CTL (Bennett i sar., 1998).

1.9. Terapeutski potencijal dendritskih ćelija

Usavršavanjem postupaka za izolaciju DĆ i njihovog stvaranja u *in vitro* uslovima od prekursora otvorene su brojne mogućnosti za primenu ovih ćelija. DĆ danas predstavljaju jedno od najznačajnijih savremenih oruđa u imunoterapiji tumora i infektivnih bolesti (van Duivenvoorde i sar., 2006; Gilboa, 2007).

Pored uloge u odbrani od patogena, DĆ imaju značajnu ulogu i u razvoju anti-tumorskog imunskog odgovora. U tome su najefikasnije DĆ koje produkuju visok nivo IL-12 (Zheng i sar., 2008). Naime, IL-12 je neophodan u imunoterapiji tumora kao treći signal (Valenzuela i sar., 2002), pored prvog signala (antigen) i drugog signala (kostimulatorni molekuli) za usmeravanje imunskog odgovora ka Th1 tipu i aktivaciju tumor specifičnih citotoksičnih T limfocita (Xu i sar., 2003). Pored jasno dokazanog povoljnog efekta Th1 odgovora u odbacivanju tumora, značajnu ulogu ima i Th17 imunski odgovor. Terapeutski potencijal Th17 odgovora

je predmet aktuelnih istraživanja (Muranski i sar., 2008; Martin-Orozco i sar., 2009). Sa obzirom da je kod obolelih od tumora imunski odgovor suprimiran istraživanja iz oblasti imunoterapije tumora su usmerena ka rekonstituciji anti-tumorskog odgovora. Naime, tumori brojnim mehanizmima izbegavaju efektorske funkcije imunskog sistema i usmeravaju razvoj DĆ u pravcu tolerogenih ćelija koje nemaju sposobnost aktivacije $CD8^+$ T limfocita (Gilboa, 2007). Dosadašnja istraživanja su pokazala da zrele DĆ efikasno stimulišu anti-tumorski odgovor i zbog toga je značajan cilj imunoterapije tumora poboljšanje protokola za stimulaciju DĆ radi obrazovanja dovoljnog broja imunogenih DĆ. Smatra se da se „zlatni standard" za pripremu DĆ u cilju imunoterapije kancera sastoji od koktela proinflamatornih citokina TNF-α, IL-1β, IL-6 i PGE_2 (Jonuleit i sar., 1997). Međutim, DĆ stimulisane ovim koktelom slabo produkuju IL-12 i samim tim nisu efikasne u pokretanju efikasnog Th1 imunskog odgovora (Pedersen i sar., 2006). Agonisti PRR, pogotovo TLR, imaju veliku primenu u modulaciji funkcija DĆ u eksperimentalnim i kliničkim studijama iz oblasti imunoterapije tumora (Cella i sar., 1999; Verdijk i sar., 1999; Rouas i sar., 2004; Gilboa, 2007). Uprkos brojnim studijama, biološki potencijal DĆ za *in vivo* stimulaciju anti-tumorskog imunskog odgovora još uvek nije maksimalno iskorišćen. U fokusu aktuelnih istraživanja je priprema zrelih DĆ kombinovanom primenom različitih agonista PRR i citokina koja se zasniva na sposobnosti DĆ da integrišu signale sa različitih receptora u jedinstven odgovor.

Sa druge strane, uprkos efikasnoj supresiji replikacije HIV-1 i rekonstituciji $CD4^+$ T ćelijske populacije, kombinovana antiretroviralna terapija ne nudi mogućnost eradikacije HIV-1 i razvoja HIV-1 specifičnog ćelijskog imunskog odgovora (Plana i sar., 1998; Ogg, i sar., 1998; Carcelain i sar., 2001; Blankson i sar., 2002). Za kontrolu replikacije HIV-1 virusa neophodna je aktivacija specifičnih citotoksičnih limfocita uz pomoć $CD4^+$ T limfocita (Ostrowski i sar., 2000; Letvin i Walker, 2003; Day i Walker, 2003). HIV-1 specifični $CD4^+$ i $CD8^+$ T limfociti produkuju IFN-γ, ali ne dolazi do replikacije $CD4^+$ T ćelija i citolitička aktivnost $CD8^+$ T limfocita je narušena (Pitcher i sar., 1999; Garcia i sar., 1999; Seder i Ahmed, 2003; Yokomaku i sar., 2004). Disfunkciji ćelijskog odgovora kod inficiranih sa HIV-

1 doprinose i imunosupresivni efekti DĆ tako da tolerogene DĆ ne samo da služe kao „rezervoar“ HIV-1 virusa već i indukuju tolerancu na HIV-1 (Chehimi i sar., 2002; Steinman i Nussenzweig, 2002; Kawamura i sar., 2003). Kao mogući terapeutski pristup koji bi imao za cilj prevazilaženje navedenih problema predložena je primena *ex vivo* pripremljenih DĆ. Ispitivanja na animalnim modelima su pokazala da DĆ pretretirane sa lizatom HIV-1, glikoproteinima omotača, inaktivisanim virusom ili nanočesticama imaju potencijal da pokrenu snažan imunski odgovor usmeren protiv HIV-1 (Lapenta i sar., 2003; Yoshida i sar., 2003; Aline i sar., 2007). U do sada publikovanim rezultatima kliničkih studija pokazano je da imunoterapija HIV-1 sa DĆ do izvesnog stepena dovodi do razvoja HIV-1 specifičnog imunskog odgovora, ali su rezultati istraživanja veoma varijabilni zbog različitog i nest iardizovanog dizajna studija (Garcia i sar., 2011).

Funkcionalna plastičnost DĆ nudi brojne mogućnosti za primenu ovih ćelija u različite terapeutske svrhe. DĆ mogu imati primenu i u transplantacionoj imunologiji i kod autoimunskih bolesti. Terapijski pristup kod ovih stanja se zasniva na primeni lekova koji nespecifično blokiraju imunski sistem što pacijente čini podložnim infekcijama (Penn, 2000a; Penn, 2000b). Za razliku od imunoterapije kancera, u transplantaciji i kod autoimunskih bolesti cilj je indukcija antigen-specifične supresije imunskog odgovora čime bi se izbegli neželjeni efekti generalne nespecifične supresije. DĆ imunosupresivnih karakteristika se mogu dobiti *in vitro* primenom biološkog, farmakološkog i genetskog pristupa (Ehser i sar., 2008).

2. HIPOTEZA I CILJEVI ISTRAŽIVANJA

Zahvaljujući specijalizaciji za prepoznavanje specifičnih struktura patogena od strane PRR, pogotovo TLR, DĆ vrše diskriminaciju vrste patogena koja ugrožava homeostazu organizma i u skladu sa primljenim informacijama aktiviraju i usmeravaju imunski odgovor. Tokom infekcije različite komponente patogena istovremeno aktiviraju veći broj PRR, a interakcija između signalnih puteva pokrenutih nakon aktivacije ovih receptora ima ključni uticaj na krajni ishod imunskog odgovora. Dakle, iako aktivacija pojedinačnog receptora dovodi do fenotipskih i funkcionalnih promena DĆ, za pokretanje efikasnog imunskog odgovora neophodna je kooperacija između više receptora (Roelofs i sar., 2005; Trinchieri i Sher, 2007; Bagchi i sar., 2007; Ouyang i sar., 2007). Za modulaciju funkcija DĆ, pored stimulacije PRR, značajnu ulogu imaju i citokini prirodnog imuniteta koji se produkuju tokom infekcije, kao i signali stečenog imuniteta koje DĆ dobijaju tokom njihove interakcije sa T limfocitima.

Sposobnost DĆ da primaju signale sa različitih receptora koje integrišu u jedinstveni odgovor se sve više koristi za generisanje zrelih DĆ kombinovanom primenom različitih agonista PRR i citokina. Na osnovu rezultata dosadašnjih istraživanja, postavljena je radna hipoteza: kombinovana primena TLR3 agonista sa TLR7 agonistom, agonistom dektin-1 receptora, citokinom urođenog imuniteta i signalima stečenog imuniteta poboljšava fenotipske i funkcionalne karakteristike humanih MoDĆ *in vitro* u odnosu na tretman pojedinačnim stimulatorima.

U cilju provere radne hipoteze postavljeni su sledeći ciljevi istraživanja:

- Ispitati fenotipske i funkcionalne karakteristike humanih MoDĆ stimulisanih TLR3 agonistom, Poly (I:C);
- Ispitati efekat kombinovane primene selektivnih TLR3 i TLR7 agonista (Poly (I:C) i loksoribina) na fenotipske i funkcionalne karakteristike humanih MoDĆ;
- Ispitati modulatorni efekat ko-aktivacije dektin-1 receptora i TLR3 na fenotipske i funkcionalne karakteristike humanih MoDĆ;

- Ispitati dozno- i vremensku- zavisnost modulatornog efekta kombinovane primene Poly (I:C) i citokina prirodnog imuniteta, TNF-α, na fenotipska i funkcionalna svojstva humanih MoDĆ;
- Ispitati uticaj signala stečenog imuniteta koje DĆ dobijaju tokom njihove interakcije sa T ćelijama, uključujući signalizaciju preko CD40 molekula i receptora za IFN-γ na funkcionalne karakteristike humanih MoDĆ diferenciranih u prisustvu Poly (I:C).

3. MATERIJALI I METODE

3.1. Medijumi, hemikalije, supstance

3.1.1. Medijumi

3.1.1.1. RPMI 1640

Kao osnovni medijum za izolaciju i kultivisanje ćelija korišćen je RPMI 1640 medijum (ICN Flow, SAD) kome je dodato 1% L-glutamina (ICN Flow, SAD), 1% gentamicina (ICN-Galenika, Beograd) i 7.5% $NaHCO_3$ (Apoteka VMA, Beograd) kao pufer.

Za izolaciju mononuklearnih ćelija periferne krvi (MNĆ) korišćen je osnovni medijum kome je dodato 0.02% NaEDTA (Apoteka VMA, Beograd).

Za ispiranje MNĆ korišćen je osnovni medijum kome je dodato 2% fetalnog telećeg seruma (Fetal calf serum, FCS, ICN Flow, SAD).

Za kultivaciju ćelija korišćen je kompletni medijum koji predstavlja osnovni medijum u koji je dodato 10% fetalnog telećeg seruma, 50μM 2-merkaptoetanola (2-ME), 50 i.j/ml penicilina, 50 mg/ml streptomicina. (0.1% penicilina i streptomicina)

3.1.1.2. PBS (Phosphate Buffered Saline)

Fiziološki rastvor puferovan fosfatnim puferom (Phosphate Buffered Saline, PBS) pravljen je u dejonizovanoj vodi po sledećoj recepturi:

14 ml 0.2M NaH_2PO_4 (Serva, Nemačka)

36 ml 0.2M Ha_2HPO_4 (Serva, Nemačka)

50 ml 16% NaCl (Zorka, Šabac)

900 ml destilovane vode

Modifikovani puferi za pripremu ćelija za fenotipsku analizu metodom protočne citofluorimetrije su pravljeni dodavanjem natrijum-azida (NaN_3) i fetalnog telećeg seruma u PBS i to:

PBS1: PBS + 0.01% NaN_3 (Apoteka VMA, Beograd)

PBS2: PBS + 0.01% NaN_3 + 2% FCS

3.1.1.3. Pufer za magnetno sortiranje

Pufer za magnetno sortiranje se priprema dodavanjem 2 mM EDTA i 0.5% goveđeg serum-albumina (Sigma-Aldrich, Nemačka) u PBS.

3.1.1.4. K-PBS

K-PBS je korišćen za pripremu pufera za ispiranje ploča u ELISA testovima, a sastojao se od 137 mM NaCl (Zorka, Šabac), 2.7 mM KCl (Serva, Nemačka), 8.1 mM Na_2HPO_4 (Serva, Nemačka) i 1.5 mM KH_2PO_4 (Serva, Nemačka) sa podešenim pH na 7.2 - 7.4.

3.1.2. Citokini

3.1.2.1. Rekombinantni humani GM-CSF (rhGM-CSF)

Rekombinantni humani GM-CSF (Leucomax, specifične aktivnosti $4.44x10^6$ IU) je nabavljen od Sandoz-Schering Plough, Švajcarska. Rastvoren je pod sterilnim uslovima u destilovanoj vodi i RPMI 1640 medijumu do koncentracije od 100 μg/ml i tako čuvan do upotrebe na -80°C. U eksperimentu je rhGM-CSF korišćen u finalnoj koncentraciji od 100 ng/ml.

3.1.2.2. Rekombinantni humani IL-4 (rhIL-4)

Rekombinantni humani IL-4 nabavljen je od Roche Diagnostics GmbH, Nemačka. Rastvoren je pod sterilnim uslovima u PBS-u obogaćenom sa 0.1% goveđim serum-albuminom do koncentracije od 25 μg/ml i tako čuvan do upotrebe na -80°C. U eksperimentu je rhIL-4 korišćen u finalnoj koncentraciji od 20 ng/ml.

3.1.2.3. Rekombinantni humani (rhTNF-α)

Rekombinantni humani TNF-α nabavljen je od Roche Diagnostics GmbH, Nemačka. Rastvoren je pod sterilnim uslovima u PBS-u obogaćenom sa 0.1%

govođim serum-albuminom do koncentracije od 100 μg/ml i tako čuvan do upotrebe na -80°C. rhTNF-α je u eksperimentu korišćen u finalnoj koncentraciji od 10 ng/ml.

3.1.2.4. Rekombinantni humani (rhIFN-γ)

Rekombinantni humani IFN-γ nabavljen je od Roche Diagnostics GmbH, Nemačka. Rastvoren je pod sterilnim uslovima u PBS-u obogaćenom sa 0.1% govođim serum-albuminom do koncentracije od 100 μg/ml i tako čuvan do upotrebe na -80°C. rhIFN-γ je u eksperimentu korišćen u finalnoj koncentraciji od 5 ng/ml.

3.1.3. Imunomodulatorne i druge supstance

3.1.3.1. Poliinosinsko-policitidilinska kiselina (Poly (I:C))

Poly (I:C) (Sigma-Aldrich-Aldrich, Nemačka) rastvoren je u RPMI 1640 medijumu do koncentracije od 10 mg/ml i čuvan do upotrebe na -20°C. Poly (I:C) je u eksperimentu korišćen u koncentraciji od 5 μg/ml, 25 μg/ml i 50 μg/ml.

3.1.3.2. Loksoribin (Loxoribine)

Loksoribin (InvivoGen, SAD) rastvoren je u RPMI 1640 medijumu do koncentracije od 2 mg/ml i čuvan do upotrebe na -20°C. Loksoribin je u eksperimentu korišćen u koncentracijama od 100 μg/ml i 250 μg/ml.

3.1.3.3. Kurdlan (Curdlan)

Kurdlan (Sigma-Aldrich-Aldrich, Nemačka) je rastvoren do koncentracije od 30 mg/ml, najpre u destilovanoj vodi i potom je dodat 3N NaOH, i čuvan do upotrebe na +4°C. Kurdlan je u eksperimentu korišćen u koncentracijama od 10 μg/ml i 1000 μg/ml.

3.1.3.4. Rekombinantni humani ligand za CD40 molekul (CD40 ligand, CD40L)

Rekombinantni humani CD40L nabavljen je od InvivoGen, SAD. Rastvoren je pod sterilnim uslovima u PBS-u obogaćenom sa 0.1% govođim serum-albuminom do koncentracije od 100 μg/ml i tako čuvan do upotrebe na -80°C. rhIFN-γ je u eksperimentu korišćen u finalnoj koncentraciji od 10 μg/ml.

3.1.3.5. 2-merkaptoetanol (2-ME)

2-merkaptoetanol (Fluka, Nemačka) je rastvoren u medijumu za kultivaciju do koncentracije od 50 mM i čuvan do upotrebe na +4°C, zaštićen od svetlosti. U eksperiementu je 2-ME korišćen u koncentraciji od 50 μM.

3.1.3.6. Radioaktivno obeležen timidin

Timidin obeležen tricijumom ([^{3}H]-timidin; Amersham International, Velika Britanija), specifične aktivnosti 5μCi/mM, u eksperimentu je korišćen u koncentraciji od 1 μCi po bazenu ploče od 96 mesta (Sarstedt, Nemačka).

3.1.3.7. Limfoprep gradijent za izdvajanje humanih mononuklearnih ćelija (Lymphoprep)

Limfoprep (PAA Laboratories GmbH, Austrija) je gradijent gustine 1.077 g/ml i korišćen je za izolovanje mononuklearnih ćelija iz humane periferne krvi.

3.1.3.8. Monenzin (monensin sodium)

Monenzin (Sigma-Aldrich-Aldrich, Nemačka) je rastvoren u dimetilsulfoksidu do koncentracije od 6mM i čuvan do upotrebe na -20°C. U eksperimentu je korišćen u koncentraciji od 3μM.

3.2. Izolacija i kultivacija humanih mononukelarnih ćelija iz periferne krvi

3.2.1. Izolacija humanih mononuklearnih ćelija iz periferne krvi

Mononuklearne ćelije su izolovane iz „buffy coat"-a (sloj leukocita dobijen centrifugiranjem pune krvi u postupku pripremanja krvnih derivata-plazme, eritrocita, trombocita) koji je dobijen iz Instituta za transfuziologiju Vojnomedicinske akademije uz pismenu saglasnost davalaca. „Buffy coat" je prvo razblažen u medijumu za izolaciju MNĆ u odnosu 1:4. Razblažena ćelijska suspenzija je u zapremini od 7 ml pipetom pažljivo nanošena na 3 ml Limfoprep gradijenta. U tako dobijenoj dvofaznoj suspenziji se posle centrifugiranja na 800xg obrtaja tokom 20 minuta na sobnoj temperaturi izdvojio prsten MNĆ u interfazi. Gornji sloj razblažene plazme je uklonjen, a MNĆ su pažljivo sakupljane i potom resuspendovane u medijumu sa 0.02% NaEDTA i ispirane od gradijenta centrifugiranjem na 2500 obrtaja tokom 15 min na sobnoj temperaturi. U cilju uklanjanja trombocita MNĆ su resuspendovane u medijumu za ispiranje i centrifugirane na malim brzinama (1000 obrtaja tokom 10 minuta na sobnoj temperaturi). Talog je resuspendovan u medijumu za kultivaciju.

Ovako dobijene ćelije su korišćene za dalju izolaciju monocita i $CD4^+$ T limfocita.

3.2.2. Izolacija monocita periferne krvi

Monociti su izolovani iz MNĆ adherencijom za plastiku flaskova za ćelijske kulture (Flow, Škotska). U svaki flask je na adherencu zasejano po 30-40x10^6 ćelija u 5 ml medijuma. Nakon inkubacije u trajanju od 1,5 sata na 37°C u atmosferi zasićenoj vodenom parom sa 5% CO_2 flaskovi su ispirani toplim medijumom u cilju uklanjanja neadherentnih ćelija. Adherentni monociti su dalje kultivisani prema potrebama eksperimenta.

3.2.3. Dobijanje i kultivacija dendritskih ćelija monocitnog porekla (MoDĆ)

Monociti dobijeni adherencom MNĆ za plastiku su kultivisani narednih 5-7 dana na 37°C u atmosferi zasićenoj vodenom parom sa 5% CO_2 u medijumu za kultivaciju ćelija kome su dodati GM-CSF u koncentraciji od 100 ng/ml i IL-4 u koncentraciji od 20 ng/ml. Nakon 5-7 dana kultivacije sakupljene su neadherentne ćelije, koje su predstavljale nezrele MoDĆ, i koje su kao takve korišćene u daljim testovima. Sazrevanje nezrelih MoDĆ je izazvano stimulisanjem ovih ćelija agonistima receptora koji su dodavani u ćelijske kulture i to Poly (I:C) (5 μg/ml, 25 μg/ml i 50 μg/ml), loksoribin (100 μg/ml i 250 μg/ml) i kurdlan (10 μg/ml i 100 μg/ml). Efekti citokina TNF-α (10 ng/ml) i IFN-γ (5 ng/ml), kao i liganda za CD40 (10 μg/ml), na ćelije u kulturama ispitivani su njihovim dodavanjem ponaosob istovremeno sa agonistima receptora ili posle 24h kultivacije ćelija. Ćelije su nakon stimulacije u trajanju od 24h ili 48h isprane dva puta u čistom medijumu, u cilju oslobađanja od egzogenih i endogenih citokina, i kao takve korišćene u daljim testovima. Supernatanti iz svih bazena u kojima su ćelije bile kultivisane su pokupljeni i zamrznuti na -20°C radi kasnijeg određivanja nivoa produkovanih citokina.

3.2.4. Izolacija alogenih $CD4^+$ T limfocita tehnikom magnetnog sortiranja

Za postavljanje kulture mešane leukocitne reakcije (engl. mixed leukocyte reaction, MLR), kao i za utvrđivanje Th citokinskog profila, korišćene su $CD4^+$ ćelije koje su dobijene kao negativna frakcija iz MNĆ primenom tehnike magnetnog sortiranja. Naime, od alogenih MNĆ, dobijenih prethodno opisanim postupkom, je odvojeno $1x10^8$ ćelija i izvedena je procedura imunomagnetnog sortiranja $CD4^+$ T limfocita, korišćenjem $CD4^+$ kita za izolaciju, a prema protokolu proizvođača ($CD4^+$ T Cell Isolation Kit II, MACS, Myltenyi Biotec, Nemačka). Ukratko, ćelije su inkubirane 15 minuta na hladnom u puferu za magnetno sortiranje sa koktelom antitela konjugovanih sa biotinom, u razblaženju 1:5. Koktel je sadržao antitela na sledeće antigene: CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR γ/δ i CD235a.

Nakon inkubacije, ćelije su dva puta isprane u puferu za sortiranje ćelija i inkubirane 15 minuta na hladnom sa anti-biotin magnetnim partikulama u finalnom razblaženju 1:5. Magnetnim partikulama obeležena ćelijska suspenzija je nakon inkubacije isprana dva puta u puferu za sortiranje, resuspendovana u 500 μl istog pufera i potom naneta na separacionu kolonu (LS Columns, MACS, Myltenyi Biotec, Nemačka) postavljenu u magnetnom polju (Midi MACS Magnet, Myltenyi Biotec, Nemačka). Nakon jednog ciklusa magnetne deplecije na koloni su se izdvojile imunomagnetno obeležene ćelije, dok su u eluatu sakupljene prečišćene $CD4^+$ T ćelije. Čistoća izolovanih $CD4^+$ ćelija je bila preko 95%, što je potvrđeno protočnom citofluorimetrijom korišćenjem anti-CD4-FITC i anti-CD3-PE antitela.

3.2.5. Izolacija alogenih naivnih ($CD45RA^+$) i memorijskih ($CD45RO^+$) T limfocita imunomagnetnim sortiranjem ukupnih $CD4^+$ T limfocita

Naivni i memorijski $CD4^+$ T limfociti su, kao pozitivna i negativna frakcija, izolovani iz prethodno sortiranih $CD4^+$ T limfocita prema ekspresiji CD45RA molekula korišćenjem magnetnih partikula konjugovanih sa mišjim anti-humanim CD45RA monoklonskim antitelom (CD45RA MicroBeads, MACS, Myltenyi Biotec, Nemačka). Usledila je inkubacija $CD4^+$ T ćelija magnetnim partikulama, u trajanju od 15 minuta, na hladnom. Posle toga suspenzija ćelija je isprana dva puta u puferu za sortiranje i resuspendovana u 500 μl istog pufera i naneta na separacionu kolonu (LS Columns, MACS, Myltenyi Biotec, Nemačka) postavljenu u magnetnom polju (Midi MACS Magnet, Myltenyi Biotec, Nemačka). Nakon jednog ciklusa magnetne deplecije na koloni su se izdvojile CD45RA imunomagnetno obeležene ćelije, dok su u eluatu kao negativna frakcija dobijene CD45RO ćelije. Čistoća izolovanih celija je bila preko 90%, što je potvrđeno protočnom citofluorimetrijom korišćenjem anti-CD4-FITC, anti-CD3-PE, anti-CD45RA-PE (Serotec, V. Britanija) i anti-CD45RO-PE antitela (Immunotools, Nemačka).

3.3. Fenotipske karakteristike MoDĆ

3.3.1. Monoklonska antitela

Diferencijacija i sazrevanje MoDĆ praćena je fenotipskom analizom diferencijalno eksprimiranih markera na površini ćelija. U tu svrhu korišćenja su monoklonska antitela u određenim razblaženjima, kao što je navedeno:

Monoklonsko antitelo	Razblaženje	Proizvođač
Mišije anti-humano CD40 (FITC)	1:5	BD Biosciences, SAD
Mišije anti-humano HLA-DR (PE)	1:10	Serotec, V. Britanija
Mišije anti-humano CD54 (PE)	1:10	Serotec, V. Britanija
Mišije anti-humano CD83 (FITC)	1:5	BD Biosciences, SAD
Mišije anti-humano CD86 (PE)	1:10	Serotec, V. Britanija
Mišije anti-humano CCR7 (FITC)	1:10	R&D Systems, SAD
Mišije anti-humano CD80 (PE)	1:10	Serotec, V. Britanija

3.3.2. Protočna citofluorimetrija

Nezrele MoDĆ kao i MoDĆ stimulisane sa Poly (I:C), loksoribinom, kurdlanom, CD40L, TNF-α i IFN-γ, su isprane u hladnom PBS1 na 1800 rpm tokom 8 minuta na +4°C. Nakon ispiranja, ćelije su resuspendovane u PBS1 do koncentracije od $1x10^5$ ćelija u 50μl po epruveti za protočnu citofluorimetriju. U suspenziju celija su dodavna monoklonska antitela u prethodno prikazanim finalnim razblaženjima. Ćelije su potom inkubirane 30 minuta na +4°C. I potom isprane centrifugiranjem na 1800 rpm, na hladnom, tokom 8 minuta i fiksirane u 4% formalinu. Kontrola se sastojala od uzoraka sa adekvatnim irelevantnim mišijim monoklonskim antitelima

specifičim za pacovske antigena konjugovanim sa odgovarajućom fluorescentnom bojom.

Fenotip obeleženih ćelija je analiziran na EPICS XL-MCS protočnom citofluorimetru (Coulter, Krefeld, Nemačka) korišćenjem programa SZSTEMTM II software. U ovom aparatu kao izvor svetlosti korišćen je argonski laser sa emisijom ekscitirajuće svetlosti u opsegu talasne dužine 450-530nm. Rezultati su predstavljeni kao numeričke vrednosti procenta pozitivnih ćelija i srednje vrednosti intenziteta fluorescence (engl. mean fluorescence intensity, mfi). Procenat pozitivnih ćelija je određivan postavljenjem graničnika na histogramu fluorescencije na osnovu kontrole. Srednji intenzitet fluerescencije predstavlja aritmetičku sredinu intenziteta fluerscencije pojedinačnih događaja (ćelija) izraženu u relativnim brojevima kanala fluorescencije (0-1024). Analizirano je najmanje 5000 ćelija po uzorku.

3.4. Funkcionalne karakteristike MoDĆ

Alostimulatorni potencijal MoDĆ i sposobnost usmeravanja T ćelijskog odgovora ka Th1, Th2, Th17 ili Treg odgovoru su ispitivani korišćenjem sledećih testova:

Test alogene mešane leukocitne reakcije – MLR

ELISA test za utvrđivanje produkovanih citokina u kulturama MoDĆ

ELISA test za utvrđivanje produkovanih citokina u od strane T limfocita kokulturama – Th profil

3.4.1. Alostimulatorni potencijal MoDĆ

Alostimulatorni potencijal MoDĆ je ispitivan MLR testom u kome su nezrele i stimulisane MoDĆ korišćene kao stimulatori, dok su kao responderi korišćeni $CD4^+$ T limfociti od alogenog davaoca. U ploču od 96 mesta sa „U“- dnom (Sarstedt, Nemačka) su dodati stimulatori kao triplikati dvostruko opadajućih razblaženja (od

$1x10^4$ do $0.125x10^4$ MoDĆ po bazenu) i konstantan broj ($1x10^5$) $CD4^+$ T ćelija u ukupnoj zapremini od 200μl po bazenu. Ćelije su kultivisane 6 dana u kompletnom medijumu u termostatu na 37°C i 5% CO_2. Osamnaest sati pre isteka kultivacije dodat je radioaktivno obeleženi timidin [^{3}H] u koncentraciji 1 μCi po bazenčiću. Nakon isteka kulture ćelije su pokupljene automatskim skidačem kulture (Titertec Cell Harvester, ICN Flow, SAD). Ugradanja [^{3}H] timidina izmerena je scintilacionim β-brojačem (LKB-1219 Rackbeta, Finska) i izražena je kao broj otkucaja u minuti (engl. counts per minute, cpm).

3.4.2. Određivanje produkovanih citokina od strane kultivisanih MoDĆ

U supernatantima kultura nezrelih i stimulisanih MoDĆ određena je koncentracija citokina IL-12p70, IL-23, IL-27, IL-10, IL-6 i TNF-α korišćenjem komercijalnih ELISA testova.

3.4.2.1. IL-12p70

Koncentracija IL-12p70 u supernatantima kultura MoDĆ određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (R&D Systems, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene mišijim monoklonskim antitelima specifičnim za humani IL-12p70, dok je za sekundarno poliklonsko kozje anti-humano antitelo bilo konjugovano biotinom. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-12p70.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 μl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na sobnoj temperaturi. Po isteku inkubacije ploča je isprana 3 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 300 μl rastvora koji sadrži K-PBS i goveđi serum albumin (reagent diluent). Posle inkubacije u trajanju od 1 sata ploča je isprana 3 puta i dodato je po 100 μl standarda u duplikatima dvostrukih opadajućih koncentracija

(2000 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 μl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 μl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 2 sata. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 μl streptavidinom konjugovanog enzima peroksidaze rena. Posle inkubacije u trajanju od 20 minuta na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 μl rastvora supstrata i usledila je inkubacija u trajanju od 20 min u mraku. Kao finalni korak dodato je po 50 μl rastvora za zaustavljanje reakcije. Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.2.2. IL-27

Koncentracija IL-27 u supernatantima kultura MoDĆ određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (R&D Systems, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene mišijim monoklonskim antitelima specifičnim za humani IL-27, dok je za sekundarno poliklonsko kozje anti-humano antitelo bilo konjugovano biotinom. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-27.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 μl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na sobnoj temperaturi. Po isteku inkubacije ploča je isprana 3 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 300 μl rastvora koji sadrži K-PBS i goveđi serum albumin (reagent diluent). Posle inkubacije u trajanju od 1 h ploča je isprana 3 puta i dodato je po 100 μl standarda u duplikatima dvostrukih opadajućih koncentracija (10000 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 μl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 μl po bazenu biotiniziranog sekundarnog antitela i

ploča je inkubirana 2 sata. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 µl streptavidinom konjugovanog enzima peroksidaze rena. Posle inkubacije u trajanju od 20 minuta na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 µl rastvora supstrata i usledila je inkubacija u trajanju od 20 min u mraku. Kao finalni korak dodato je po 50 µl rastvora za zaustavljanje reakcije. Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.2.3. IL-23

Koncentracija IL-23 u supernatantima kultura MoDĆ određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani IL-23, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-23. Senzitivnost metode je 15 pg/ml.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 µl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 µl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 µl standarda u duplikatima dvostrukih opadajućih koncentracija (2000 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 µl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 µl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 µl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne

inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 µl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku. Kao finalni korak dodato je po 50 µl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.2.4. IL-10

Koncentracija IL-10 u supernatantima kultura MoDĆ određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani IL-10, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan sa streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-10. Senzitivnost metode je 2 pg/ml.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 µl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 µl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 µl standarda u duplikatima dvostrukih opadajućih koncentracija (300 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 µl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 µl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 µl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato

po 100 µl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku. Kao finalni korak dodato je po 50 µl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.2.5. IL-6

Koncentracija IL-6 u supernatantima kultura MoDĆ određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani IL-6, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan sa streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-6. Senzitivnost metode je 2 pg/ml.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 µl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 µl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 µl standarda u duplikatima dvostrukih opadajućih koncentracija (200 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 µl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 µl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 µl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 µl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku.

Kao finalni korak dodato je po 50 μl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.2.6. TNF-α

Koncentracija TNF-α u supernatantima kultura MoDĆ određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani TNF-α, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani TNF-α. Senzitivnost metode je 4 pg/ml.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 μl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 μl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 μl standarda u duplikatima dvostrukih opadajućih koncentracija (500 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 μl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 μl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 μl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 μl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku. Kao finalni korak dodato je po 50 μl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm

talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.3. Određivanje citokina produkovanih od strane T limfocita u kokulturi sa MoDĆ

U supernatantima kultura $CD4^+$ T limfocita i MoDĆ određena je koncentracija citokina IFN-γ, IL-17, IL-5, IL-2 i IL-10 primenom komercijalnih ELISA testova i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije (Th1/Th2 11plex Kit, Bender MedSystems, Vienna, Austrija), prema uputstvu proizvođača.

U bazene ploče sa 96 mesta sa „U"-dnom (Sarstedt, Nemačka) dodato je po $1x10^4$ nezrelih i stimulisanih MoDĆ. U iste bazene dodati su i sortirani $CD4^+$ T limfociti, kao i naivne i memorijske podvrste $CD4^+$ T limfocita, i to $1x10^5$ po bazenu i to u zapremini od 200 μl medijuma za kultivaciju ćelija po bazenu. Nakon petodnevne inkubacije u svaki bazen su dodati forbol-12-miristat-13-acetat (engl. phorbol 12-myristate 13-acetate, PMA, Sigma-Aldrich, Nemačka) u koncentraciji od 20 ng/ml i jonomicin (Sigma-Aldrich, Nemačka) u koncentraciji od 500 ng/ml. Ćelije su inkubirane još 24h, a zatim su u supernatantima svakog bazena određivane koncentracije citokina.

3.4.3.1. IL-17

Koncentracija IL-17 u supernatantima kokultura MoDĆ i $CD4^+$ T limfocita određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani IL-17, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-17.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 µl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 µl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 µl standarda u duplikatima dvostrukih opadajućih koncentracija (1000 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 µl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 µl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 µl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 µl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku. Kao finalni korak dodato je po 50 µl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.3.2. IL-5

Koncentracija IL-5 u supernatantima kokultura MoDĆ i $CD4^+$ T limfocita određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani IL-5, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-5. Senzitivnost metode je 4 pg/ml.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 µl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 µl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 µl standarda u duplikatima dvostrukih opadajućih koncentracija (500 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 µl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 µl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 µl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 µl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku. Kao finalni korak dodato je po 50 µl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.3.3. IL-10

Koncentracija IL-10 u supernatantima kokultura MoDĆ i $CD4^+$ T limfocita određivana je komercijalnim ELISA testom, sledeći uputstva proizvođača (eBioscience, SAD). U ovom testu, mikroploče od 96 mesta (Corning Costar 9018 ELISA plate, Sigma-Aldrich, Nemačka) su bile obložene antitelima specifičnim za humani IL-10, dok je sekundarno anti-humano antitelo bilo konjugovano biotinom. Enzim peroksidaza rena je bio konjugovan streptavidinom, što je omogućilo specifično prepoznavanje i vezivanje za biotinom obeleženo sekundarno antitelo. Kao supstrat za ovaj enzim je je korišćen vodonik peroksid (H_2O_2) i tetrametilbenzidin, dok je kao standard u testu korišćen rekombinantni humani IL-10. Senzitivnost metode je 2 pg/ml.

Procedura testa je sledeća:

U svaki bunar ploče dodato je 100 μl antitela za oblaganje ploče (coating antibody) i inkubirano preko noći na +4 ^{o}C. Po isteku inkubacije ploča je isprana 5 puta sa puferom za ispiranje (wash buffer, K-PBS sa 0.05% Tween 20) i blokirana dodavanjem 200 μl rastvora koji sadrži PBS i goveđi serum (assay buffer). Posle inkubacije u trajanju od 1 sata ploča je isprana 5 puta i dodato je po 100 μl standarda u duplikatima dvostrukih opadajućih koncentracija (300 pg/ml - 0 pg/ml) i u odgovarajuće bazene po 100 μl uzoraka u kojima smo želeli da odredimo koncentraciju citokina. Po isteku inkubacije u trajanju od 2 sata i ispiranja ploče, dodato je 100 μl po bazenu biotiniziranog sekundarnog antitela i ploča je inkubirana 1 sat. Nakon toga, ploča je isprana i u svaki bazen je dodato po 100 μl streptavidinom konjugovanog enzima peroksidaze rena. Posle tridesetominutne inkubacije na sobnoj temperaturi i ponovljenog ispiranja, u svako polje je dodato po 100 μl rastvora supstrata i usledila je inkubacija u trajanju od 15 min u mraku. Kao finalni korak dodato je po 50 μl rastvora za zaustavljanje reakcije (stop solution, 2N H_2SO_4). Intenzitet boje je meren na spektrofotometru na 450 nm talasne dužine, a tražena koncentracija citokina u svakom od uzoraka je određena na osnovu standardne krive.

3.4.3.4. IFN-γ i IL-2

IFN-γ i IL-2 su određeni u supernatantima kokultura MoDĆ i $CD4^+$ T limfocita korišćenjem humanog Th1/Th2 11plex-a. Prvo su pripremljeni svi reagensi neophodni za izvođenje testa. Esej pufer (assay buffer) je pripremljen rastvaranjem koncentrata sa destilovanom vodom u odnosu 1:10 i stavljen je u frižider do početka izvođenja testa. Liofizirani standardi su rekonstituisani dodavanjem destilovane vode do oznake naznačene na bočici standarda čime je dobijena koncentracija 400 ng/ml i za IL-2 i IFN-γ. Bočice su ostavljene desetak minuta, centrifugirane i po 10 μl svakog rekonstituisanog standarda je dodato u epruvetu označenu kao „standard 1“ i dopunjeno esej puferom do 200 μl finalne zapremine. U epruvete označene kao „standard 2“-„standard-7“ dodato je po 100 μl esej pufera. Potom je usledilo serijsko razblaženje standarda prebacivanjem po 50 μl standarda 1 u epruvetu 2, sadržaj je vorteksiran, pa je zatim 50 μl standarda 2 prebačeno u epruvetu 3 i postupak je nastavljen do sedme epruvete. Nakon pripreme standarda

pripremljena je mešavina kuglica(bead mixture). Za analizu svakog citokina korišćena je određena vrsta kuglica obložena odgovarajućim antitelom. Mešavina kuglica je pripremljena tako da ukupni volumen bude bude jednak proizvodu 25 μl kuglica i ukupnog broja testova. Bočice sa kuglicama su vorteksirane 5 sekundi i iz svake je uzeto po 1/20 ukupnog volumena koji je dodat u novu epruvetu. Epruveta je dopunjena sa puferom za razređivanje (reagent dilution puffer) i centrifugirana na 3000xg tokom 5 minuta. Tečnost je pažljivo odlivena tako da u epruveti ostane oko 50 μl tečnosti. Nakon toga je u epruvetu ponovo dodat pufer za razređivanje i epruveta je centrifugirana 5 sekundi. Mešavina sekundarnih antitela konjugovanih biotinom (biotin-conjugate mixture) je pripremljena na sličan način kao i mešavina kuglica. Ukupni volumen je dobijen množenjem broja testova sa 50 μl smeše biotinom-konjugovanih sekundarnih antitela. Uzeto je po 1/20 ukupnog volumena i prebačeno u novu epruvetu koja je dopunjena sa puferom za razređivanje. Rastvor sa streptavidin-fikoeritrinom (streptavidin-phycoerythrin solution), koji se vezuje za biotinom-konjugovana sekundarna antitela, pripremljen je rastvaranjem u esej puferu prema proporciji: za 96 testova potrebno je 176 μl strepravidin-fikoeritrin rastvora i 5324 μl esej diluenta. Na osnovu ove proporcije određene su zapremine prema broju testova u našem eksperimentu. Kada su svi reagensi pripremljeni u ploču od 96 mesta sa „V"-dnom (Sardstedt, Nemačka) dodato je po 25 μl esej pufera u bunare za blank, po 25 μl standarda u odgovarajuće bunare (opseg koncentracija standarda je 27-20000 pg/ml, a senzitivnost testa je za IL-2 16.4 pg/ml, a za IFN-γ 1.6 pg/ml) i po 25 μl uzorka. Nakon toga dodato je po 25 μl mešavine kuglica i po 50 μl mešavine sekundarnih antitela konjugovanih biotinom. Usledila je inkubacija u trajanju od 2 sata na sobnoj temperaturi na mešalici. Po isteku inkubacije, dodato je po 100 μl esej pufera u svaki bunarić i ploča je centrifugirana 5 minuta na 200xg. Nakon toga iz svakog bunara je uzeto po 150 μl tečnosti. Potom je dodato po 150 μl esej pufera i ploča je ponovo centrifugirana tokom 5 minuta na 200xg. Nakon uzimanja po 150 μl tečnosti iz svakog bunara dodato je po 50 μl rastvora sa streptavidin-fikoeritrinom i ploča je inkubirana 1 sat na sobnoj temperaturi na mešalici. Po isteku inkubacije ponovljen je prethodno opisan postupak ispiranja i sadržaj svakog bunarića je prebačen u epruvete za protočnu citofluorimetriju i svaka je dopunjena sa po 500 μl esej pufera. Uzorci su analizirani na protočnom

citofluorimetru (EPICS XL-MCS, Coulter, Krefeld, Nemačka), a rezultati su obrađeni softverom koji je dobijen uz kit (FlowCytomix Pro Software).

3.4.3.5. Intracitoplazmatska detekcija citokina aktivisanih T limfocita

Procena Th polarizujuće aktivnosti MoDĆ je dodatno izvršena određivanjem produkcije IL-17 i IFN-γ u kokulturi sa alogenim $CD4^+$ T ćelijama. Purifikovane $CD4^+$ T ćelije ($1x10^5$ ćelija po bunariću) su kultivisane u prisustvu alogenih MoDĆ ($1x10^4$ ćelija po bunariću) tokom 5 dana u kompletnom medijumu u ploči od 96 mesta sa „U"- dnom (Sarstedt, Nemačka). Nakon toga su ćelije sakupljene i inkubirane sa monenzinom (3 μM) tokom 6h. Potom je usledila inkubacija u prisustvu anti-IFN-γ-FITC (R&D Systems; SAD) i anti-IL-17-PE (BD Biosciences Pharmingen; SAD) uz primenu FIX & PERM® Cell Fixation & Cell Permeabilization Kit (Invitrogen, SAD) prateći uputstva proizvođača. Ćelijska fluorescence je analizirana na EPICS XL-MCS protočnom citofluorimetru (Coulter, Krefeld, Nemačka) korišćenjem programa SZSTEMTM II software.

3.5. Statistička obrada podataka

Podaci su statistički obrađeni korišćenjem Studentovog T-testa u okviru softverskog programa PRIMER.

4. REZULTATI

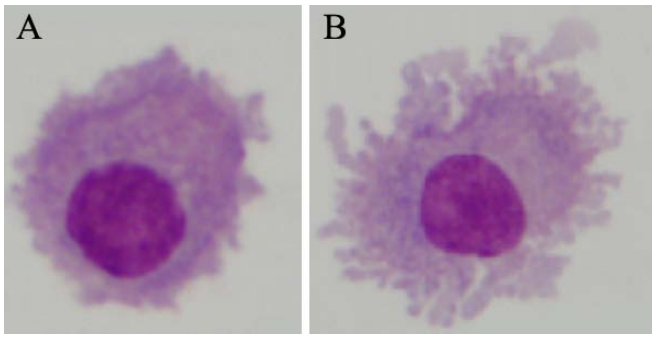
Poglavlje Rezultati je podeljeno u pet delova, tako da je u prvom delu prikazan efekat Poly (I:C) na funkcionalna i fenotipska svojstva MoDĆ, a u narednim efekti drugih modulatora i njihovih kombinacija sa Poly (I:C). U drugom delu prikazani su efekti loksoribina i njegove kombinacije sa Poly (I:C), u trećem delu efekti kurdlana i njegove kombinacije sa Poly (I:C), u četvrtom delu efekti citokina IFN-γ i liganda za CD40 molekul i njihove kombinacije sa Poly (I:C) i u petom delu efekti TNF-α i njegove kombinacije sa Poly (I:C). U različitim delovima istraživanja korišćene su MoDĆ dobijene od različitih zdravih donora.

4.1. Uticaj Poly (I:C) na diferencijaciju nezrelih humanih MoDĆ

Prvi cilj ovog istraživanja je bio da se ispita efekat dejstva Poly (I:C) na diferencijaciju nezrelih humanih MoDĆ. U preliminarnim eksperimentima nezrele MoDĆ smo kultivisali u prisustvu različitih koncentracija Poly (I:C) (5 μg/ml, 25 μg/ml i 50 μg/ml). Na osnovu fenotipskih i funkcionalnih karakteristika MoDĆ stimulisanih na ovaj način, procenjeno je da je optimalna koncentracija Poly (I:C) za aktivaciju nezrelih humanih MoDĆ 25 μg/ml. U narednim eksperimentima su nezrele MoDĆ tokom 48h kultivisane u prisustvu optimalne koncentracije Poly (I:C), dok su kontrolnu grupu predstavljale MoDĆ diferencirane pod istovetnim uslovima u medijumu koji nije sadržao Poly (I:C).

4.1.1. Uticaj Poly (I:C) na morfološke i fenotipske karakteristike MoDĆ

Morfološkom analizom citospin preparata obojenih MGG metodom ustanovljeno je da se MoDĆ stimulisane sa optimalnom koncentracijom Poly (I:C) odlikuju izraženim citoplazmatskim nastavcima što je odlika zrelih MoDĆ (*Slika 7*).



Slika 7. Morfološke karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C)

Prikazane su slike citospin preparata MoDĆ kultivisanih u kontrolnom medijumu (A) i u prisustvu Poly (I:C) (B) koji su obojeni po MGG

Fenotipske karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C) analizirane su metodom protočne citometrije korišćenjem specifične kombinacije monoklonskih antitela, postupkom koji je detaljno opisan u poglavlju Materijal i metode. Rezultati su prikazani kao procenat pozitivnih ćelija i srednja vrednost intenziteta fluorescence (*Tabela 1*).

Tabela 1. Fenotipske karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C)

		Kontrola	Poly (I:C)
HLA-DR	*%*	98.0 ± 1.6	99.0 ± 0.6
	mfi	28.8 ± 4.3	62.9 ± 9.5***
CD86	*%*	75.4 ± 10.9	96.5 ± 1.4***
	mfi	13.6 ± 2.0	37.6 ± 5.6***
CD40	*%*	92.8 ± 5.3	97.5 ± 1.1
	mfi	15.0 ± 1.8	41.6 ± 5.0***
CD54	*%*	83.9 ± 13.2	94.2 ± 3.3
	mfi	23.5 ± 3.5	55.9 ± 8.4***
CD83	*%*	33.2 ± 5.0	75.9 ± 11.4***
	mfi	3.4 ± 0.5	6.4 ± 1.0*
CCR7	*%*	2.7 ± 0.4	11.3 ± 1.6***
	mfi	2.6 ± 0.4	2.9 ± 0.5

MoDĆ su dobijene iz humanih monocita nakon šestodnevne kultivacije u prisustvu GM-CSF (100 ng/ml) i IL-4 (20 ng/ml) i stimulacije sa Poly (I:C) tokom 48h. Neadherentne ćelije su skupljene i obeležene antitelima specifičnim za ključne markere DĆ (anti-HLA-DR-PE, anti-CD86-PE, CD83-FITC, anti-CD40-FITC, anti-CD54-PE i anti-CCR7-FITC), a potom analizirane na protočnom citofluorimetru. Rezultati iz jednog reprezentativnog eksperimenta su predstavljeni kao procenat pozitivnih ćelija (% ± SD) i kao srednja vrednost intenziteta fluorescence (engl. mean fluorescence intensity, mfi ± SD).
* p <0.05; *** p<0.005 u odnosu na kontrolne MoDĆ

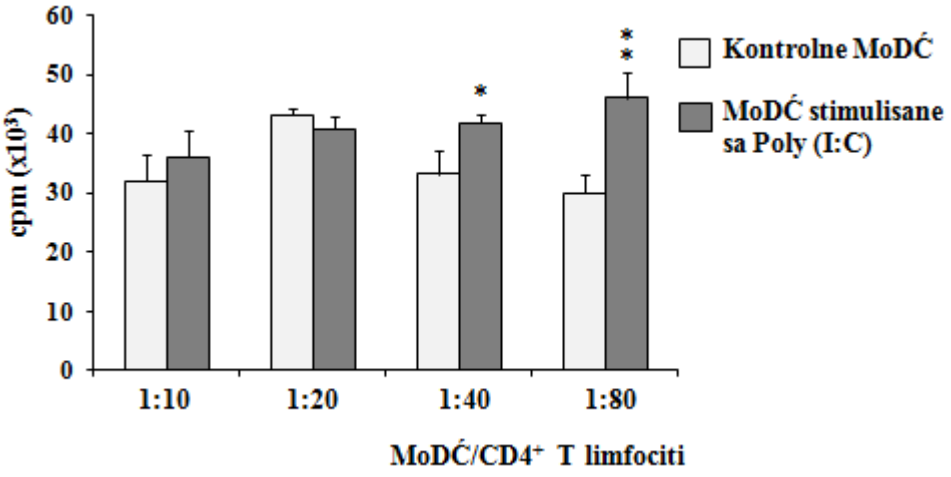
Rezultati fenotipske analize kontrolnih MoDĆ pokazali su da su na značajnom procentu ovih ćelija bili ispoljeni molekuli HLA-DR, CD40, CD54 i CD86, dok je procenat CD83 pozitivnih ćelija bio manji. Procenat ćelija koje ispoljavaju

CCR7 je bio izuzetno nizak. Najveće srednje vredosti intenziteta fluorescence su bile za HLA-DR i CD54 molekul, dvostruko niže za CD40 i CD86 i niske za CD83 i CCR7.

Fenotipskom analizom MoDĆ diferenciranih u prisustvu Poly (I:C) ustanovljeno je statistički značajno povećanje procenta ćelija koje ispoljavaju CD86, CD83 i CCR7 molekule, u poređenju sa kontrolnim MoDĆ. Takođe, u poređenju sa kontrolnim MoDĆ, stimulacija MoDĆ sa Poly (I:C) je dovela do povećanja srednje vrednosti intenziteta fluorescence za HLA-DR, CD86, CD40, CD54 i CD83.

4.1.2. Alostimulatorna aktivnost MoDĆ stimulisanih sa Poly (I:C)

U cilju daljeg proučavanja uticaja Poly (I:C) na diferencijaciju MoDĆ ispitivane su funkcionalne karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C). Jedna od funkcionalnih karakteristika MoDĆ je sposobnost da stimulišu proliferaciju alogenih $CD4^+$ T limfocita u mešanoj kulturi leukocita, postupkom koji je detaljno opisan u poglavlju Materijal i metode. Proliferativni odgovor $CD4^+$ T limfocita u kokulturi sa kontrolnim i MoDĆ stimulisanim Poly (I:C) je prikazan na *Grafikonu 1.*



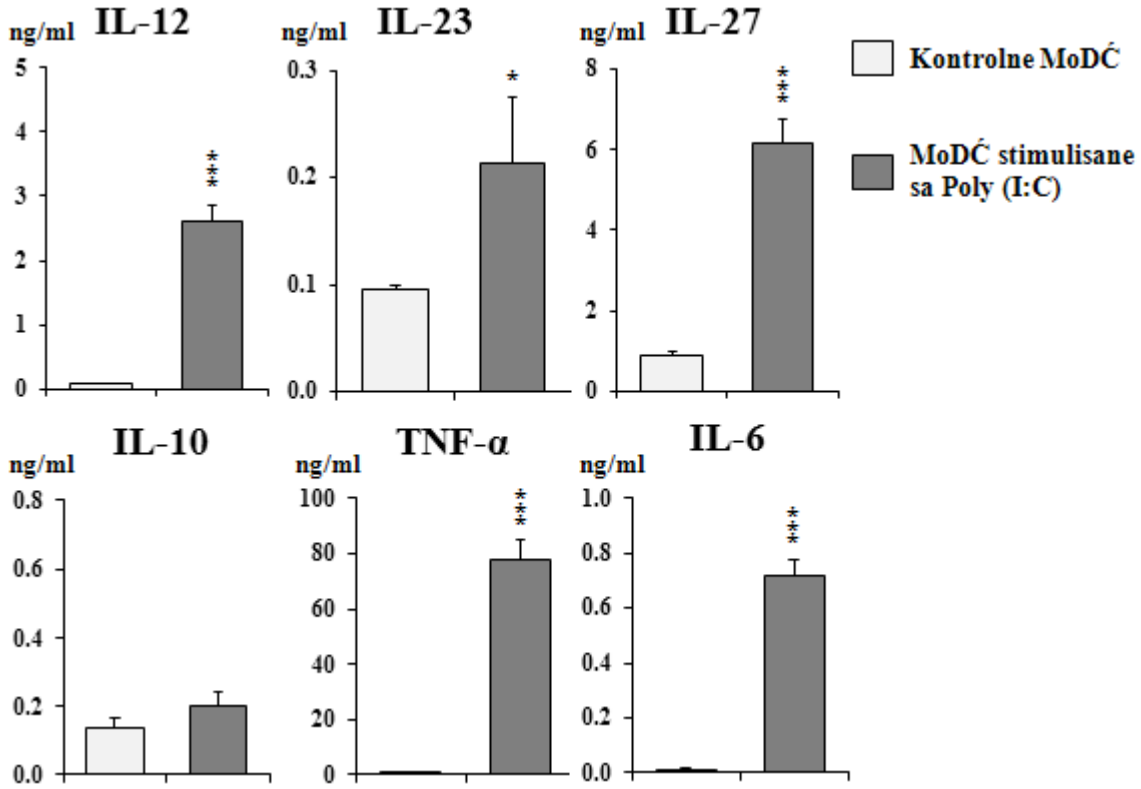
Grafikon 1. Alostimulatorni kapacitet MoDĆ diferenciranih u prisustvu Poly (I:C) u kokulturi sa alogenim CD4 T limfocitima

Na grafikonu je prikazan alostimulatorni potencijal MoDĆ diferenciranih u kontrolnom medijumu (kontrolne MoDĆ) ili u prisustvu Poly (I:C). MoDĆ su kultivisane su u opadajućim dvostrukim razblaženjima ($1x10^4$-$0.125x10^4$) sa alogenim $CD4^+$ T limfocitima ($1x10^5$) u toku 5 dana u 200 µl kompletnog medijuma. U medijum za kultivaciju ćelija 18h pre merenja proliferativnog odgovora je dodat [H^3] timidin. Proliferativan odgovor je izražen kao broj otkucaja u minuti (engl. counts per minute, cpm). Na grafiku su prikazani rezultati jednog reprezentativnog od šest sličnih eksperimenata kao srednja vrednost cpm triplikata kulture ± SD.
* p <0.05; ** p<0.01 u odnosu na kontrolne MoDĆ

Kontrolne MoDĆ su pokazale umeren potencijal za stimulaciju proliferacije alogenih $CD4^+$ T limfocita. MoDĆ kultivisane u prisustvu Poly (I:C) imale su povećan alostimulatorni kapacitet i to pri nižim odnosima MoDĆ i alogenih $CD4^+$ T limfocita (1:40 i 1:80), dok pri ostalim odnosima nije došlo do statistički značajne promene alostimulatornog kapaciteta.

4.1.3. Produkcija citokina od strane MoDĆ stimulisanih sa Poly (I:C)

Sledeći korak u ispitivanju funkcionalnih karakteristika MoDĆ diferenciranih u prisustvu Poly (I:C) ili u kontrolnom medijumu bio je određivanje produkcije citokina u supernatantima kultura MoDĆ primenom ELISA metode, kao što je opisano u poglavlju Materijal i metode. Rezultati su standardizovani za $1x10^6$ MoDĆ/ml kulture i prikazani su kao srednje vrednosti koncentracija, izraženih u ng/ml, iz tri reprezentativna eksperimenta (*Grafikon 2*).



Grafikon 2. Produkcija citokina od strane MoDĆ diferenciranih bez i u prisustvu Poly (I:C)

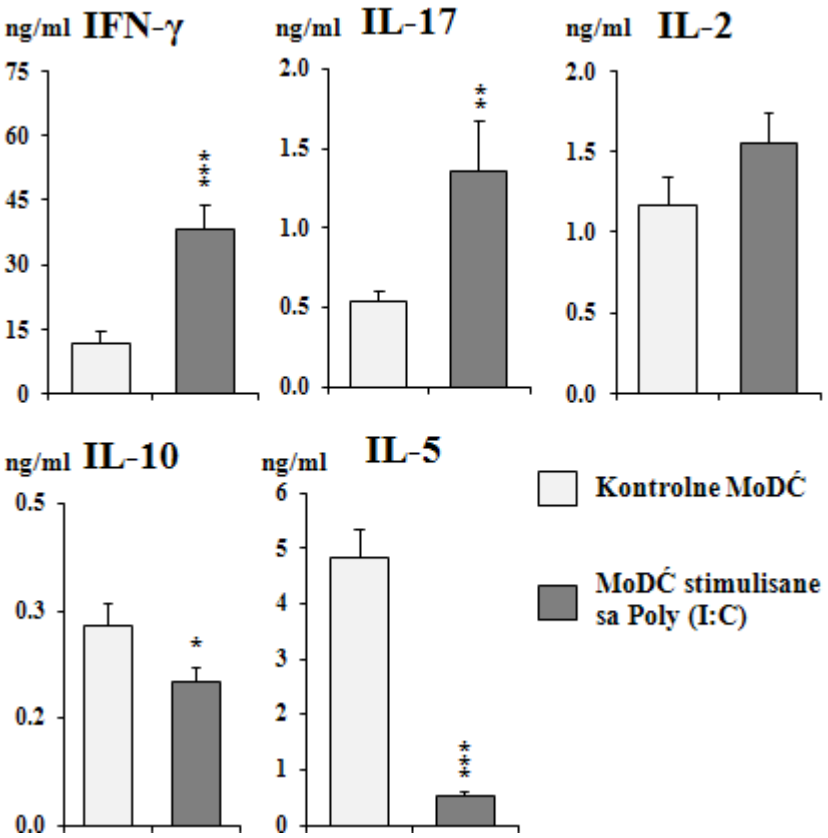
Koncentracije IL-12, IL-23, IL-27, IL-6, TNF-α i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih bez i u prisustvu Poly (I:C) tokom 2 dana. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* p <0.05; *** p<0.005 u odnosu na kontrolne MoDĆ

Kontrolne nezrele MoDĆ su produkovale niske nivoe IL-10 i IL-27, dok su nivoi IL-12, IL-23, TNF-α i IL-6 bili nedetektabilni. MoDĆ stimulisane u prisustvu Poly (I:C) pokazale su statistički značajno povećanje produkcije IL-12, IL-27, TNF-α i IL-6, umereno povećanje nivoa IL-23, dok je produkcija IL-10 bila nepromenjena, u poređenju sa kontrolnim MoDĆ.

4.1.4. Produkcija citokina u kokulturi alogenih $CD4^+$ T limfocita i MoDĆ stimulisanih u prisustvu Poly (I:C)

Uticaj Poly (I:C) na sposobnost MoDĆ da usmere $CD4^+$ T ćelijski imunski odgovor procenjen je na osnovu produkcije citokina IFN-γ, IL-17, IL-5, IL-2 i IL-10 u

kokulturi alogenih $CD4^+$ T limfocita i MoDĆ postupkom koji je detaljno opisan u poglavlju Materijal i metode. Rezultati su prikazani kao srednje vrednosti koncentracija, izraženih u ng/ml, iz šest reprezentativnih eksperimenata (*Grafikon 3*).



Grafikon 3. Citokinski profil $CD4^+$ T limfocita stimulisanih sa MoDĆ koje su diferencirane u prisustvu Poly (I:C)

Nezrele MoDĆ ($1x10^4$), kultivisane 2 dana u prisustvu Poly (I:C) ili u kontrolnom medijumu, su potom kultivisane sa $CD4^+$ sortiranim alogenim T limfocitima ($1x10^5$) tokom 5 dana, u 200 µl kompletnog medijuma. Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracija citokina merena je ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* p <0.05; ** p<0.01; *** p<0.005 u odnosu na kontrolne MoDĆ

Prikazani rezultati pokazuju da alogeni $CD4^+$ T limfociti stimulisani sa kontrolnim MoDĆ produkuju izvesne nivoe svih ispitivanih citokina. MoDĆ koje su stimulisane sa Poly (I:C) u kokulturi sa $CD4^+$ T limfocitima dovode do povećanja

produkcije IFN-γ i IL-17, odnosno do smanjenja produkcije IL-5 i IL-10, u poređenju sa produkcijom ovih citokina u kokulturi sa kontrolnim MoDĆ.

4.2. Uticaj kombinovane primene Poly (I:C) i loksoribina na diferencijaciju nezrelih humanih MoDĆ

U preliminarnim eksperimentima nezrele MoDĆ su kultivisane u prisustvu različitih kombinacija suboptimalnih (10 μg/ml) i optimalnih (25 μg/ml) koncentracija Poly (I:C) i suboptimalnih (34 μg/ml) i optimalnih (85 μg/ml) koncentracija loksoribina. Na osnovu fenotipskih i funkcionalnih karakteristika MoDĆ stimulisanih na ovaj način procenjeno je da su efekti kombinovane primene optimalne koncentracije Poly (I:C) i suboptimalne koncentracije loksoribina slični efektima primene optimalne koncentracije samog Poly (I:C). Iz tog razloga, u narednim eksperimentima smo koristili obe suboptimalne, obe optimalne i kombinaciju suboptimalne koncentracije Poly (I:C) i optimalne koncentracije loksoribina.

4.2.1. Efekti kombinacije Poly (I:C) i loksoribina na fenotipske karakteristike MoDĆ

Fenotipske karakteristike MoDĆ stimulisanih kombinacijom Poly (I:C) i loksoribina analizirane su metodom protočne citometrije korišćenjem specifične kombinacije monoklonskih antitela, postupkom koji je detaljno opisan u poglavlju Materijal i metode. Rezultati su prikazani kao procenat pozitivnih ćelija i srednja vrednost intenziteta fluorescence (*Tabela 2*).

Tabela 2. Fenotipske karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C), loksoribina i njhovih kombinacija

		Kontrola	Poly (I:C) (μg/ml)		Loksoribin (μg/ml)		Poly (I:C)/Loksoribin		
			10	25	34	85	10/34	10/85	25/85
HLA-DR	%	96.2 ± 2	98.4 ± 1	97.1 ± 1	97.0 ± 1	80.4 ± 3*	98.6 ± 0	91.0 ± 1$^{\Delta}$	90.2 ± 1 $^{\#\#\#}$
	mfi	15.0 ± 1	20.6 ± 1*	20.8 ± 2*	14.3 ± 1.	7.31 ±1***	21.7 ± 2	9.95 ± 1	10.0 ± 1
CD86	%	70.8 ± 4	84.2 ± 5*	92.5 ± 3**	70.2 ± 4	72.8 ± 3	89.2 ± 1	93.0 ± 1$^{\Delta}$	85.7 ± 1
	mfi	5.6 ± 0.4	14.3 ± 1***	15.3 ± 1***	5.1 ± 1	8.4 ± 1	20.3 ± 2	26.6 ± 2	14.1 ± 2
CD83	%	14.2 ± 3.6	52.0 ± 4***	58.6 ± 5***	21.6 ± 1*	27.2 ± 1*	88.1 ± 2$^{\Delta\Delta\Delta}$	47.6 ± 4	38 ± 2 $^{\#\#\#}$
	mfi	2.0 ± 0.3	4.6 ± 0.4***	4.11 ± 0.9*	2.4 ± 0.8	2.5 ± 0.6	6.4 ± 0.5$^{\Delta\Delta}$	5.2 ± 1.0	6.39 ± 0.7
CD54	%	88.1 ± 4.2	91.4 ± 3.6	94.5 ± 2.4	90.2 ± 3.0	75.9 ± 4.6*	95.1 ± 1.6	90.4 ± 1.7	90.7 ± 1.9
	mfi	14.9 ± 1.0	28.1 ± 2.1*	21.1 ± 2.4*	16.9 ± 2.2	10.6 ± 1.6	34.1 ± 1.6$^{\Delta}$	25.0 ± 3.9	29.0 ± 1.6 $^{\#\#}$
CD40	%	98.2 ± 0.4	97.1 ± 1.0	97.9 ± 0.6	96.6 ± 1.0	94.5 ± 1.4	98.8 ± 0.4	95.4 ± 1.0	72.7 ± 1 $^{\#\#\#}$
	mfi	10.8 ± 1.0	30.5 ± 2***	29.9 ± 3*	15.1 ± 2*	14.5 ± 2.0	37.7 ± 4.6	28.6 ± 1.9	8.4 ± 1 $^{\#\#\#}$

U tabeli su prikazane fenotipske karakteristike kontrolnih MoDĆ i MoDĆ stimulisanih u prisustvu Poly (I:C), loksoribina ili njihovih kombinacija obeleženih antitelima specifičnim za ključne markere DĆ (anti-HLA-DR-PE, anti-CD86-PE, anti-CD83-FITC, anti-CD40-FITC i anti-CD54-PE), a potom analizirane na protočnom citofluorimetru. Rezultati jednog reprezentativnog eksperimenta su predstavljeni kao procenat pozitivnih ćelija (% ± SD) i kao srednja vrednost intenziteta fluorescence (engl. mean fluorescence intensity, mfi ± SD).
* p <0.05; ** p<0.01 ; *** p<0.005 u poređenju sa kontrolnim MoDĆ
$^{\Delta}$ p < 0.05; $^{\Delta\Delta}$ p < 0.01; $^{\Delta\Delta\Delta}$ p < 0.005 u poređenju sa MoDĆ tretiranim Poly (I:C) 10 μg/ml
$^{\#\#}$ p< 0.01; $^{\#\#\#}$ p < 0.005 u poređenju sa MoDĆ tretiranim Poly (I:C) 25 μg/ml

Stimulacija MoDĆ suboptimalnim i optimalnim koncentracijama Poly (I:C) je dovela do povećanja ekspresije HLA-DR, CD40, CD54, CD83 i CD86 molekula.

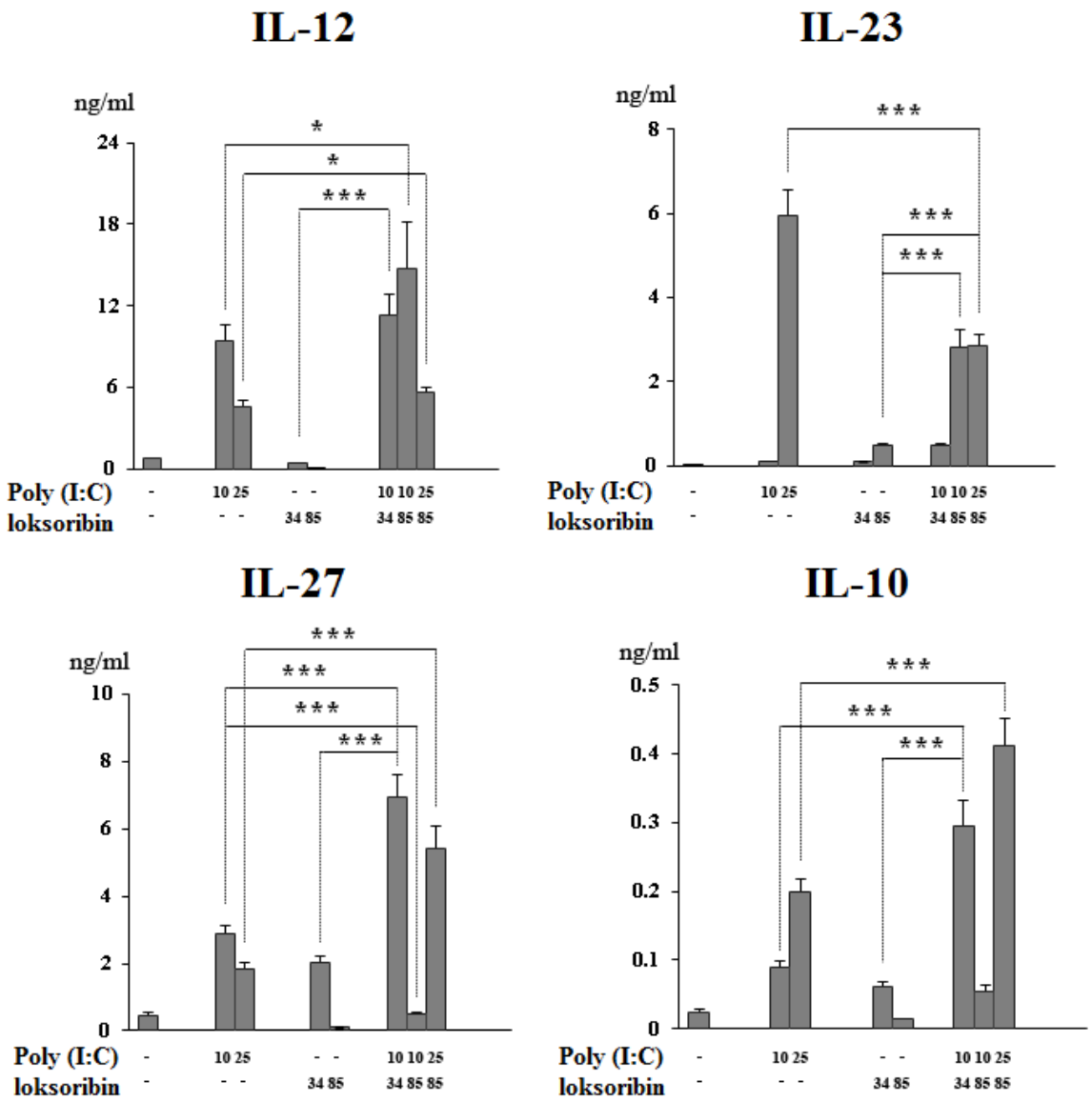
Kultivacija MoDĆ u prisustvu suboptimalne koncentracije loksoribina stimulisala je povećanje ekspresije CD40 i CD83 molekula, u poređenju sa ekspresijom na kontrolnim MoDĆ. Sa druge strane, kod MoDĆ tretiranih optimalnom koncentracijom loksoribina uočeno je povećanje ekspresije CD83 molekula i značajno smanjeno ispoljavanje HLA-DR i CD54 molekula.

Kultivacija MoDĆ u prisustvu suboptimalnih koncentracija Poly (I:C) i loksoribina dovela je do blagog povećanja ekspresije svih ispitivanih markera u poređenju sa odgovarajućim kontrolama. Stimulacija MoDĆ suboptimalnom koncentracijom Poly (I:C) i optimalnom koncentracijom loksoribina dovela je do

povećanja ekspresije CD86 i smanjenja ekspresije HLA-DR. Tretman MoDĆ optimalnim koncentracijama oba TLR agonista rezultirao je smanjenjem ekspresije HLA-DR, CD83 i CD40 molekula u poređenju sa odgovarajućim kontrolama.

4.2.2. Efekat kombinovane primene Poly (I:C) i loksoribina na produkciju citokina od strane MoDĆ

Na *Grafikonu 4* je prikazana produkcija IL-12, IL-23, IL-27 i IL-10 u supernatantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C) i loksoribina određena ELISA metodom. Rezultati su standardizovani za $1x10^6$ MoDĆ/ml kulture i prikazani kao srednje vrednosti koncentracija, izraženih u ng/ml, iz šest reprezentativnih eksperimenata.



Grafikon 4. Produkcija citokina od strane MoDĆ diferenciranih u prisustvu Poly (I:C), loksoribina i njhovih kombinacija

Koncentracije IL-12, IL-23, IL-27 i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C), loksoribina i njihovih kombinacija, kao i u kontrolnom medijumu tokom 2 dana. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* p <0.05; *** p<0.005 u odnosu na odgovarajuće kontrole

MoDĆ kultivisane u prisustvu obe ispitivane koncentracije Poly (I:C) imaju povećanu produkciju IL-12, IL-23, IL-27 i IL-10. Uočene razlike u efektima različitih koncentracija Poly (I:C) su se ogledale u tome da je tretman MoDĆ suboptimalnom

koncentracijom stimulisao produkciju IL-12 i IL-27, dok je tretman opimalnom koncentracijom doveo do značajnijeg povećanja produkcije IL-23 i IL-10.

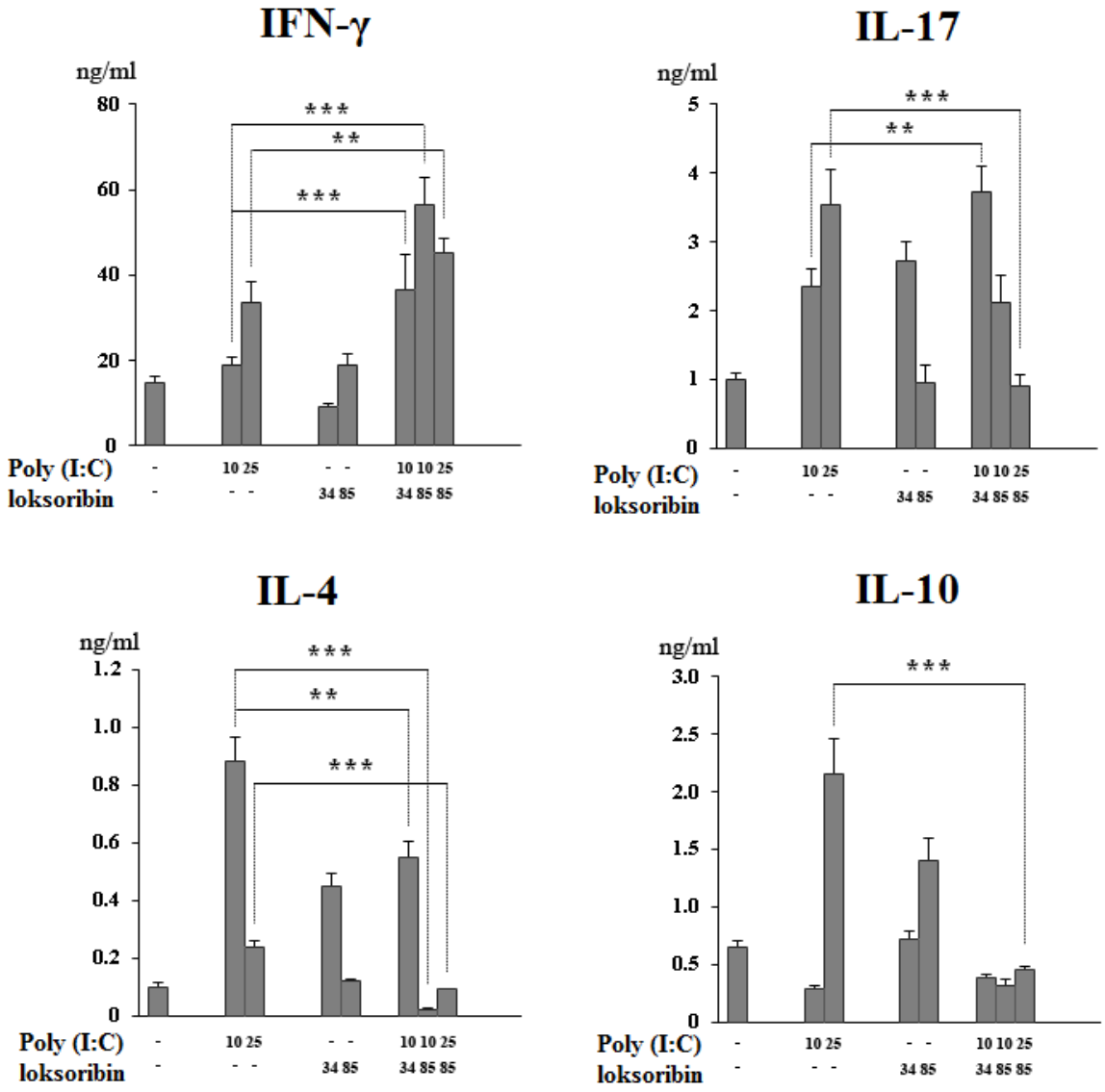
MoDĆ kultivisane u prisustvu obe koncentracije loksoribina nisu produkovale IL-12. Dozno-zavisan efekat loksoribina se ogledao u povećanju produkcije IL-10 i IL-27 kada je primenjena suboptimalna koncentracija, odnosno povećanju produkcije IL-23 od strane MoDĆ stimulisanih sa optimalnom koncentracijom.

Efekat kombinovane primene suboptimalnih koncentracija ova dva ispitivana TLR agonista se ogledao u povećanoj produkciji IL-27, IL-23 i IL-10. Sa druge strane, istovremena primena suboptimalne koncentracije Poly (I:C) i optimalne koncentracije loksoribina uticala je na povećanje produkcije IL-12 i IL-23 i smanjenje nivoa IL-10 i IL-27. Stimulacija MoDĆ sa optimalnim koncentracijama oba TLR agonista pospešila je produkciju IL-12, IL-27 i IL-10 u poređenju sa odgovarajućim kontrolama. Ovaj tretman je doveo do značajnog sniženja nivoa IL-23 u poređenju sa produkcijom ovog citokina od strane MoDĆ stimulisanih optimalnom koncentracijom Poly (I:C).

Kultivacija MoDĆ u prisustvu svih ispitivanih kombinacija Poly (I:C) i loksoribina je dovela do smanjenja produkcije IL-12 u poređenju sa efektom suboptimalne koncentracije Poly (I:C) i optimalne koncentracije loksoribina. Suprotan efekat je uočen u pogledu produkcije IL-10.

4.2.3. Produkcija citokina u kokulturi alogenih $CD4^+$ T limfocita i MoDĆ stimulisanih kombinacijom Poly (I:C) i loksoribina

Uticaj kombinovane primene Poly (I:C) i loksoribina na sposobnost MoDĆ da usmere imunski odgovor procenjen je na osnovu produkcije citokina IFN-γ, IL-17, IL-4 i IL-10 u kokulturama alogenih $CD4^+$ T limfocita i MoDĆ. Rezultati su predstavljeni na *Grafikonu 5* kao srednje vrednosti koncentracija, izraženih u ng/ml, iz šest reprezentativnih eksperimenata.



Grafikon 5. Citokinski profil u kokulturi $CD4^+$ T limfocita i MoDĆ diferenciranih u prisustvu Poly (I:C), loksoribina i njhovih kombinacija

Nezrele MoDĆ ($1x10^4$), kultivisane 2 dana u prisustvu Poly (I:C), loksoribina i njihovih kombinacija, ili u kontrolnom medijumu, su potom kultivisane sa $CD4^+$ alogenim T limfocitima ($1x10^5$) tokom 5 dana, u 200 μl kompletnog medijuma. Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracija citokina merena je ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
** $p<0.01$; *** $p<0.005$ u odnosu na odgovarajuće kontrole

Stimulacija MoDĆ u prisustvu obe ispitivane koncentracije Poly (I:C) pokazala je dozno-zavisan stimulatorni efekat na produkciju IFN-γ i IL-17 u

kokulturi sa alogenim $CD4^+$ T limfocitima, Suboptimalna koncentracija Poly (I:C) je indukovala veću produkciju IL-4 u poređenju sa MoDĆ stimulisanim optimalnom koncentracijom. Suprotan efekat je uočen u pogledu produkcije IL-10.

MoDĆ stimulisane sa suboptimalnom koncentracijom loksoribina su stimulisale povećanje produkcije IL-4 i IL-17 od strane alogenih $CD4^+$ T limfocita, dok se efekat optimalne koncentracije ogledao u povećanju produkcije IFN-γ i IL-10 u alogenoj kulturi.

MoDĆ kultivisane u prisustvu suboptimalnih koncentracija oba TLR agonista su dovele do povećanja produkcije IFN-γ i IL-17 u alogenoj kulturi, u poređenju sa relevantnim kontrolama. Sa druge strane, nivoi IL-4 i IL-10 u supernatantima ovih kultura su bili znatno niži u poređenju sa vrednostima u supernatantima kokultura alogenih $CD4^+$ T limfocita i MoDĆ tretiranih pojedinačnim agonistima.

Efekat kombinovane primene suboptimalne koncentracije Poly (I:C) i optimalne koncentracije loksoribina se ogledao u povećanju produkcije IFN-γ, za razliku od IL-4 i IL-10 čiji su nivoi znatno niži, u poređenju sa nivoom u kokulturi kontrolnih MoDĆ i $CD4^+$ T limfocita.

Stimulacija MoDĆ optimalnim koncentracijama oba TLR agonista ispoljila je povoljan efekat na produkciju IFN-γ i negativan na produkciju IL-4, IL-10 i IL-17 u kokulturi u poređenju sa efektom MoDĆ tretiranih optimalnim koncentracijama pojedinačno primenjenih agonista.

4.3. Uticaj kurdlana i njegove kombinacije sa Poly (I:C) na diferencijaciju nezrelih humanih MoDĆ

U preliminarnim eksperimentima nezrele MoDĆ su kultivisane u prisustvu različitih koncentracija Poly (I:C) (5 μg/ml, 25 μg/ml i 50 μg/ml) odnosno kurdlana (10 μg/ml, 50 μg/ml, 100 μg/ml i 200 μg/ml). Na osnovu fenotipskih i funkcionalnih karakteristika MoDĆ stimulisanih na ovaj način, procenjeno je da je optimalna koncentracija Poly (I:C) za aktivaciju MoDĆ 25 μg/ml, a kurdlana 100 μg/ml.

Optimalne koncentracije agonista su korišćene za stimulaciju MoDĆ u svim narednim eksperimentima.

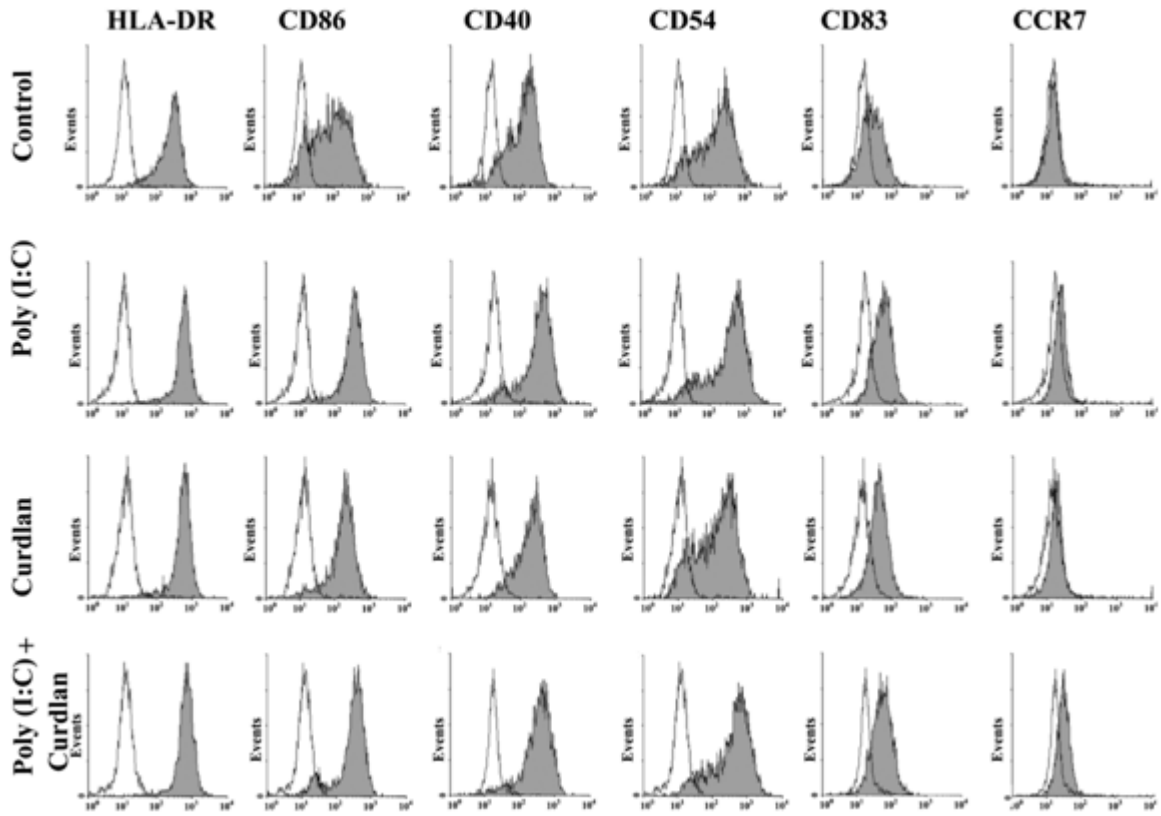
4.3.1. Efekat kurdlana i njegove kombinacije sa optimalnom koncentracijom Poly (I:C) na fenotipske karakteristike MoDĆ

Fenotipske karakteristike MoDĆ diferenciranih u pristustvu Poly (I:C) i kurdlana analizirane su metodom protočne citometrije. Rezultati su prikazani u *Tabeli 3 i na Grafikonu 6* kao procenat pozitivnih ćelija i srednja vrednost intenziteta fluorescence.

Tabela 3. Fenotipske karakteristike MoDĆ diferenciranih u prisustvu optimalnih koncentracija Poly (I:C), kurdlana i njihove kombinacije

		Kontrola	Poly (I:C)	Kurdlan	Poly (I:C)+ kurdlan
HLA-DR	%	98.0 ± 1.6	99.0 ± 0.6	99.1 ± 0.8	99.6 ± 0.2
	mfi	28.8 ± 4.3	62.9 ± 9.5***	61.2 ± 9.2***	75.7 ± 11.4***
CD86	%	75.4 ± 10.9	96.5 ± 1.4***	95.4 ± 2.0***	92.4 ± 3.0***
	mfi	13.6 ± 2.0	37.6 ± 5.6***	21.6 ± 3.2*	36.5 ± 5.5***
CD40	%	92.8 ± 5.3	97.5 ± 1.1	98.6 ± 0.9	97.8 ± 1.4
	mfi	15.0 ± 1.8	41.6 ± 5.0***	22.6 ± 2.7**	44.0 ± 5.3***
CD54	%	83.9 ± 13.2	94.2 ± 3.3	83.2 ± 8.4	93.6 ± 3.4
	mfi	23.5 ± 3.5	55.9 ± 8.4***	24.5 ± 3.7	59.5 ± 9.0***
CD83	%	33.2 ± 5.0	75.9 ± 11.4***	56.9 ± 8.5***	70.9 ± 10.6***
	mfi	3.4 ± 0.5	6.4 ± 1.0*	4.7 ± 0.7	6.2 ± 0.8**
CCR7	%	2.7 ± 0.4	11.3 ± 1.6***	4.0 ± 0.6*	18.5 ± 2.9***, Δ
	mfi	2.6 ± 0.4	2.9 ± 0.5	2.2 ± 0.4	5.2 ± 0.6***, ΔΔ

MoDĆ su dobijene iz humanih monocita nakon šestodnevne kultivacije u prisustvu GM-CSF (100 ng/ml) i IL-4 (20 ng/ml) i stimulisane optimalnim koncentracijama Poly (I:C), kurdlana i njihovom kombinacijom tokom 48h. Neadherentne ćelije su skupljene i obeležene antitelima specifičnim za ključne markere DĆ (anti-HLA-DR-PE, anti-CD86-PE, anti-CD83-FITC, anti-CD40-FITC, anti-CD54-PE i anti-CCR7-FITC), a potom analizirane na protočnom citofluorometru. Rezultati iz jednog reprezentativnog eksperimenta su predstavljeni kao procenat pozitivnih ćelija (% ± SD) i kao srednja vrednost intenziteta fluorescence (engl. mean fluorescence intensity, mfi ± SD).
* p <0.05; *** p<0.005 u odnosu na kontrolne MoDĆ
$^{\Delta}$ p <0.05; $^{\Delta\Delta}$ p<0.01 u odnosu na MoDĆ stimulisane sa Poly (I:C)



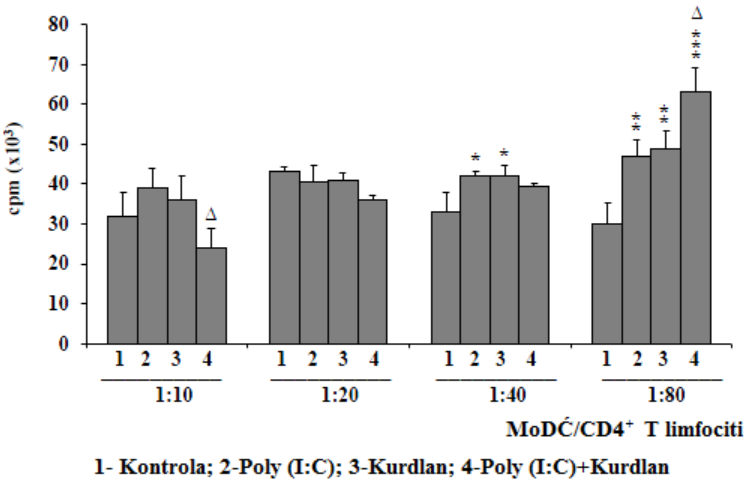
Grafikon 6. Fenotipske karakteristike MoDĆ diferenciranih u prisustvu optimalnih koncentracija Poly (I:C), kurdlana i njihove kombinacije

MoDĆ su dobijene iz humanih monocita nakon šestodnevne kultivacije u prisustvu GM-CSF (100 ng/ml) i IL-4 (20 ng/ml) i stimulisane sa optimalnim koncentracijama Poly (I:C), kurdlana i njihovom kombinacijom tokom 48h. Neadherentne ćelije su skupljene i obeležene antitelima specifičnim za ključne markere DĆ (anti-HLA-DR-PE, anti-CD86-PE, anti-CD83-FITC, anti-CD40-FITC, anti-CD54-PE i anti-CCR7-FITC), a potom analizirane na protočnom citofluorometru. Prikazani su rezultati jednog reprezentativnog od šest eksperimenata.

Fenotipskom analizom MoDĆ stimulisanih optimalnom koncentracijom Poly (I:C) ustanovljeno je povećanje ekspresije HLA-DR, CD86, CD40, CD54, CD83 i CCR7 molekula u poređenju sa kontrolnim MoDĆ. MoDĆ diferencirane u prisustvu optimalne koncentracije kurdlana su imale povećanu ekspresiju HLA-DR, CD86, CD83, CD40 i CCR7 molekula u poređenju sa MoDĆ koje su kultivisane samo u medijumu, ali je efekat kurdlana bio slabiji u poređenju sa efektom Poly (I:C). Istovremena stimulacija MoDĆ optimalnim koncentracijama oba agonista dovela je do povećanja ekspresije CCR7 molekula u poređenju sa MoDĆ diferenciranim u prisustvu Poly (I:C).

4.3.2. Alostimulatorni potencijal MoDĆ stimulisanih kurdlanom i njegovom kombinacijom sa Poly (I:C)

Na *Grafikonu 7* prikazana je proliferacija alogenih $CD4^+$ T limfocita u kokulturi sa MoDĆ prethodno stimulisanih kurdlanom i njegovom kombinacijom sa optimalnom koncentracijom Poly (I:C). MoDĆ direrencirane u prisustvu optimalne koncentracije Poly (I:C) odnosno kurdlana su stimulisale proliferaciju alogenih $CD4^+$ T limfocita pri nižim odnosima MoDĆ/$CD4^+$ T limfocita (1:40 i 1:80). MoDĆ stimulisane sa oba agonista su inhibirale proliferaciju alogenih $CD4^+$ T limfocita pri najvišem odnosu (1:10), dok su najveći alostimulatorni potencijal imale pri najnižem odnosu (1:80), u poređenju sa alostimulatornim potencijalom MoDĆ stimulisanih samo sa Poly (I:C).

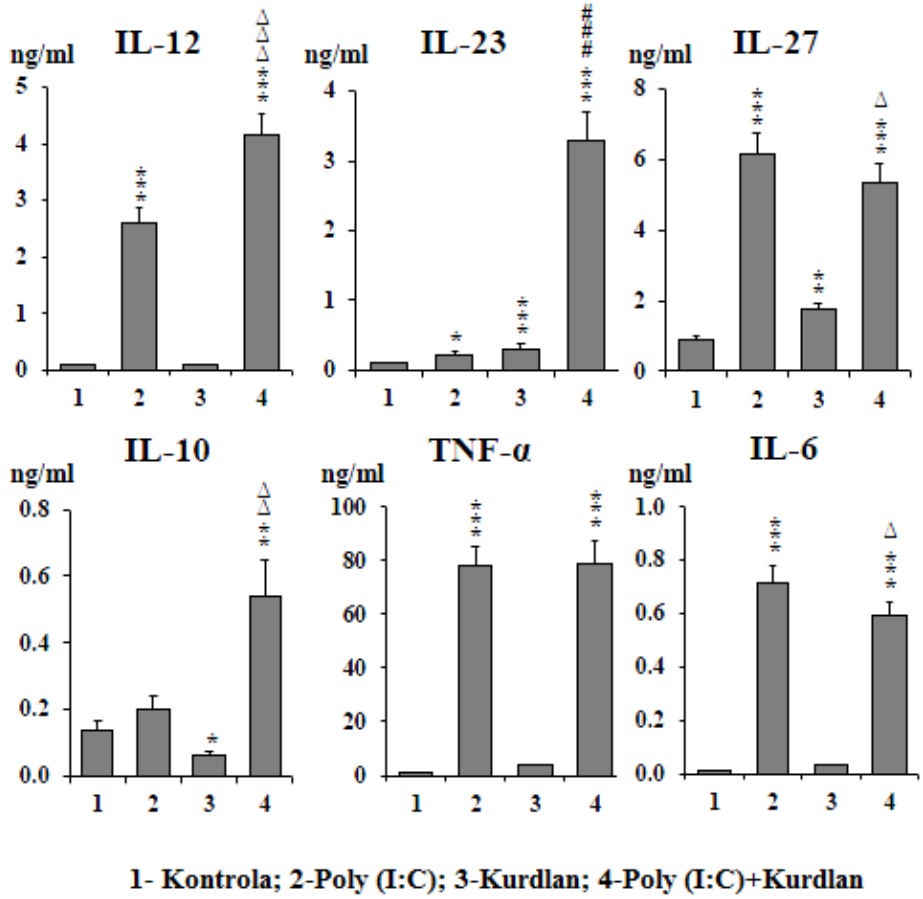


Grafikon 7. Alostimulatorni kapacitet MoDĆ diferenciranih u prisustvu optimalnih koncentracija Poly (I:C), kurdlana i njihove kombinacije u kokulturi sa alogenim $CD4^+$ T limfocitima

Na grafikonu je prikazan alostimulatorni potencijal MoDĆ diferenciranih u kontrolnom medijumu (kontrolne MoDĆ) ili u prisustvu Poly (I:C), kurdlana i njihove kombinacije. MoDĆ su kultivisane su u opadajućim dvostrukim razblaženjima ($1x10^4$-$0.125x10^4$) sa alogenim $CD4^+$ T limfocitima ($1x10^5$) u toku 5 dana u 200 µl kompletnog medijuma. U medijum za kultivaciju ćelija 18h pre merenja proliferativnog odgovora je dodat [H^3] timidin. Proliferativan odgovor je izražen kao broj otkucaja u minuti (engl. counts per minute, cpm). Na grafiku su prikazani rezultati jednog reprezentativnog od šest sličnih eksperimenata kao srednja vrednost cpm triplikata kulture ± SD.
* $p<0.05$; ** $p<0.01$; *** $p<0.005$ u odnosu na kontrolne MoDĆ
$^{\Delta}$ $p<0.05$ u poređenju sa MoDĆ tretiranim sa Poly (I:C)

4.3.3. Kombinovani efekat Poly (I:C) i kurdlana na produkciju citokina od strane MoDĆ

Koncentracije IL-12, IL-23, IL-27, IL-6, TNF-α i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C), kurdlana i njihove kombinacije. Rezultati su predstavljeni na *Grafikonu 8* kao srednja vrednost koncentracija, izraženih u ng/ml, iz šest reprezentativna eksperimenta sa različitim donorima.



Grafikon 8. Produkcija citokina od strane MoDĆ diferenciranih u prisustvu optimalnih koncentracija Poly (I:C), kurdlana i njihove kombinacije

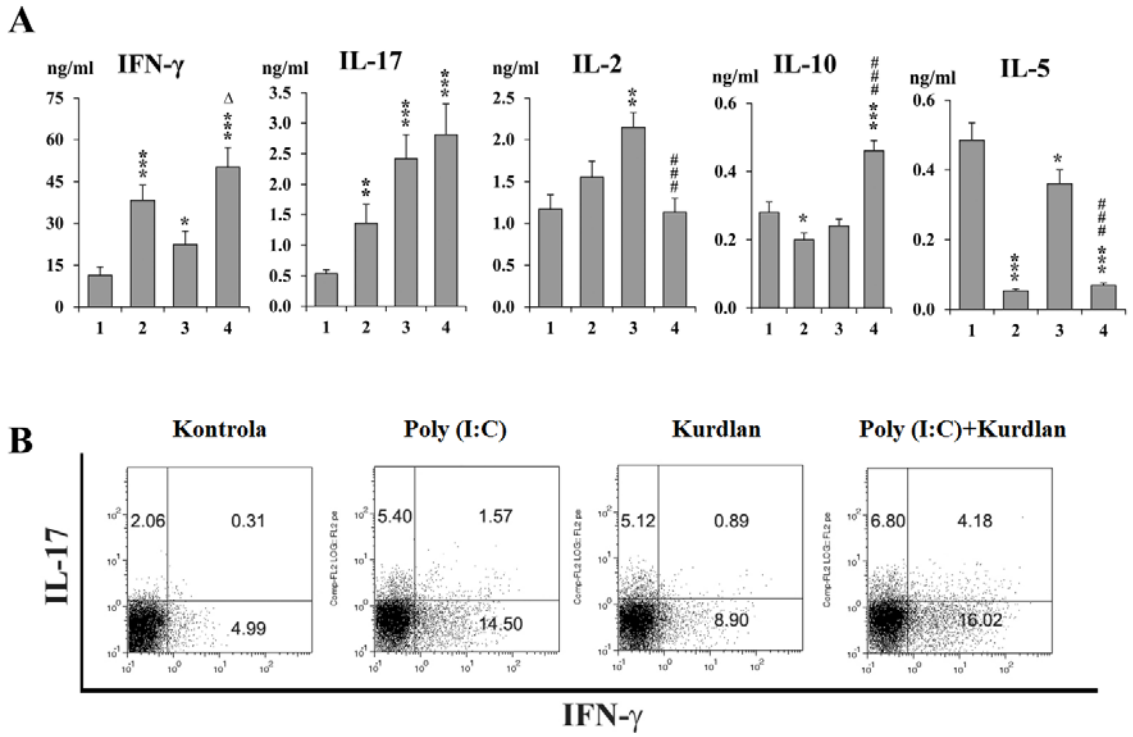
Koncentracije IL-12, IL-23, IL-27, IL-6, TNF-α i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C), kurdlana i njihove kombinacije kao i u kontrolnom medijumu tokom 2 dana. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* p<0.05; ** p<0.01; *** p<0.005 u poređenju sa kontrolnim MoDĆ
$^{\Delta}$ p<0.05; $^{\Delta\Delta}$ p<0.01; $^{\Delta\Delta\Delta}$ p<0.005 u poređenju sa MoDĆ tretiranim sa Poly (I:C)
$^{\#}$ p<0.05 u poređenju sa MoDĆ tretiranim sa kurdlanom

Kontrolne MoDĆ su produkovale niske nivoe IL-10 i IL-27, dok su nivoi IL-12, IL-23, TNF-α i IL-6 bili gotovo nedetektabilni. Stimulacija MoDĆ optimalnom koncentracijom Poly (I:C) dovela je do značajnog povećanja produkcije IL-12, IL-27, TNF-α i IL-6, umerenog povećanja produkcije IL-23 dok je nivo produkcije IL-10 bio nepromenjen u poređenju sa kontrolnim MoDĆ. MoDĆ kultivisane u prisustvu kurdlana produkovale su povećane nivoe IL-23 i IL-27 i smanjen nivo IL-10 u poređenju sa kontrolnim MoDĆ. Stimulacija MoDĆ sa kombinacijom TLR3 i dektin-1 agonista je dovela do sinergističkog povećanja produkcije IL-12, IL-23 i IL-10, dok su nivoi IL-6 i IL-27 bili sniženi u poređenju sa produkcijom citokina od strane MoDĆ stimulisanih samo sa Poly (I:C).

4.3.4. Uticaj kombinovane primene Poly (I:C) i kurdlana na potencijal MoDĆ da usmere efektorske funkcije $CD4^+$ T limfocita

U prvom delu ispitivanja efekta kombinovane primene Poly (I:C) i kurdlana na potencijal MoDĆ da usmere efektorske funkcije $CD4^+$ T limfocita određena je produkcija citokina u supernatantima i intracitoplazmatska ekspresija citokina kod $CD4^+$ T limfocita u kokulturama diferenciranih MoDĆ i alogenih $CD4^+$ T limfocita.

Rezultati produkcije citokina u supernatantima kokultura alogenih $CD4^+$ T limfocita i MoDĆ stimulisanih sa Poly (I:C) i kurdlanom prikazani su na *Grafikonu 9*A.



Grafikon 9. Citokinski profil CD4⁺ T limfocita stimulisanih sa MoDĆ koje su diferencirane u prisustvu Poly (I:C), kurdlana i njihove kombinacije

Nezrele MoDĆ (1x104), kultivisane 2 dana u prisustvu Poly (I:C), kurdlana i njihove kombinacije, ili u kontrolnom medijumu, su potom kultivisane sa alogenim $CD4^+$ T limfocitima (1x105) tokom 5 dana, u 200 μl kompletnog medijuma. A) Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracija IFN-γ, IL-17, IL-2, IL-10 i IL-5 u supernatantima kokultura merena je ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD. B) Radi procene intracitoplazmatske ekspresije citokina sakupljene su ćelije iz $CD4^+$ T/MoDĆ kokultura, postavljenih na identičan način kao za određivanje nivoa produkcije citokina u supernatantima, i inkubirane sa monenzinom tokom 6h. Ćelije su potom inkubirane sa anti-IFN-γ-FITC i anti-IL-17-PE i analizirane na protočnom citofluorometru. Rezultati dvostruke imunofenotipske analize su predstavljeni histogramima. Procenti jednostruko i dvostruko pozitivnih ćelija su prikazani na histogramima (jedan reprezentativni eksperiment od tri sa sličnim rezultatima).
1-kontrola; 2-Poly (I:C); 3-kurdlan; 4-Poly (I:C)+kurdlan
* p<0.05; ** p<0.01; *** p<0.005 u poređenju sa kontrolnim MoDĆ
$^{\Delta}$ p<0.05 u poređenju sa MoDĆ tretiranim sa Poly (I:C)
$^{\#\#\#}$ p<0.005 u poređenju sa MoDĆ tretiranim sa kurdlanom

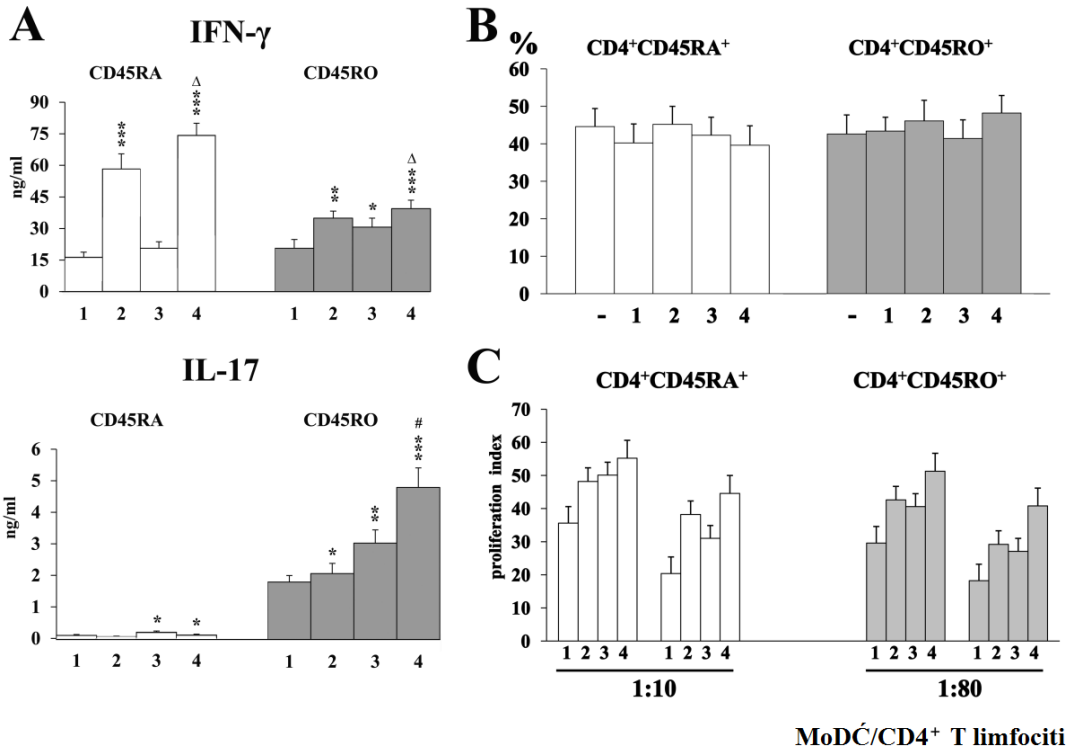
MoDĆ kultivisane u prisustvu Poly (I:C) su stimulisale produkciju IFN-γ i IL-17 i smanjile produkciju IL-10 i IL-5 od strane alogenih $CD4^+$ T limfocita, u poređenju sa kontrolnim MoDĆ. $CD4^+$ T ćelije u kokulturi sa MoDĆ tretiranim kurdlanom su produkovale visoke nivoe IFN-γ, IL-17 i IL-2 i niske nivoe IL-5, u poređenju sa nivoima u kokulturama sa kontrolnim MoDĆ. Stimulatorni efekat

MoDĆ tretiranih kurdlanom na produkciju IL-17 od strane alogenih $CD4^+$ T ćelija je bio veći u poređenju sa efektom MoDĆ tretiranih sa Poly (I:C), dok je obratno zapaženo u slučaju produkcije IFN-γ. U kokulturi $CD4^+$ T ćelija sa MoDĆ stimulisanih optimalnim koncentracijama oba agonista u optimalnim koncentracijama detektovani su povišeni nivoi IFN-γ i IL-10, trend povećanja produkcije IL-17, kao i sniženje produkcije IL-2 od strane $CD4^+$ T ćelija, u poređenju sa efektom pojedinačnih agonista.

Rezultati analize intracitoplazmatske ekspresije IFN-γ i IL-17 kod $CD4^+$ T limfocita u kokulturama pretretiranih MoDĆ i alogenih $CD4^+$ T limfocita prikazani su na *Grafikonu 9B.* MoDĆ stimulisane sa kombinacijom Poly (I:C) i kurdlana stimulisale su ekspanziju i jednostruko i dvostruko IFN-γ i IL-17 pozitivnih alogenih $CD4^+$ T limfocita. Nasuprot tome, MoDĆ tretirane pojedinačnim agonistima su stimulisale ekspanziju jednostruko pozitivnih efektorskih IFN-γ^+ ili $IL\text{-}17^+$ T limfocita.

Naredni cilj našeg istraživanja bilo je ispitivanje uticaja kombinovane primene Poly (I:C) i kurdlana na produkciju citokina, procentualnu zastupljenost i proliferaciju $CD4^+CD45RA^+$ i $CD4^+CD45RO^+$ T limfocita u kokulturi sa MoDĆ diferenciranim u prisustvu agonista.

Produkcija IFN-γ i IL-17, određenih u supernatantima kokultura pretretiranih MoDĆ i naivnih i memorijskih ($CD4^+CD45RA^+$ i $CD4^+CD45RO^+$) $CD4^+$ T limfocita, se razlikovala (*Grafikon 10A*).



Grafikon 10. Uticaj kombinovane primene Poly (I:C) i kurdlana na potencijal MoDĆ da utiču na produkciju citokina (A), procentualnu zastupljenost (B) i proliferaciju (C) $CD4^+CD45RA^+$ i $CD4^+CD45RO^+$ T limfocita u kokulturi

Nezrele MoDĆ ($1x10^4$), kultivisane 2 dana u prisustvu Poly (I:C), kurdlana i njihove kombinacije ili u kontrolnom medijumu, su potom kultivisane sa sortiranim alogenim $CD4^+CD45RA^+$ i $CD4^+CD45RO^+$ T limfocitima ($1x10^5$) tokom 5 dana, u 200 μl kompletnog medijuma. A) Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracije IFN-γ i IL-17 u supernatantima kokultura merene su ELISA testom i metodom za detekciju citokina pomoću imunofluorescentnih kuglica. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD. B) Procenat $CD4^+CD45RA^+$ i $CD4^+CD45RO^+$ T limfocita u kokulturama sa ukupnim $CD4^+$ T limfocitima je određen pre i 5 dana nakon kokultivacije sa MoDĆ diferenciranim u prisustvu agonista primenom anti-CD45RA-FITC i anti-CD45RO-PE monoklonskih antitela. Ćelijska fluorescenca je analizirana na protočnom citofluorimetru. Rezultati su prikazani kao srednja vrednost procenta pozitivnih ćelija ± SD iz tri eksperimenta. C) U cilju određivanja stope proliferacije naivnih i memorijskih $CD4^+$ T limfocita, purifikovani alogeni $CD45RA^+$ i $CD4^+CD45RO^+$ T limfociti ($1x10^5$) su kokultivisani sa MoDĆ pretretiranim sa agonistima pri različitim odnosima MoDĆ/$CD4^+$ T limfocita (1:10 i 1:80) tokom 5 dana. U medijum za kultivaciju ćelija 18h pre merenja proliferativnog odgovora je dodat [H^3] timidin. Proliferativan odgovor je izmeren kao broj otkucaja u minuti (engl. counts per minute, cpm). Na grafiku su prikazani rezultati jednog reprezentativnog od tri slična eksperimenata kao indeks proliferacije ± SD.

- pre kultivacije; 1-kontrola; 2-Poly (I:C); 3-kurdlan; 4-Poly (I:C)+kurdlan

* p<0.05; ** p<0.01; *** p<0.005 u poređenju sa kontrolnim MoDĆ

$^{\Delta}$ p<0.05 u poređenju sa MoDĆ tretiranim sa Poly (I:C)

$^{\#}$ p<0.05 u poređenju sa MoDĆ tretiranim sa kurdlanom

MoDĆ tretirane sa Poly (I:C) su stimulisale produkciju IFN-γ od strane naivnih i memorijskih $CD4^+$ T limfocita, ali je odgovor naivnih $CD4^+$ T ćelija bio jači. Nasuprot tome, MoDĆ tretirane kurdlanom su stimulisale produkciju IFN-γ samo od strane memorijskih $CD4^+$ T limfocita. MoDĆ stimulisane sa Poly (I:C) podstakle su produkciju IL-17 od strane memorijskih $CD4^+$ T limfocita. MoDĆ tretirane kurdlanom su stimulisale produkciju IL-17 od strane obe podvrste $CD4^+$ T ćelija, ali je efekat na memorijske $CD4^+$ T ćelije bio izraženiji. MoDĆ kultivisane u prisustvu oba agonista su dovele do povećanja produkcije IFN-γ od strane naivnih i memorijskih $CD4^+$ T ćelija i IL-17 od strane memorijskih $CD4^+$ T ćelija. Uticaj MoDĆ stimulisanih kombinacijom Poly (I:C) i kurdlana na efektorske fukcnije $CD4^+$ T limfocita nije bio rezultat promene procentualne zastupljenosti $CD4^+CD45RA^+$ i $CD4^+CD45RO^+$ T limfocita (*Grafikon 10B*) kao ni različite stope proliferacije ove dve podvrste (*Grafikon 10C*) $CD4^+$ T limfocita.

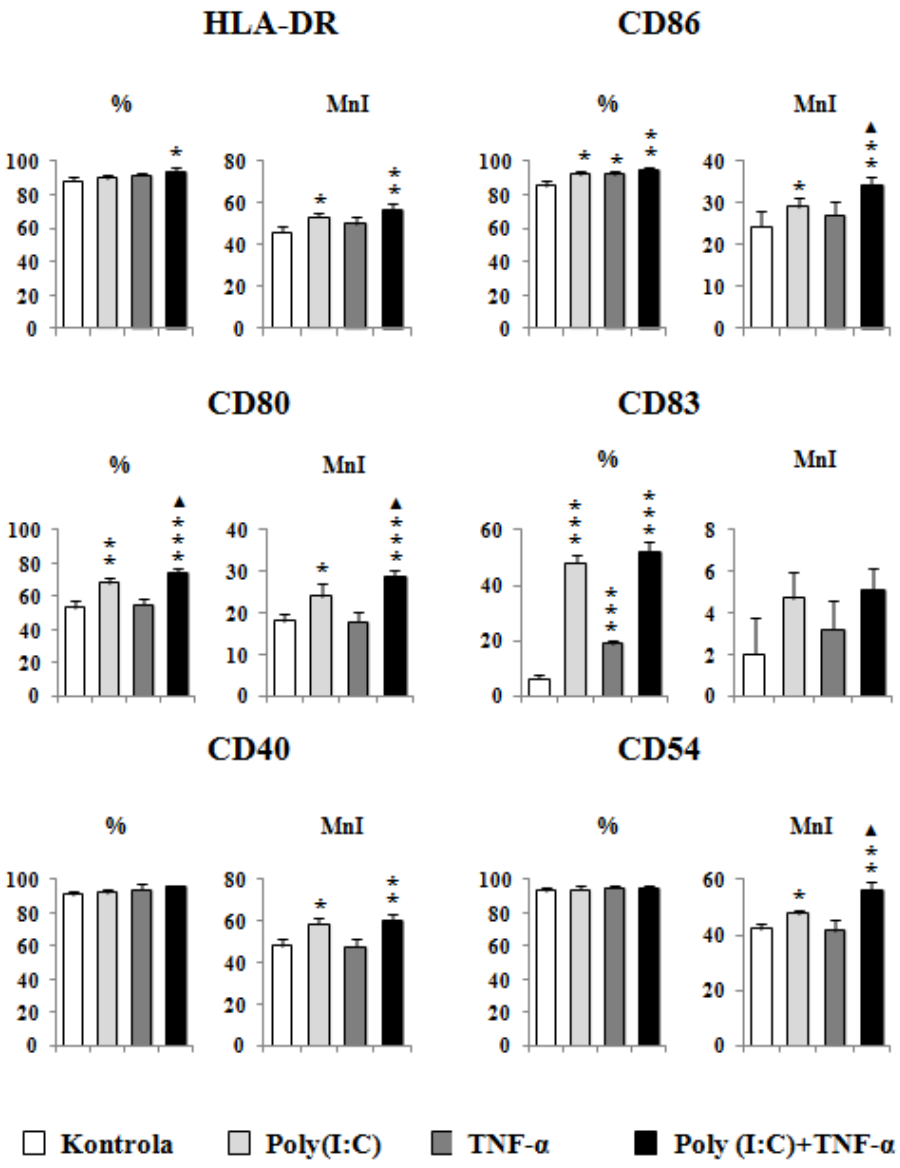
4.4. Efekat kombinovane primene Poly (I:C) i TNF-α na diferencijaciju MoDĆ

Kombinacija Poly (I:C) i TNF-α je korišćena za pripremu DĆ anti-tumorskih vakcina jer dovodi do snažne indukcije Th1 odgovora. Međutim, funkcionalne karakteristike MoDĆ stimulisanih na ovaj način nisu detaljno ispitane niti upoređene sa efektima pojedinačnih stimulatora. Stoga je jedan od ciljeva ovog istraživanja bio ispitivanje fenotipskih i funkcionalnih karakteristika MoDĆ tretiranih kombinacijom Poly (I:C) i TNF-α.

4.4.1. Efekat kombinovane primene optimalne koncentracije Poly (I:C) i TNF-α na fenotipska svojstva MoDĆ

Fenotipske karakteristike MoDĆ stimulisanih kombinacijom Poly (I:C) i TNF-α analizirane su metodom protočne citometrije, a rezultati su prikazani kao

procenat pozitivnih ćelija i srednja vrednost intenziteta fluorescence na *Grafikonu 11.*



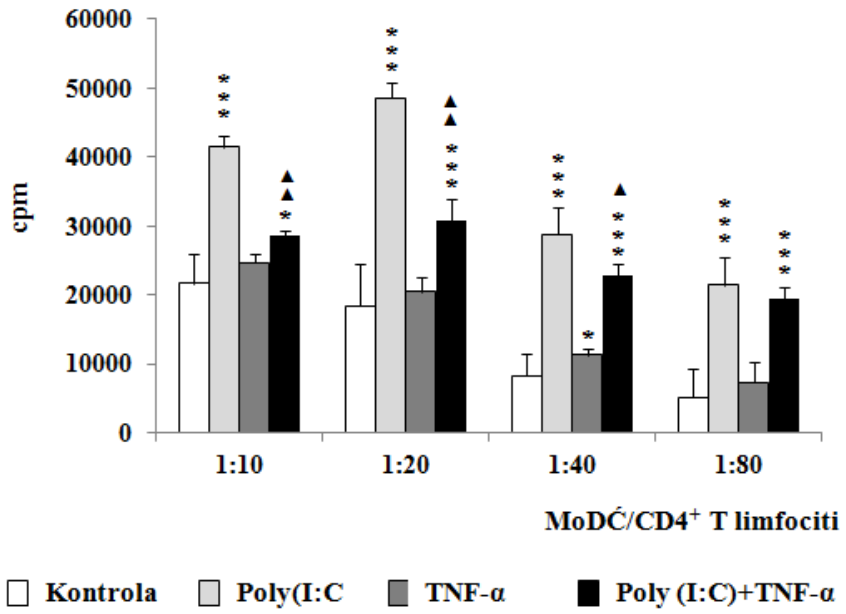
Grafikon 11. Fenotipske karakteristike MoDĆ stimulisanih optimalnom koncentracijom Poly (I:C), TNF-α i njihovom kombinacijom

MoDĆ su dobijene iz humanih monocita nakon šestodnevne kultivacije u prisustvu GM-CSF (100 ng/ml) i IL-4 (20 ng/ml) i stimulacije optimalnom koncentracijom Poly (I:C), TNF-α i njihovom kombinacijom tokom 48h. Neadherentne ćelije su skupljene i obeležene antitelima specifičnim za ključne markere DĆ (anti-HLA-DR-PE, anti-CD86-PE, anti-CD80-FITC, anti-CD83-FITC, anti-CD40-FITC, anti-CD54-PE i anti-CCR7-FITC), a potom analizirane na protočnom citofluorometru. Rezultati iz jednog reprezentativnog eksperimenta od šest sa sličnim rezultatima su predstavljeni kao procenat pozitivnih ćelija (% ± SD) i kao srednja vrednost intenziteta fluorescence (engl. mean fluorescence intensity, mfi ± SD).
* p<0.05; ** p<0.01; *** p<0.005 u poređenju sa kontrolnim MoDĆ
▲ p<0.05 u poređenju sa MoDĆ tretiranim sa Poly (I:C)

Stimulacija MoDĆ sa Poly (I:C) dovela do povećanja ekspresije HLA-DR, CD80, CD86, CD83, CD40 i CD54. Fenotipskom analizom MoDĆ tretiranih sa TNF-α utvrđeno je da je stimulacija MoDĆ samo sa ovim citokinom dovela do umerenog povećanja ekspresije CD83 i CD86, dok je TNF-α u prisustvu Poly (I:C) doveo do dodatnog povećanja ekspresije CD80, CD86 i CD54.

4.4.2. Alostimulatorni potencijal MoDĆ stimulisanih kombinacijom optimalne koncentracije Poly (I:C) i TNF-α

Proliferativni odgovor alogenih $CD4^+$ T limfocita u mešanoj kulturi leukocita sa MoDĆ stimulisanih kombinacijom Poly (I:C) i TNF-α prikazan je na *Grafikonu 12.*



Grafikon 12. Alostimulatorni kapacitet MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α i njihove kombinacije u kokulturi sa alogenim $CD4^+$ T limfocitima

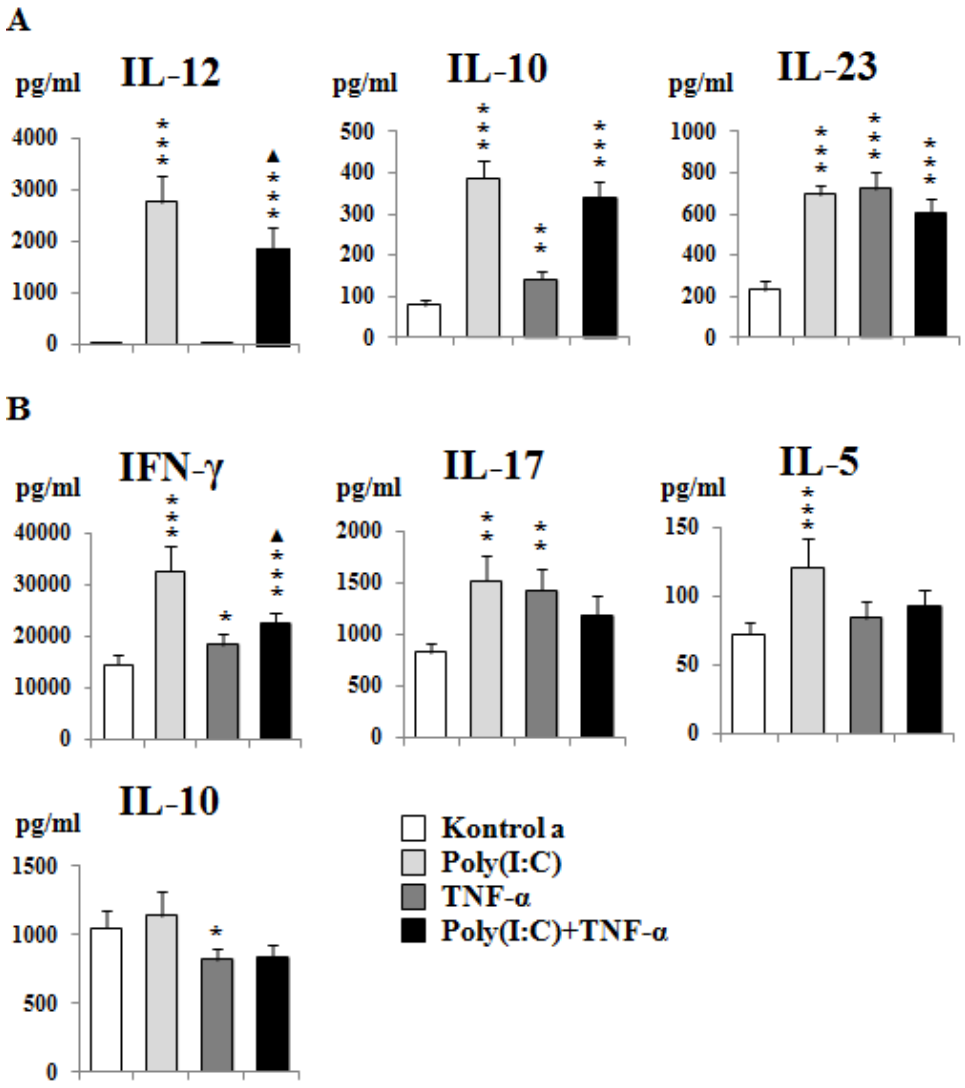
Na grafikonu je prikazan alostimulatorni potencijal MoDĆ diferenciranih u kontrolnom medijumu (kontrolne MoDĆ) ili u prisustvu Poly (I:C), TNF-α i njihove kombinacije. MoDĆ su kultivisane su u opadajućim dvostrukim razblaženjima ($1x10^4$-$0.125x10^4$) sa alogenim $CD4^+$ T limfocitima ($1x10^5$) u toku 5 dana u 200 µl kompletnog medijuma. U medijum za kultivaciju ćelija 18h pre merenja proliferativnog odgovora je dodat [H^3] timidin. Proliferativan odgovor je izražen kao broj otkucaja u minuti (engl. counts per minute, cpm).
Na grafiku su prikazani rezultati jednog reprezentativnog od šest sličnih eksperimenata kao srednja vrednost cpm triplikata kulture ± SD.
* $p<0.05$; *** $p<0.005$ u poređenju sa kontrolnim MoDĆ
▲ $p<0.05$ u poređenju sa MoDĆ tretiranim sa Poly (I:C)

MoDĆ kultivisane u prisustvu Poly (I:C) su stimulisale proliferaciju alogenih $CD4^+$ T limfocita. Sa druge strane, MoDĆ koje su kultivisane u prisustvu TNF-α su imale slab alostimulatorni potencijal. MoDĆ tretirane kombinacijom Poly (I:C) i TNF-α su inhibirale proliferaciju alogenih $CD4^+$ T limfocita, pogotovo pri višim odnosima (1:10, 1:20 i 1:40) MoDĆ i $CD4^+$ T limfocita, u poređenju sa efektom Poly (I:C).

4.4.3. Produkcija citokina od strane MoDĆ stimulisanih kombinacijom optimalne koncentracije Poly (I:C) i TNF-α

Produkcija IL-12, IL-23 i IL-10 u kulturama MoDĆ stimulisanih kombinacijom optimalne koncentracije Poly (I:C) i TNF-α prikazana je na *Grafikonu 13A*. Stimulacija MoDĆ sa Poly (I:C) pospešila je produkciju IL-12, IL-23 i IL-10. Tretman MoDĆ sa TNF-α je stimulisao produkciju IL-23 i IL-10. Kombinovani efekat Poly (I:C) i TNF-α na produkciju citokina od strane MoDĆ se nije značajnije razlikovao od efekta samog Poly (I:C) sem u pogledu produkcije IL-12 koja je bila smanjena.

Uticaj kombinovane primene Poly (I:C) i TNF-α na sposobnost MoDĆ da usmere imunski odgovor procenjen je na osnovu produkcije citokina IFN-γ, IL-5, IL-10 i IL-17 u kokulturama alogenih $CD4^+$ T limfocita i MoDĆ. Rezultati su predstavljeni na *Grafikonu 13B* kao srednje vrednosti koncentracija, izraženih u ng/ml. MoDĆ stimulisane sa TNF-α stimulisale su povećanje produkcije IFN-γ i IL-17 i smanjenje produkcije IL-10 od strane $CD4^+$ T ćelija u alogenoj kulturi. Efekat kombinovane primene Poly (I:C) i TNF-α na Th polarizujući potencija MoDĆ se ogledao u smanjenju produkcije svih ispitivanih citokina, pri čemu je inhibitorni uticaj bio najizraženiji u pogledu produkcije IFN-γ.

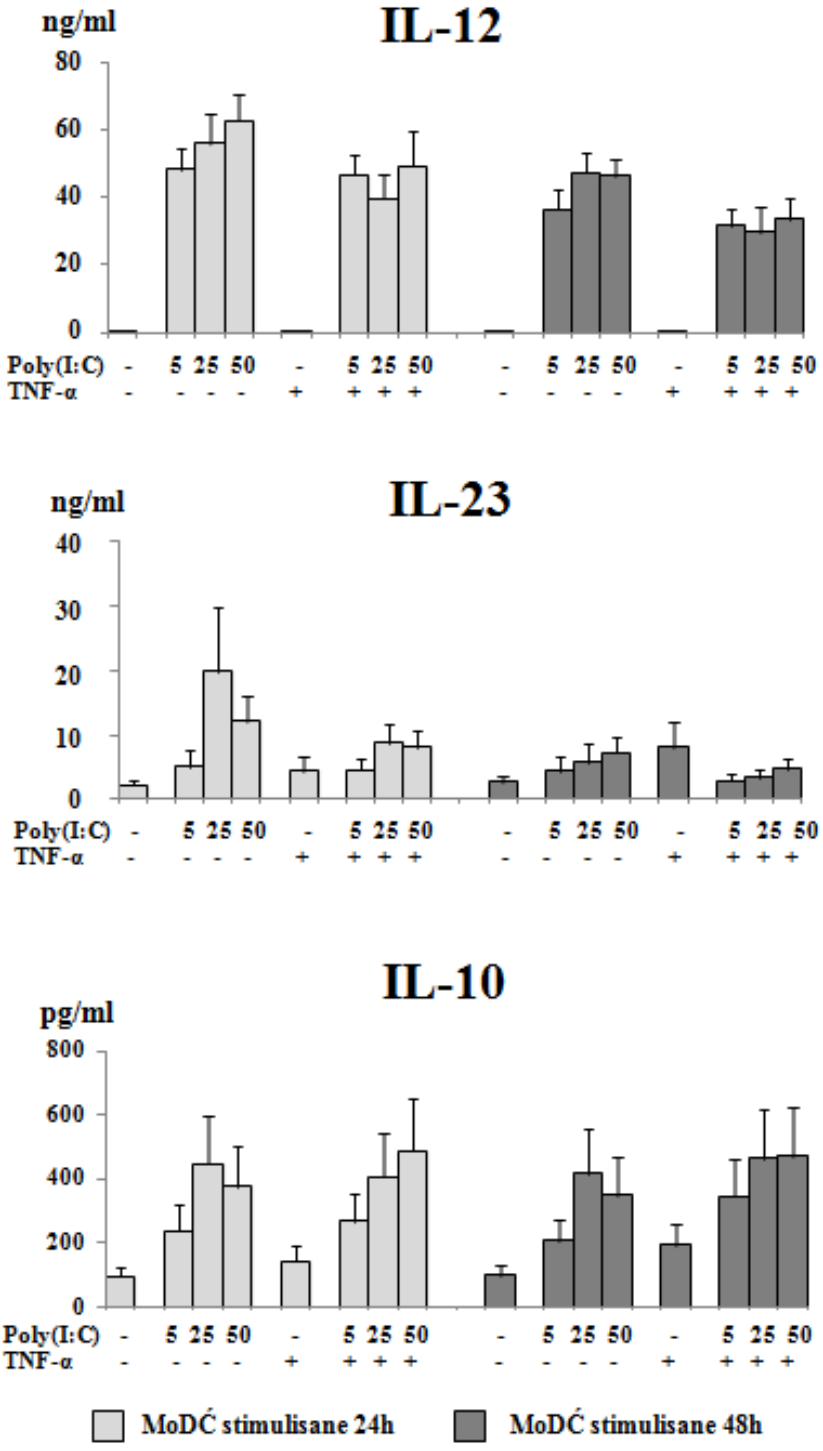


Grafikon 13. Uticaj kombinovane primene Poly (I:C) i TNF-α na produkciju citokina i Th polarizujući kapacitet MoDĆ

MoDĆ su dobijene iz humanih monocita nakon šestodnevne kultivacije u prisustvu GM-CSF (100 ng/ml) i IL-4 (20 ng/ml) i stimulacije sa optimalnom koncentracijom Poly (I:C), TNF-α i njihovom kombinacijom tokom 48h. A)Koncentracije IL-12, IL-23 i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α i njihove kombinacije, kao i u kontrolnom medijumu tokom 2 dana. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ±SD. B)Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracije IFN-γ, IL-5, IL-10 i IL-17 u supernatantima kokultura merene su ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* $p<0.05$; ** $p<0.01$; *** $p<0.005$ u poređenju sa kontrolnim MoDĆ
▲ $p<0.05$ u poređenju sa MoDĆ tretiranim sa Poly (I:C)

4.4.4. Dozno- i vremenski- zavisan efekat kombinacije Poly (I:C) i TNF-α na produkciju citokina od strane MoDĆ

Sledeći cilj istraživanja bilo je ispitivanje dozno- i vremenski-zavisnog efekta Poly (I:C) primenjenog u tri koncentracije (5 μg/ml, 25 μg/ml i 50 μg/ml), TNF-α i njihove kombinacije na produkciju citokina od strane MoDĆ. Rezultati su prikazani na *Grafikonu 14.*



Grafikon 14. Produkcija citokina od strane MoDĆ diferenciranih u prisustvu različitih koncentracija Poly (I:C), TNF-α i njihovih kombinacija

Koncentracije IL-12, IL-23 i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α i njihovih kombinacija, kao i u kontrolnom medijumu tokom 2 dana. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ±SD.

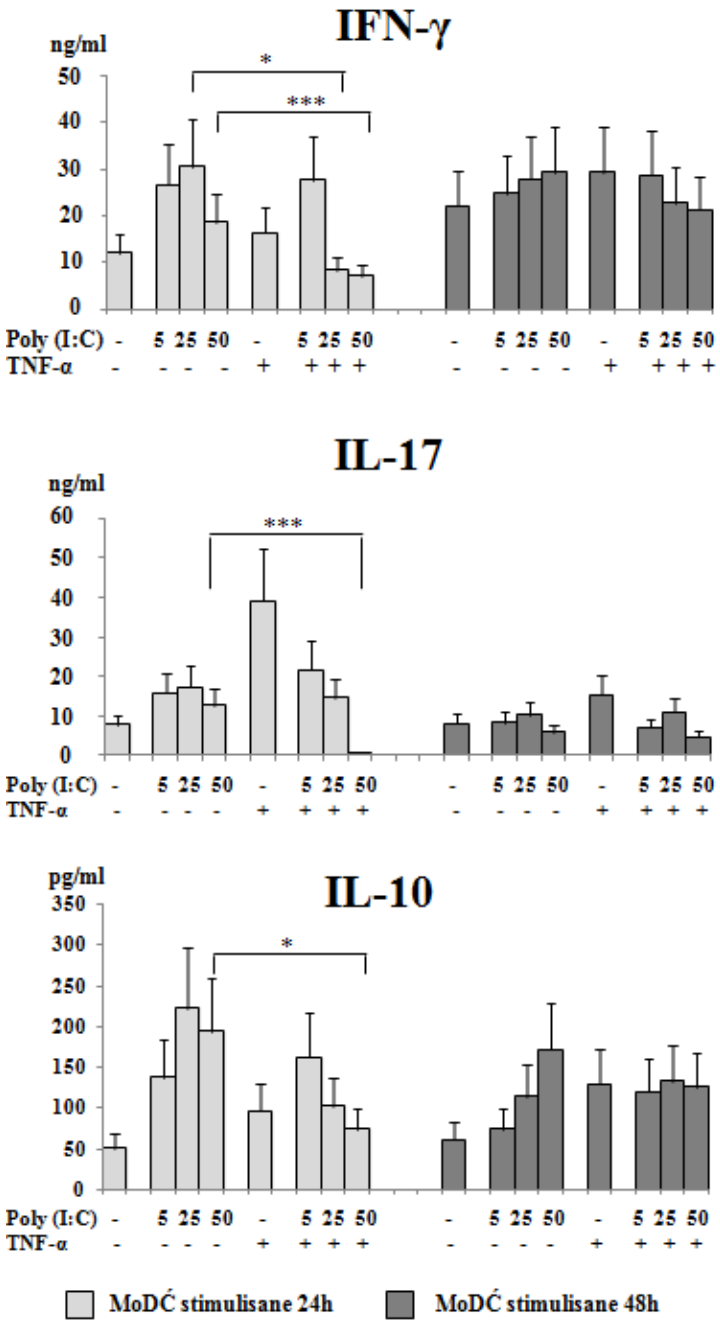
Poly (I:C) je ispoljio dozno-zavisni efekat na produkciju IL-12 posle stimulacije MoDĆ u toku 24h. Nakon prolongirane stimulacije MoDĆ sa Poly (I:C) u trajanju od 48h došlo je do smanjenja produkcije IL-12 i dozno-zavisni efekat više nije bio toliko izražen. Dodatak TNF-α tokom kultivacije MoDĆ stimulisanih sa Poly (I:C) je ispoljio inhibitorni efekat na produkciju IL-12, pogotovo kada su MoDĆ stimulisane višim koncentracijama Poly (I:C) (25 μg/ml i 50 μg/ml).

Poly (I:C) je takođe stimulisao povećanje produkcije IL-23 od strane MoDĆ. Ovaj efekat je bio izraženiji nakon 24h stimulacije MoDĆ sa Poly (I:C), a maksimum stimulacije je postignut u prisustvu koncentracije 25 μg/ml. Stimulacija MoDĆ kombinacijom Poly (I:C) i TNF-α je dovela do smanjenja produkcije IL-23 nakon 24h i 48h kultivacije sa tim da je efekat bio izraženiji ukoliko su primenjene više koncentracije Poly (I:C).

Kultivacija MoDĆ u prisustvu Poly (I:C) i TNF-α je dovela do povećanja produkcije IL-10 bez značajne razlike nakon 24h i 48h kultivacije. Više koncentracije Poly (I:C) (25 μg/ml i 50 μg/ml) su se pokazale kao snažniji stimulatori produkcije IL-10 od strane MoDĆ.

4.4.5. Dozno- i vremenski- zavisan efekat kombinovane primene Poly (I:C) i TNF-α na Th polarizacionu aktivnost MoDĆ

Dozno- i vremenski- zavisan efekat kombinovane primene Poly (I:C) i TNF-α na sposobnost MoDĆ da usmere imunski odgovor procenjen je na osnovu nivoa citokina IFN-γ, IL-17 i IL-10 u supernatantima kultura alogenih $CD4^+$ T limfocita i MoDĆ postupkom koji je detaljno opisan u poglavlju Materijal i metode. Rezultati su prikazani na *Grafikonu 15* kao srednje vrednosti koncentracija, izraženih u pg/ml, iz šest reprezentnativnih eksperimenta.



Grafikon 15. Citokinski profil $CD4^+$ T limfocita stimulisanih sa MoDĆ diferenciranim u prisustvu različitih koncentracija Poly (I:C), TNF-α i njihovih kombinacija

Nezrele MoDĆ ($1x10^4$) su kultivisane 24h u kontrolnom medijumu ili u prisustvu Poly (I:C), TNF-α ili njihovih kombinacija tokom narednih 24h ili 48h. Nakon 2 dana kultivacije MoDĆ tretirane na opisan način su potom kultivisane sa sortiranim alogenim $CD4^+$ T limfocitima ($1x10^5$) tokom 5 dana, u 200 µl kompletnog medijuma. Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracija citokina merena je ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* $p<0.05$; *** $p<0.005$ u odnosu na odgovarajuće kontrole

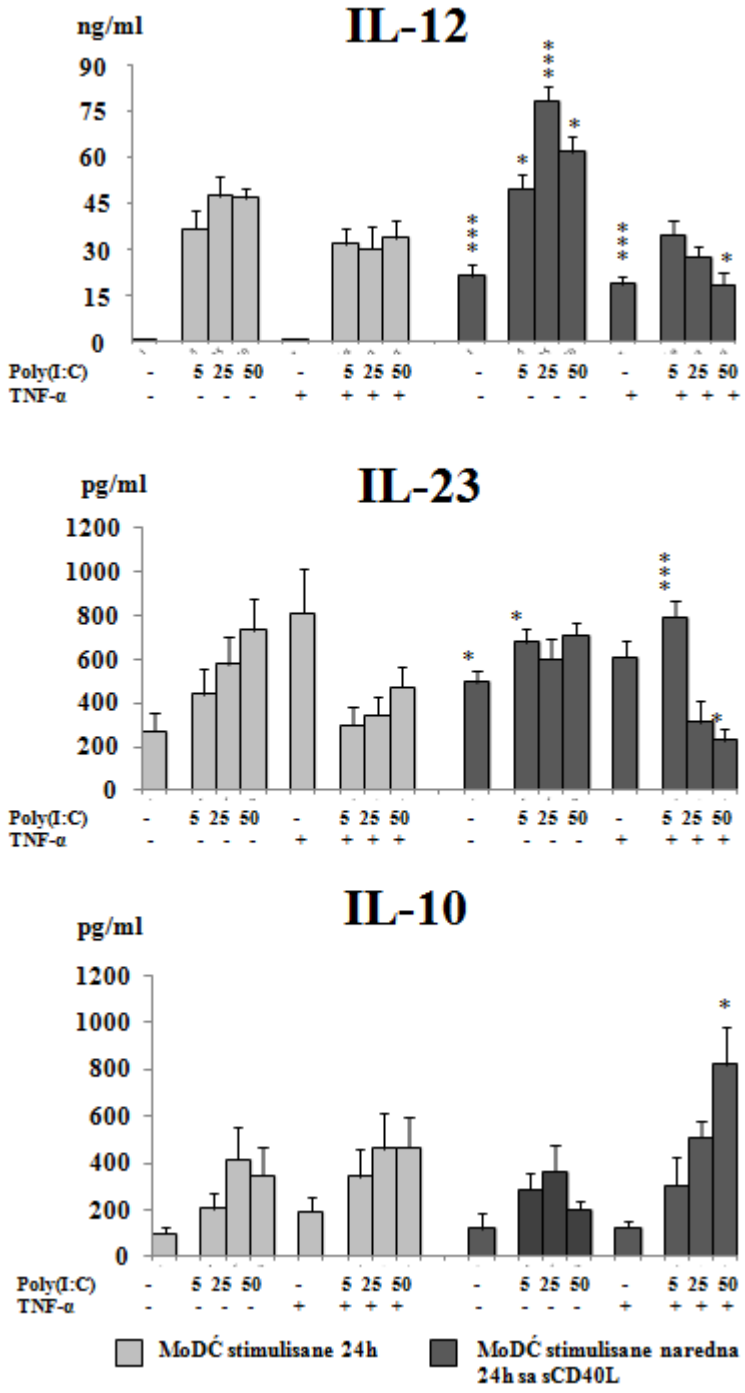
MoDĆ koje su stimulisane nižim koncentracijama Poly (I:C) (5 μg/ml i 25 μg/ml) tokom 24h su snažnije stimulisale produkciju IFN-γ od strane alogenih $CD4^+$ T limfocita u poređenju sa najvišom koncentracijom Poly (I:C) (50 μg/ml). Ove razlike nisu uočene nakon stimulacije MoDĆ sa Poly (I:C) tokom 48h. Kada su MoDĆ stimulisane sa TNF-α i Poly (I:C) tokom 24h uočeno je značajno smanjenje produkcije IFN-γ u kokulturama sa 25 μg/ml i 50 μg/ml Poly (I:C). U kokulturama u kojima su MoDĆ stimulisane u prisustvu oba agonista tokom 48h nisu uočene statistički značajne razlike u pogledu produkcije IFN-γ kod svih primenjenih koncentracija Poly (I:C).

MoDĆ kultivisane u prisustvu TNF-α tokom 24h su bile bolji stimulatori produkcije IL-17 u kokulturi, u poređenju sa MoDĆ tretiranim svim ispitivanim koncentracijama Poly (I:C). MoDĆ stimulisane najvišom koncentracijom Poly (I:C) u kombinaciji sa TNF-α tokom 24h su dovele do gotovo kompletnog sniženja produkcije IL-17 od strane alogenih $CD4^+$ T limfocita.

Produkcija IL-10 u kokulturama je pratila isti trend kao i produkcija IFN-γ, uključujući i uticaj MoDĆ stimulisanih najvišom koncentracijom Poly (I:C) (50 μg/ml) u kombinaciji sa TNF-α tokom 24h na smanjenje produkcije IL-10. Sa druge strane, MoDĆ tretirane svim ispitivanim koncentracijama Poly (I:C) i TNF-α i njihovim kombinacijama tokom 48h nisu ispoljile ovakav efekat na nivo produkovanog IL-10 od strane alogenih $CD4^+$ T limfocita.

4.4.6. Efekat povezivanja CD40 molekula na produkciju citokina i Th polarizacionu aktivnost MoDĆ pretretiranih kombinacijom Poly (I:C) i TNF-α

Sledeći korak u našem istraživanju je bio ispitivanje modulacije funkcije MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α i njihovih kombinacija tokom interakcije sa T limfocitima posredovanoj CD40 molekulom. MoDĆ kultivisane u prisustvu Poly (I:C), TNF-α i njihovih kombinacija tokom 24h su stimulisane solubilnim CD40L tokom naredna 24h, postupkom koji je detaljno opisan u poglavlju Materijal i metode. Rezultati su prikazani na *Grafikonu 16.*



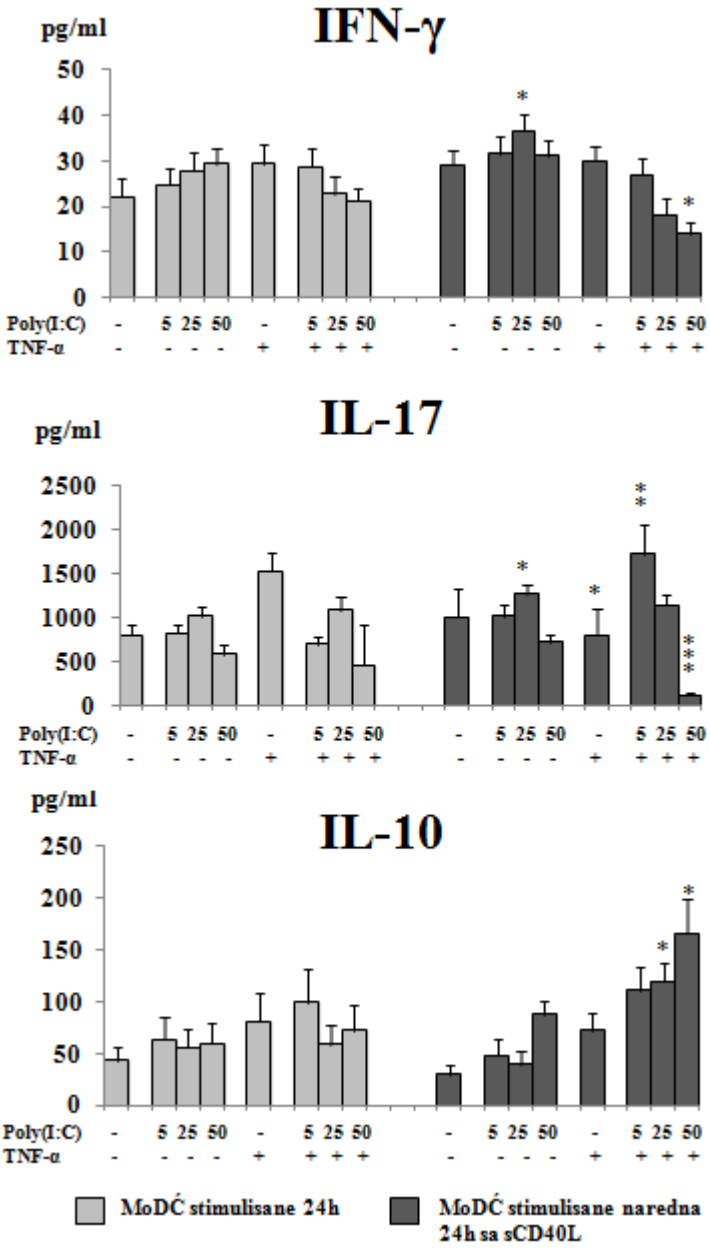
Grafikon 16. Uticaj povezivanja CD40 molekula na produkciju citokina od strane MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α i njihovih kombinacija

Koncentracije IL-12, IL-23 i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α, njihovih kombinacija i sCD40L, kao i u kontrolnom medijumu postupkom koji je detaljno opisan u poglavlju Materijali i metode. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
* $p<0.05$; *** $p<0.005$ u odnosu na odgovarajuće kontrole

Uticaj interakcije CD40:CD40L na produkciju citokina od strane MoDĆ pretretiranih sa Poly (I:C), TNF-α i njihovom kombinacijom je bio najizraženiji u slučaju produkcije IL-12. Naime, nivoi IL-12 su bili povišeni u kulturama MoDĆ tretiranih sa sve tri primenjene koncentracije Poly (I:C) i samo sa TNF-α. Međutim, do povećanja produkcije IFN-γ je došlo samo u kokulturama alogenih $CD4^+$ T limfocita i MoDĆ stimulisanih optimalnom koncentracijom Poly (I:C) (25 μg/ml) (*Grafikon 17*). Kombinacija TNF-α i najviše koncentracije Poly (I:C) je dovela do značajnog smanjenja produkcije IL-12 što se odrazilo na smanjenje produkcije IFN-γ od strane alogenih $CD4^+$ T limfocita kokultivisanih sa MoDĆ tretiranim ovom kombinacijom.

Povezivanje CD40 molekula na MoDĆ dovelo je do povećanja produkcije IL-23 u kulturama nezrelih kao i MoDĆ stimulisanih suboptimalnom koncentracijom Poly (I:C) (5μg/ml). Stimulacija MoDĆ samo sa optimalnom koncentracijom Poly (I:C) i samo sa TNF-α nije dovela do promene u produkciji IL-23, ali je u kokulturi ovih ćelija sa $CD4^+$ T limfocitima došlo do povećanja produkcije IL-17. Interakcija CD40 i CD40L je pospešila produkciju IL-23 od strane MoDĆ diferenciranih u prisustvu najniže ispitivane koncentracije Poly (I:C) i TNF-α što je bilo praćeno povećanjem produkcije IL-17 u kokulturi sa $CD4^+$ T limfocitima. Povezivanje CD40 molekula na MoDĆ pretretiranim najvišom ispitivanom koncentracijom Poly (I:C) i TNF-α je dovelo do smanjenja produkcije IL-23 što se odrazilo na značajno smanjenje produkcije IL-17 u kokulturi MoDĆ stimulisanih na ovaj način i alogenih $CD4^+$ T limfocita.

Povezivanje CD40 molekula na MoDĆ nije dovelo do značajne promene u pogledu produkcije IL-10 sa izuzetkom MoDĆ pretretiranih najvišom ispitivanom koncentracijom Poly (I:C) i TNF-α. Naime, u ovim kulturama je detektovano značajno povećanje nivoa IL-10. Ovaj efekat se ispoljio i u kokulturi ovih ćelija sa alogenim $CD4^+$ T limfocitima. Povećanje produkcije IL-10 je uočeno i u alogenoj kulturi MoDĆ stimulisanih srednjom ispitivanom koncentracijom Poly (I:C) i TNF-α.



Grafikon 17. Citokinski profil u kokulturi CD4$^+$ T limfocita i MoDĆ diferenciranih u prisustvu Poly (I:C), TNF-α, njihovih kombinacija i sCD40L

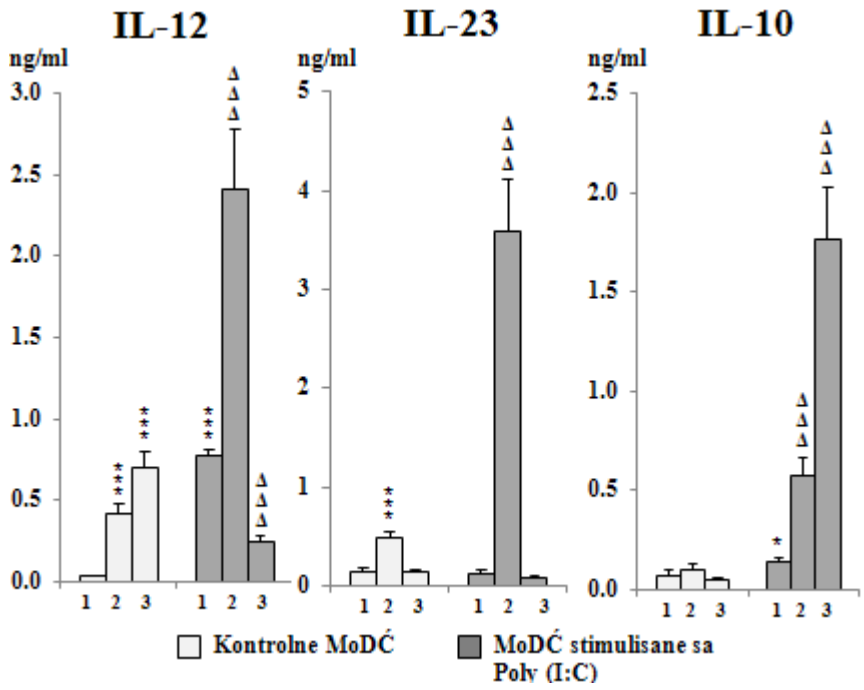
Nezrele MoDĆ (1x10^4) su kultivisane 24h u kontrolnom medijumu ili u prisustvu Poly (I:C), TNF-α ili njihove kombinacije i tokom naredna 24h u prisustvu solubilnog CD40L. Nakon 2 dana kultivacije MoDĆ tretirane na opisan način su potom kultivisane sa sortiranim alogenim CD4$^+$ T limfocitima (1x10^5) tokom 5 dana, u 200 µl kompletnog medijuma. Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracija citokina merena je ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ±SD.
* p<0.05; ** p<0.01; *** p<0.005 u odnosu na odgovarajuće kontrole

4.5. Uticaj IFN-γ i povezivanja CD40 molekula na funkcionalne karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C)

Ispitivanje modulacije funkcije MoDĆ tokom interakcije sa T limfocitima posredovanoj CD40 molekulom i IFN-γ je bio sledeći cilj našeg istraživanja. Nezrele MoDĆ i MoDĆ koje su stimulisane optimalnom koncentracijom Poly (I:C) tokom 24h su kultivisane naredna 24h u prisustvu J558 ćelija transfektovanim sa CD40L, odnosno u prisustvu IFN-γ.

4.5.1. Uticaj IFN-γ i povezivanja CD40 molekula na produkciju citokina od strane MoDĆ stimulisanih sa Poly (I:C)

U supernatantima kultura MoDĆ diferenciranih u prisustvu optimalne koncentracije Poly (I:C) ili u medijumu, sa ili bez J558 ćelija odnosno IFN-γ, su određeni nivoi IL-10, IL-12 i IL-23. Rezultati prikazani na *Grafikonu 18* pokazuju da su nezrele MoDĆ produkovale veoma niske nivoe sva tri ispitivana citokina. MoDĆ diferencirane u prissustvu Poly (I:C) su u poređenju sa nezrelim MoDĆ produkovale povećane nivoe IL-10 i IL-12, dok je nivo IL-23 bio nepromenjen. Povezivanje CD40 molekula na nezrelim MoDĆ je bilo praćeno povećanjem produkcije IL-12 i IL-23. Nasuprot tome, aktivacija CD40 molekula na MoDĆ pretretiranim sa Poly (I:C) je dovela do dvostrukog povećanja produkcije IL-12, dvadesetostrukog povećanja produkcije IL-23 i trostrukog povećanja produkcije IL-10. Kultivacija nezrelih MoDĆ u prisustvu IFN-γ je stimulisala produkciju IFN-γ na isti način kao i tretman MoDĆ sa Poly (I:C), a nivoi IL-23 i IL-10 su ostali nepromenjeni. Dodatak IFN-γ kulturama MoDĆ pretretiranih sa Poly (I:C) je doveo do smanjenja produkcije IL-12 i sedmostrukog povećanja produkcije IL-10.

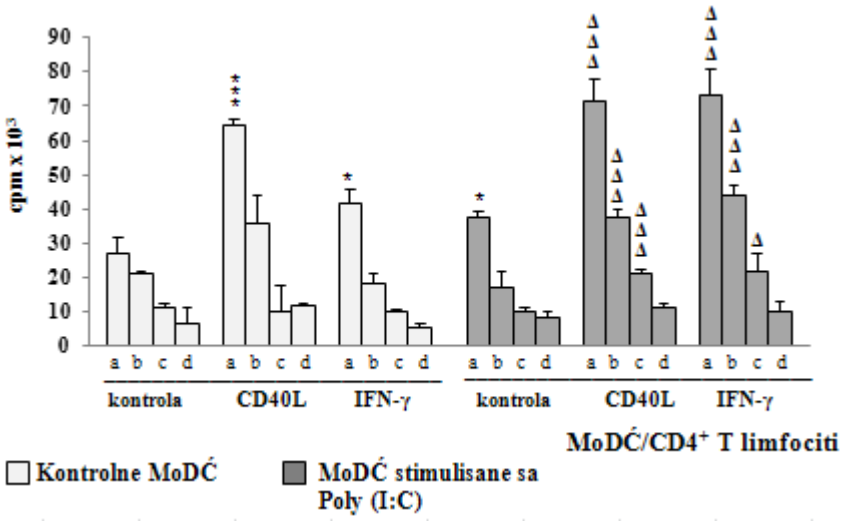


Grafikon 18. Uticaj IFN-γ i povezivanja CD40 molekula na produkciju citokina od strane MoDĆ diferenciranih bez i u prisustvu Poly (I:C)

Koncentracije IL-12, IL-23 i IL-10 određivane su ELISA testovima u superantantima kultura MoDĆ diferenciranih u prisustvu optimalne koncentracije Poly (I:C) ili u medijumu, sa ili bez J558 ćelija odnosno IFN-γ (5 ng/ml), kao i u kontrolnom medijumu tokom 2 dana. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ± SD.
1-kontrola; 2-CD40L; 3- IFN-γ
* p<0.05; *** p<0.005 u poređenju sa kontrolnim MoDĆ
$^{\Delta\Delta\Delta}$ p<0.005 u poređenju sa MoDĆ tretiranim sa Poly (I:C)

4.5.2. Uticaj IFN-γ i povezivanja CD40 molekula na alostimulatornu aktivnost MoDĆ diferenciranih u prisustvu Poly (I:C)

Proliferativan odgovor alogenih $CD4^+$ T limfocita u kokulturama sa MoDĆ diferenciranih u prisustvu optimalne koncentracije Poly (I:C) ili u medijumu, sa ili bez J558 ćelija odnosno IFN-γ prikazan je na *Grafikonu 19.*



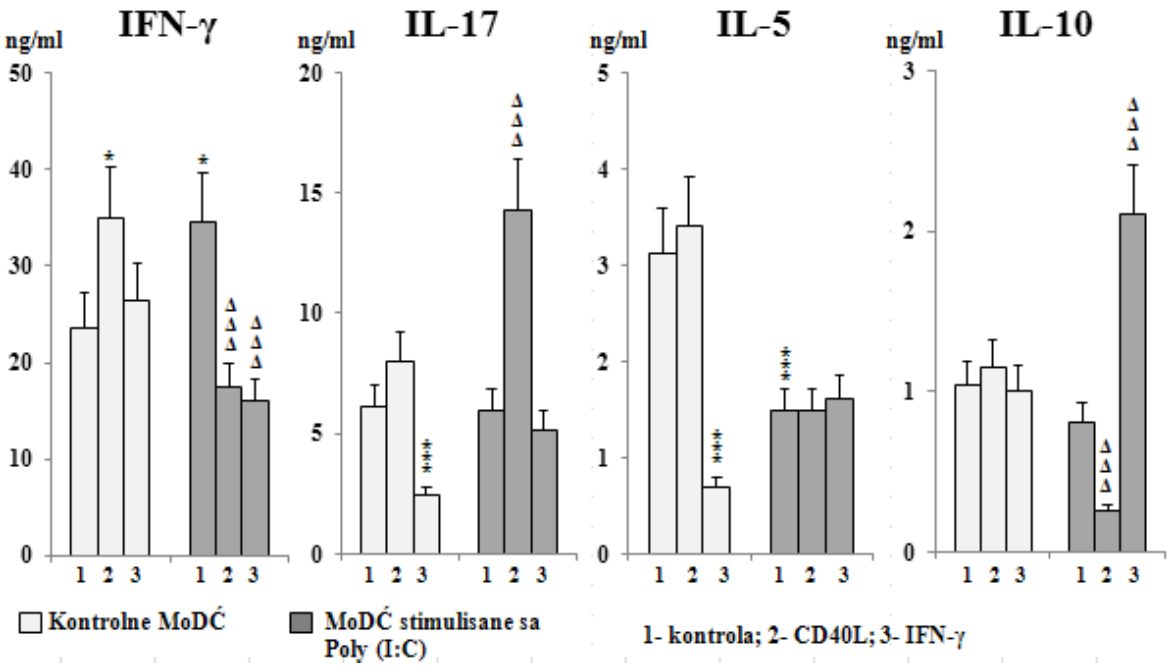
Grafikon 19. Uticaj IFN-γ i povezivanja CD40 molekula na alostimulatorni kapacitet MoDĆ diferenciranih bez i u prisustvu Poly (I:C)

Na grafikonu je prikazan alostimulatorni potencijal MoDĆ diferenciranih u medijumu (kontrolne MoDĆ) ili u prisustvu Poly (I:C), sa ili bez J558 ćelija odnosno IFN-γ. MoDĆ su kultivisane su u opadajućim dvostrukim razblaženjima (1x104-0.125x104) sa alogenim $CD4^+$ T limfocitima (1x105) u toku 5 dana u 200 µl kompletnog medijuma. U medijum za kultivaciju ćelija 18h pre merenja proliferativnog odgovora je dodat [H^3] timidin. Proliferativan odgovor je izražen kao broj otkucaja u minuti (engl. counts per minute, cpm). Na grafiku su prikazani rezultati jednog reprezentativnog od šest sličnih eksperimenata kao srednja vrednost cpm triplikata kulture ± SD.
a-1:10; b-1:20; c-1:40; d-1:80
* $p<0.05$; *** $p<0.005$ u poređenju sa kontrolnim MoDĆ
$^{\Delta}p<0.05$; $^{\Delta\Delta\Delta}p<0.005$ u poređenju sa MoDĆ tretiranim sa Poly (I:C)

Nezrele MoDĆ su pokazale umereni potencijal za stimulaciju proliferacije alogenih $CD4^+$ T limfocita koji je progresivno opadao sa povećanjem odnosa MoDĆ/$CD4^+$ T limfocita. MoDĆ diferencirane u prisustvu Poly (I:C) su imale najvišu alostimulatornu aktivnost pri najvećem odnosu MoDĆ/$CD4^+$ T limfocita. Povezivanje CD40 molekula na nezrelim MoDĆ ili tretman MoDĆ sa IFN-γ je doveo do značajnog povećanja njihovog alostimulatornog potencijala. Aktivacija CD40 molekula na MoDĆ kultivisanih u prisustvu Poly (I:C) odnosno dodatak IFN-γ ovim kulturama su doveli do dodatnog povećanja proliferacije alogenih $CD4^+$ T limfocita.

4.5.3. Uticaj IFN-γ i povezivanja CD40 molekula na Th polarizacionu aktivnost MoDĆ diferenciranih u prisustvu Poly (I:C)

Efekti povezivanja CD40 molekula i IFN-γ na Th polarizacionu aktivnost kontrolnih i MoDĆ tretiranih optimalnom koncentracijom Poly (I:C) prikazani su na *Grafikonu 20.*



Grafikon 20. Uticaj IFN-γ i povezivanja CD40 molekula na Th polarizacionu aktivnost MoDĆ diferenciranih bez i u prisustvu Poly (I:C)

Nezrele MoDĆ ($1x10^4$), kultivisane 2 dana u prisustvu Poly (I:C) ili u medijumu, sa ili bez J558 ćelija odnosno IFN-γ, su potom kultivisane sa $CD4^+$ sortiranim alogenim T limfocitima ($1x10^5$) tokom 5 dana, u 200 μl kompletnog medijuma. Forbol-12-miristat-13-acetat (PMA) (20ng/ml) i jonomicin (500 ng/ml) su dodati 8h pre isteka kulture. Koncentracija citokina merena je ELISA testovima i metodom za detekciju citokina pomoću imunofluorescentnih kuglica i protočne citofluorimetrije. Rezultati su predstavljeni kao srednja vrednost koncentracija iz šest eksperimenata sa različitim donorima ±SD.
*p<0.05; *** p<0.005 u poređenju sa kontrolnim MoDĆ
$^{\Delta\Delta\Delta}$ p<0.005 u poređenju sa MoDĆ tretiranim sa Poly (I:C)

Stimulacija MoDĆ optimalnom koncentracijom Poly (I:C) je dovela do povećanja produkcije IFN-γ i smanjenja produkcije IL-5, dok su nivoi IL-17 i IL-10 ostali nepromenjeni. Aktivacija CD40 molekula na nezrelim MoDĆ je ispoljila sličan efekat kao tretman sa Poly (I:C), sa tim da nije došlo do promene nivoa IL-5. Nasuprot tome, aktivacija CD40 molekula na MoDĆ koje su pretretirane sa Poly (I:C)

dovela je do povećanja produkcije IL-17 kao i sniženja nivoa IFN-γ i IL-10. Prisustvo IFN-γ u kulturama nezrelih MoDĆ je dovelo do smanjenja produkcije IL-17 i IL-5 i nije uticalo na produkciju IFN-γ od strane ovih ćelija. Dodatna stimulacija sa IFN-γ MoDĆ pretretiranih sa Poly (I:C) je dovela do značajnog povećanja produkcije IL-10 i smanjenja nivoa IFN-γ dok su nivoi IL-17 i IL-5 ostali nepromenjeni.

5. DISKUSIJA

Zahvaljujući svojoj jedinstvenoj ulozi u stimulaciji specifičnog imuniteta, uključujući i razvoj anti-tumorskog imunskog odgovora, DĆ predstavljaju jedno od najznačajnijih savremenih oruđa u imunoterapiji tumora. Uprkos tome što je sproveden veliki broj studija u kojima je opisano korišćenje DĆ za terapiju različitih vrsta malignih tumora još uvek nisu standardizovani protokoli za pripremu DĆ za kliničku upotrebu. Za poboljšanje protokola imunoterapije tumora primenom DĆ neophodna su dalja temeljna proučavanja imunobiologije DĆ, postupaka za produkciju ovih ćelija, kao i protokola za njihovu stimulaciju.

Kako je procenat DĆ u perifernoj krvi veoma mali (oko 0,5% od ukupnih mononuklearnih ćelija) usavršavanjem postupaka za izolaciju DĆ i njihovo nastajanje u *in vitro* uslovima od prekursora razvijeni su različiti načini pripreme ovih ćelija. Najpogodniji metod u pogledu jednostavnosti i velikog prinosa DĆ u humanom sistemu je dobijanje DĆ monocitnog porekla kultivacijom adherentne frakcije mononuklearnih ćelija iz *buffy coat*-a u prisustvu GM-CSF i IL-4 (Sallusto i Lanzavecchia, 1994). Ovaj metod predstavlja pogodan model za ispitivanje uticaja različitih faktora na diferencijaciju i maturaciju DĆ u *in vivo* uslovima (Zou i Tam, 2002). Stoga smo ovaj metod koristili u našim istraživanjima. U prethodnoj studiji naše istraživačke grupe (Colic i sar., 2003) kao i u brojnim publikacijama (Verdijk i sar., 1999; Lapointe i sar., 2000; Rouas i sar., 2004; Roses i sar., 2008) je pokazano da su MoDĆ dobijene na ovaj način nezrele, sa umerenim potencijalom da stimulišu proliferaciju alogenih $CD4^+$ T limfocita u mešanoj lekocitnoj kulturi i polarizuju imunski odgovor ka Th1 i Th17 pravcu. Ove karakteristike *in vitro* dobijenih nezrelih MoDĆ su u skladu sa saznanjima da nezrele MoDĆ imaju slab potencijal da stimulišu imunski odgovor što ograničava njihovu primenu u kliničkim ispitivanjima, pogotovo kao anti-tumorskih vakcina (McIlroy i Gregoire, 2003) .

Biološki potencijal DĆ za *in vivo* stimulaciju anti-tumorskog imunskog odgovora još uvek nije maksimalno iskorišćen. Zbog toga je značajan cilj imunoterapije tumora poboljšanje protokola za stimulaciju DĆ u cilju stvaranja dovoljnog broja imunogenih DĆ stabilnih fenotipskih karakteristika koje produkuju visok nivo IL-12, ključnog citokina za stimulaciju efikasnog Th1 odgovora i nastanak citotoksičnih T limfocita koji su od presudnog značaja za efikasnu

imunoterapiju tumora (Ikeda i sar., 2002; DeVries i sar., 2003). DĆ se odlikuju izuzetnom funkcionalnom plastičnošću koja zavisi od velikog broja faktora uključujući vrstu patogena sa kojima se sreću, receptora za prepoznavanje molekulskih obrazaca patogena koji se aktiviraju, subpopulaciju DĆ, mikrosredinu u kojoj se DĆ nalaze, ka i povratne signale poreklom od T ćelija (Banchereau i sar., 2000). U tom kontekstu, sposobnost DĆ da primaju signale sa različitih receptora koje potom integrišu i realizuju kroz jedinstveni odgovor se sve više koristi za generisanje zrelih DĆ kombinovanom primenom različitih agonista PRR i citokina, što je i bio predmet ovog istraživanja. U ovom radu su ispitivane fenotipske i funkcionalne karakteristike humanih DĆ monocitnog porekla stimulisanih sa agonistima endozomnih TLR i to pretežno TLR3. Pored detaljnog proučavanja modulatornog svojstva TLR3 agoniste, ispitivani su i modulatorni efekti kombinacije TLR3 i TLR7 agonista, kombinacija agonista TLR3 i dektin-1 receptora kao i kombinacija TLR3 agoniste, liganda za CD40 molekula i citokina (TNF-α i IFN-γ).

5.1. Uticaj Poly (I:C) na diferencijaciju nezrelih humanih MoDĆ

Prvi korak u ovom istraživanju bilo je detaljno ispitivanje modulatornog efekta Poly (I:C) na fenotipska i i funkcionalna svojsta MoDĆ. Poly (I:C) je sintetski analog dvolančane RNK koji se vezuje za TLR3, PRR koji je značajno ispoljen na nezrelim MoDĆ (Muzio i sar., 2000). Ovaj TLR3 agonist oponaša molekulski obrazac patogena i po vezivanju za receptor signalizira prisustvo infektivnog agensa što je praćeno aktivacijom DĆ i pokretanjem mehanizama za zaštitu od virusne infekcije (Cella i sar., 1999b).

Poznato je da Poly (I:C) dovodi do fenotipskog sazrevanja MoDĆ koje se ogleda u povećanju ekspresije HLA-DR molekula, kostimulatornih (CD80, CD86 i CD40) i adhezivnih molekula (CD54) kao i markera maturacije (CD83) (Cella i sar., 1999b; Rouas i sar., 2004), što smo pokazali i u našem istraživanju. Navedeni molekuli koji se povećano eksprimiraju učestvuju u formiranju imunološke sinapse između DĆ i T limfocita preko koje se integrišu svi signali i obezbeđuje adekvatan imunski odgovor posredovan efektorskim T limfocitima (Grakoui i sar., 1999; Dustin

i sar., 2006). HLA-DR molekul zajedno sa kostimulatornim i adhezivnim molekulima učestvuje u prezentaciji antigena, prenosu aktivacionih signala i stabilizaciji interakcije DĆ i $CD4^+$ T limfocita (Dubey i sar., 1995; Vremec i Shortman, 1997; Banchereau i Steinman, 1998; Banchereau i sar., 2000; Lipscomb i Masten, 2002). HLA-DR prezentuju antigene $CD4^+$ T limfocitima, obezbeđujući antigensku specifičnost ćelijskog imunskog odgovora i prenose signal 1 za aktivaciju T limfocita. Za aktivaciju T limfocita neophodan je i kostimulatorni signal, signal 2, koga obezbeđuju CD80 i CD86. Stepen ekspresije CD80 i CD86 zavisi od stadijuma zrelosti DĆ i povećava se stepenom njihove zrelosti (Lutz i Schuler, 2002). Vezivanje CD80 i CD86 za odgovarajuće receptore na T limfocitima, CD28 ili CTLA-4 (CD152), je od ključnog značaja za aktivaciju ili regulaciju T ćelijskog imunskog odgovora, ali ostvaruje i povratne efekte na DĆ (Logue i Sha, 2004; Bhatia i sar., 2006). CD40 se konstitutivno eksprimira na površini DĆ, ali se njegova ekspresija veoma povećava po aktivaciji ćelija (Steinman i Hemmi, 2006). CD54 doprinosi povezivanju DĆ i T limfocita i obezbeđuje stabilan kontakt između T-ćelijskog receptora i MHC-peptid kompleksa (Hart i Prickett, 1993). Takođe, u ovom istraživanju smo pokazali da Poly (I:C) dovodi do povećanja ekspresije CCR7 na MoDĆ, što je još jedna odlika zrelih DĆ prethodno pokazana od strane Sallusto i saradnika (Sallusto i sar., 1998). Specifični ligandi CCR7 molekula, CCL19 i CCL21, eksprimirani su od strane endotelnih ćelija limfnih venula, u postkapilarnim venulama sa visokim endotelom u limfnim čvorovima i T ćelijskim zonama limfoidnih organa (Gunn i sar., 1998; Willimann i sar., 1998; Luther i sar., 2000). Dakle, CCL19 i CCL21 usmeravaju migraciju zrelih DĆ koje ispoljavaju CCR7 ka perifernim limfoidnim organima u kojima dolazi do njihove interakcije sa antigen specifičnim, naivnim ili memorijskim T lifocitima (Sozzani, 2005; Alvarez i sar., 2008; Lukacs-Kornek i sar., 2008; Förster i sar., 2008).

U cilju daljeg proučavanja uticaja Poly (I:C) na diferencijaciju MoDĆ ispitivana je njihova sposobnost da stimulišu proliferaciju alogenih $CD4^+$ T limfocita u MLR. Aloreaktivnost se zasniva na prepoznavanju alogenih epitopa peptid-MHC kompleksa na površini DĆ od strane T-ćelijskog receptora T limfocita genetski različitih jedinki iste vrste. Usled velike polimorfnosti MHC gena aloreaktivnost predstavlja jednu od najjačih imunskih reakcija. Stoga se alogena MLR koristi za

ispitivanje stimulatornog potencijala DĆ (Jeras i sar., 2005). Tokom MLR, DĆ i $CD4^+$ T limfociti stupaju u međusobne interakcije posredovane membranskim i solubilnim molekulima, što rezultuje odgovarajućim proliferativnim odgovorom T limfocita. T limfociti integrišu signale koji im omogućavaju aktivaciju sa T-ćelijskog receptora, kostimulatornih molekula i citokinskih receptora. U zavisnosti od jačine ukupnog signala, koji zavisi od broja DĆ, koncentracije antigena, aviditeta T-ćelijskog receptora za odgovarajući peptid-MHC kompleks i trajanja interakcije DĆ i T limfocita, T limfociti sukcesivno prolaze kroz procese proliferacije, diferencijacije i ćelijske smrti. Slab signal dovodi do proliferacije naivnih T limfocita koji se ne diferenciraju u efektorske ćelije, dok previše jak signal dovodi do aktivacijom indukovane ćelijske smrti (Langenkamp i sar., 2002; Sallusto i Lanzavecchia, 2002). Naši rezultati ukazuju na to da kontrolne, netretirane, MoDĆ imaju umeren potencijal za stimulaciju proliferacije alogenih $CD4^+$ T limfocita. Stimulacija MoDĆ sa Poly (I:C) je dovela do povećanja alostimulatornog kapaciteta MoDĆ i to pri nižim odnosima MoDĆ i alogenih $CD4^+$ T limfocita (1:40 i 1:80). Pri ostalim odnosima nije došlo do statistički značajne promene alostimulatornog kapaciteta. Povećani alostimulatorni potencijal MoDĆ stimulisanih sa Poly (I:C) je odraz opisanih fenotipskih karakteristika MoDĆ.

Rezultati dosadašnjih istraživanja su pokazali da MoDĆ kultivisane u prisustvu Poly (I:C) produkuju povišen nivo IL-12 i tako usmeravaju imunski odgovor u Th1 pravcu, kako *in vitro* (Verdijk i sar., 1999) tako i *in vivo* (Rouas i sar., 2004). Sa druge strane, produkuju nizak nivo IL-10, anti-inflamatornog citokina koji usmerava efektorske funkcije $CD4^+$ T limfocita ka Th2 (Taga i Tosato, 1992) i imunoregulatornom profilu (Smits i sar., 2005). Rezultati našeg istraživanja su saglasni sa prethodno publikovanim i dodatno je pokazano da MoDĆ stimulisane u prisustvu Poly (I:C) produkuju visoke nivoe IL-23 i IL-27, članova IL-12 familije, kao i proinflamatornih citokina, TNF-α i IL-6. Pored izrazite strukturne homologije članova IL-12 familije, svaki od ovih citokina ima sebi svojstvene funkcionalne karakteristike (Hunter, 2005). Stimulišući produkciju IFN-γ i pospešujući citolitičku aktivnost NK ćelija, IL-12 ima ključnu ulogu u aktivaciji ranog urođenog imunskog odgovora protiv intracelularnih patogena i predstavlja ključni faktor aktivacije

ćelijskog imunskog odgovora pospešivanjem diferencijacije $CD4^+$ T limfocita u Th1 limfocite (Steinman i Hemmi, 2006). Pored toga, IL-12 je od ključnog značaja za anti-tumorski imunski odgovor kao signal 3 za aktivaciju T limfocita (Valenzuela i sar., 2002) jer zajedno sa signalom 1 i signalom 2 omogućava polarizaciju imunskog odgovora u Th1 pravcu i razvoj tumor-specifičnih $CD8^+$ T limfocita sa citotoksičnim svojstvima (Xu i sar., 2003). IL-27 ima ulogu u potenciranju efekta IL-12 koju ostvaruje direktno, povećanjem ekspresije IL-12Rβ2 na površini ovih ćelija (Lucas i sar., 2003; Kamiya i sar., 2004) i indirektno, povećanjem ekspresije IL-18 od strane aktiviranih monocita, koji deluje sinergistički sa IL-12 u indukciji diferencijacije Th1 ćelija (Pflanz i sar., 2004). Sa druge strane, IL-23 indukuje proliferaciju memorijskih i efektorskih Th17 limfocita (McKenzie i sar., 2005; Sallusto i Lanzavecchia, 2009; Yu i sar., 2010). Postoje indikacije da IL-23, pored IL-12, može ispoljiti povoljne efekte u imunoterapiji tumora (Zheng i sar., 2008).

U skladu sa profilom produkovanih citokina MoDĆ tretirane sa Poly (I:C) su stimulisale Th1 i Th17 imunski odgovor, a inhibirale Th2 odgovor, što je procenjeno na osnovu produkcije IFN-γ, IL-17 i IL-5 u kokulturi sa alogenim $CD4^+$ T limfocitima. Ovi nalazi su u skladu sa konceptom recipročne regulacije Th1/Th2 odgovora (Glimcher i Murphy, 2000) i prethodno publikovanim rezultatima o uticaju dvolančane RNK na odnos Th1/Th2 odgovora (Benwell i sar., 2010). Funkcionalni značaj Th17 odgovora stimulisanog usled prisustva TLR3 agonista predstavlja novi fenomen koji bi trebalo dalje ispitivati.

5.2. Uticaj kombinovane primene Poly (I:C) i loksoribina na diferencijaciju nezrelih humanih MoDĆ

Sinergistički efekat kombinovane primene TLR agonista je predmet brojnih eksperimentalnih i kliničkih studija u kojima se traga za najboljim protokolima za produkciju DĆ vakcina u terapiji različitih tipova malignih tumora. Sprovedena su brojna istraživanja u cilju ispitivanja funkcionalnih i fenotipskih karakteristika MoDĆ nakon istovremene aktivacije većeg broja TLR (Napolitani i sar., 2005; Gautier, i sar., 2005; Warger i sar., 2006; Mäkelä i sar., 2009). Pokazano je da

kooperacija TLR kod DĆ stimuliše povećanje ekspresije gena za TNF-α, IL-1β, IL-6, IL-10, IL-12, IL-23 i ciklooksigenaze 2 (engl. cyclooxygenase-2, COX2) kao i njihovu sintezu i oslobađanje, u poređenju sa efektima pojedinačnih agonista (Gautier i sar., 2005; Napolitani i sar., 2005; Trinchieri i Sher, 2007). Napolitani i saradnici su među prvima opisali snažan sinergistički efekat kombinovane primene TLR agonista na produkciju citokina od strane MoDĆ. Rezultati njihovog istraživanja su pokazali sinergistički efekat kombinovane primene TLR3 i TLR4 agonista sa TLR7/8 agonistom u produkciji IL-12 i IL-23 od strane humanih MoDĆ koje su ispoljavale izraženiji Th1 polarizujući potencijal od MoDĆ stimulisanih sa pojedinačnim agonistima (Napolitani i sar., 2005).

TLR3, zajedno sa TLR7, TLR8 i TLR9 čine TLR9 subfamiliju receptora koja predstavlja endozomne TLR specifične za nukleinske kiseline. TLR7 i TLR8, receptori za koje se vezuje jednolančana RNK i određene male interferirajuće RNK, zajedno sa TLR3 aktiviraju urođeni imunski odgovor nakon infekcije virusima (Diebold i sar., 2004) (Heil i sar., 2003). Pored jednolančane RNK i pojedina sintetska jedinjenja, poput rezikvimoda, CL075 i CL097 predstavljaju TLR7/8 agoniste (Lee i sar., 2003). MoDĆ stimulisane ovim jedinjenjima se diferenciraju u zrele ćelije koje polarizuju imunski odgovor u Th1 pravcu (Duraisingham i sar., 2009).

U cilju razgraničavanja efekata aktivacije TLR7 i TLR8 receptora bilo je neophodno ispitati efekte primene selektivnih TLR7 i TLR8 agonista. Do sada su identifikovane dve grupe selektivnih TLR7 agonista, imidazokvinolini (imikvimod i gardikvimod) i derivati guanozina (7-alil-7,8-dihidro-8-okso-guanozin (loksoribin) i 7-tia-8-okso-guanozin (7-TOG)) (Lee i sar., 2003; Heil i sar., 2003). Podaci koji se odnose na efekte ovih agonista na MoDĆ su kontraverzni kao i pitanje ekspresije TLR7 u MoDĆ. Naime, ekspresija TLR7 je visoka u pDĆ (Gorden i sar., 2005), ali su nivoi ekspresije ovog receptora u MoDĆ niski ili nedetektabilni (Jarrossay i sar., 2001; Kadowaki i sar., 2001). Takođe, pokazano je da selektivni TLR7 ligandi mogu da stimulišu samo preaktivirane MoDĆ (Severa i sar., 2007; Lombardi i sar., 2009). Rezultati naših istraživanja u kojima smo koristili selektivni TLR7 agonist, loksoribin, su pokazali da primena loksoribina dovodi do diferencijacije, sazrevanja i stimulacije alostimulatorne sposobnosti MoDĆ uz polarizaciju ka Th1 i Th17

imunskom odgovoru, pri čemu su ovi efekti delom rezultat povećanja ekspresije TLR7 u tako tretiranim MoDĆ (Dzopalic i sar., 2010).

Imajući u vidu rezultate istraživanja Napolitani i saradnika (Napolitani i sar., 2005) želeli smo da primenom selektivnih agonista detaljno ispitamo na koji način koaktivacija TLR3 i TLR7 moduliše fenotipske karakteristike, produkciju citokina i Th polarizujući potencijal MoDĆ. MoDĆ su stimulisane sa kombinacijama suboptimalne (10 μg/ml) i optimalne (25 μg/ml) koncentracije Poly (I:C) i suboptimalne (34 μg/ml) i optimalne (85 μg/ml) koncentracije loksoribina.

Stimulisanje MoDĆ suboptimalnim koncentracijama Poly (I:C) i loksoribina je dovelo do njihovog diferenciranja u fenotipski zrele ćelije koje polarizuju imunski odgovor ka Th1 pravcu što je procenjeno na osnovu povećane produkcije IFN-γ u kokulturi ovako tretiranih MoDĆ i alogenih $CD4^+$ T ćelija. Povećanje produkcije IFN-γ u kokulturi se može objasniti povećanom produkcijom IL-27 i IL-12 od strane samih tretiranih MoDĆ, za koje je pokazano udruženo dejstvo u ekspanziji i preživljavanju Th1 ćelija (Lucas i sar., 2003; Kamiya i sar., 2004; Pflanz i sar., 2004; Owaki i sar., 2006). Ovako pripremljene MoDĆ su indukovale i povećanu produkciju IL-17 od strane $CD4^+$ T ćelija, što je u skladu sa detektovanim značajnim povećanjem produkcije IL-23 od strane MoDĆ stimulisanih sa kombinacijom suboptimalnih koncentracija oba agonista u poređenju sa vrednostima IL-23 koji su produkovale MoDĆ stimulisane pojedinačnim agonistima. Naime, poznato je da je IL-23 neophodan za održanje Th17 ćelija (McKenzie i sar., 2005; Sallusto i Lanzavecchia, 2009; Yu i sar., 2010).

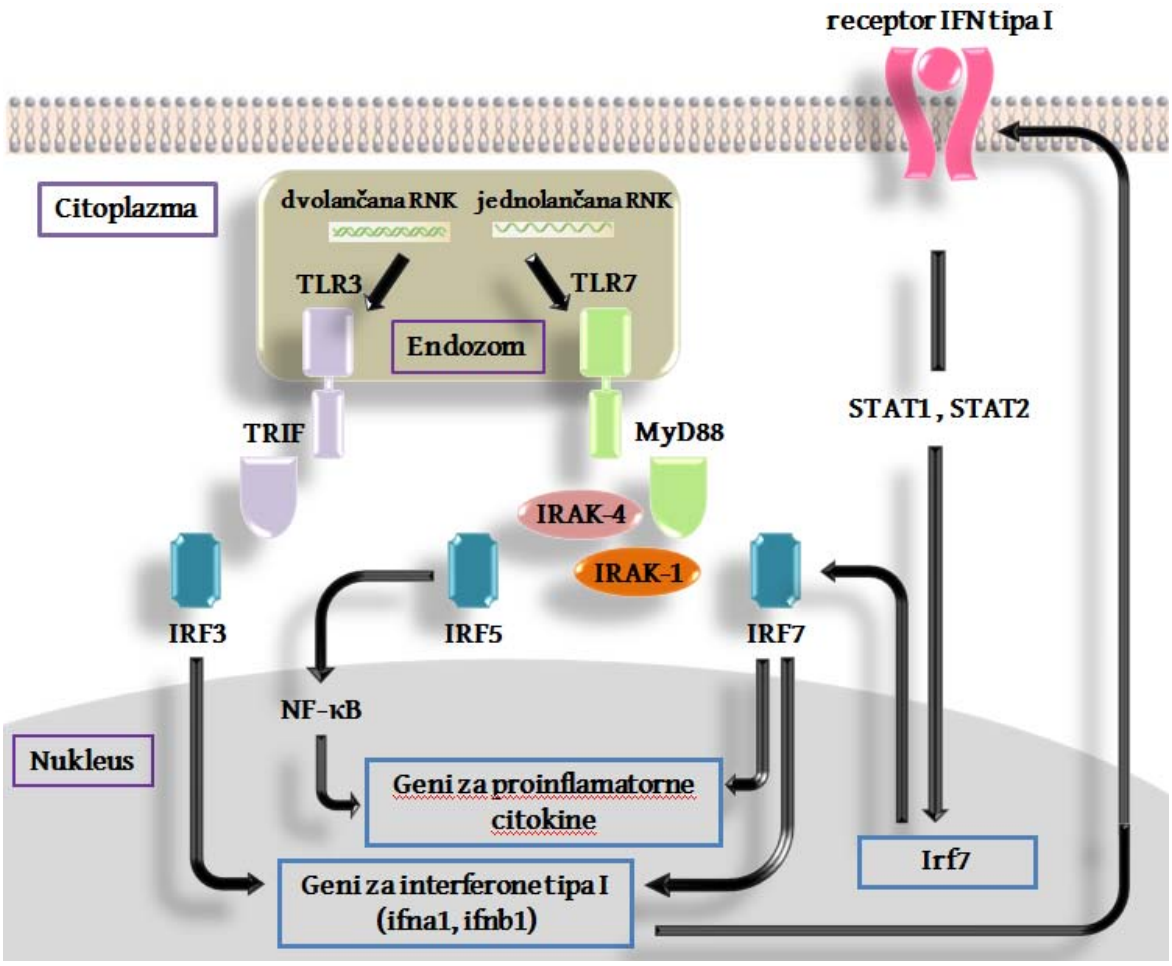
MoDĆ diferencirane u prisustvu optimalnih koncentracija Poly (I:C) i loksoribina usmeravale su imunski odgovor ka Th1 pravcu indukujući produkciju značajne količine IFN-γ od strane alogenih $CD4^+$ T ćelija. Takođe, na ovaj način stimulisane MoDĆ pokazale su smanjen potencijal za usmeravanje $CD4^+$ T limfocita ka Th2 i Th17 pravcu, u poređenju sa efektom pojedinačnih agonista. Pokazani efekti kombinovane primene optimalnih koncentracija Poly (I:C) i loksoribina na Th polarizacioni potencijal MoDĆ su u skladu sa povećanom produkcijom IL-12 i IL-27 i smanjenom produkcijom IL-23 od strane MoDĆ tretiranih na ovaj način. Pored

pokazane uloge IL-27 u potenciranju uticaja IL-12 na usmeravanje $CD4^+$ T limfocita ka Th1 profilu (Lucas i sar., 2003; Kamiya i sar., 2004; Pflanz i sar., 2004; Owaki, i sar., 2006; Molle i sar., 2007), visok nivo IL-27 utiče na smanjenje produkcije IL-4, a time i diferencijaciju Th2 ćelija (Lucas i sar., 2003). Takođe, povećan nivo IL-27 utiče na smanjenje produkcije IL-23, a samim tim i smanjenja potencijala MoDĆ za usmeravanje $CD4^+$ T limfocita ka Th17 pravcu (Yoshimura i sar., 2006). Sa druge strane, efekat kombinovane primene optimalnih koncentracija Poly (I:C) i loksoribina na fenotip MoDĆ se ogledao u smanjenju ekspresije HLA-DR i kostimulatornih molekula, u poređenju sa efektima pojedinačnih agonista. Sličan efekat su opisali Napolitani i saradnici koji su ukazali na moguću ulogu koligacije kao "sigurnosne šifre" koja moduliše antigen-prezentujući potencijal MoDĆ i dovodi do razvoja efektorskih T limfocita samo samo u slučaju prisustva patogena (Napolitani i sar., 2005).

MoDĆ diferencirane u prisustvu suboptimalne koncentracije Poly (I:C) i optimalne koncentracije loksoribina su ispoljile najizraženiji Th1 polarizujući potencijal što je u skladu sa detektovanim povećanjem produkcije IL-12 od strane ovako stimulisanih MoDĆ. Ovi rezultati potvrđuju da MoDĆ imaju veliki potencijal za produkciju IL-12 koji se ispoljava samo kao odgovor na višestuke stimuluse koji deluju istovremeno (Napolitani i sar., 2005).

Smatra se da je sinergistički efekat koaktivacije TLR3 i TLR7 ostvaren na nivou signalnih puteva. Naime, stimulacija TLR na DĆ, sa izuzetkom TLR3, dovodi do konformacionih promena receptora, angažovanja adaptorskog proteina MyD88 (engl. myeloid differentiation primary response gene 88) i pokretanja signalne kaskade koja rezultuje aktivacijom transkripcionih faktora NF-κB i regulatora transkripcije interferona (engl. IFN regulatory factor 5, IRF5). Alternativni signalni put se pokreće aktivacijom TLR-7,-8 i -9, koji takođe angažuju MyD88, ali pored NF-κB aktiviraju i IRF7. Sa druge strane, aktivacija TLR3 pokreće signalni put nezavisan od MyD88 u kome se umesto ovog molekula angažuje adaptor koji sadrži Toll/IL-1R domen (engl. Toll/IL-1R domain-containing adaptor, TICAM-1; TRIF). U MyD88-nezavisnom putu dolazi do aktivacije NF-κB, aktivacionog proteina-1 (engl. activator protein, 1 AP-1), IRF3 i ekspresije IFN-β i IFN-inducibilnih gena (Gautier i

sar., 2005; Honda i sar., 2005; Beutler i sar., 2006; Matsumoto i Seya, 2008; Mäkelä i sar., 2009; Kawai i Akira, 2010). Značaju ulogu u kooperaciji TLR receptora mogu imati IFN tipa I koji delovanjem na autokrini, odnosno parakrini način pojačavaju efekte koaktivacije TLR *(Slika 8).* Naime, sinteza IFN-β zavisna od aktivacije TLR3 indukuje sintezu IRF7. IRF7 se aktivira nakon stimulacije TLR7 i pospešuje dalju produkciju IFN tipa I čime se uspostavlja pozitivna povratna sprega i uspostavlja stabilna, pojačana produkcija ovih citokina (Gautier i sar., 2005; Malissen i Ewbank, 2005).



Slika 8. FN tipa I delovanjem na autokrini, odnosno parakrini način pojačavaju efekte koaktivacije TLR

Dakle, MyD88-nezavisni put pospešuje MyD88-zavisne funkcija DĆ i aktivaciju T ćelija. Naime, signalni put nezavisan od MyD88 odgovara signalu 2 neophodnom za aktivaciju T ćelija, dok signalni put zavisan od MyD88 predstavlja signal 3 koji određuje tip T ćelijskog odgovora (Zhu i sar., 2008). Pretpostavlja se da

je ova interakcija uspostavljena da bi se izbegla nepotrebna aktivacija imunskog odgovora, ali da se u isto vreme može uspostaviti anti-tumorski imunski odgovor.

5.3. Uticaj kurdlana i njegove kombinacije sa Poly (I:C) na diferencijaciju nezrelih humanih MoDĆ

U sklopu ovog istraživanja ispitivan je i uticaj koaktivacije TLR3 i dektin-1 receptora primenom odgovarajućih agonista, Poly (I:C) i kurdlana. Primena ove kombinacije agonista PRR ima prevashodno imunoterapeutski značaj jer do sada nije detektovan patogen koji ispoljava ligande za ova dva PRR.

Transmembranski receptor dektin-1 prepoznaje β-glukane (Ariizumi i sar., 2000; Taylor i sar., 2002) od kojih je izgrađeno oko 50% ćelijskog zida gljivica. Uloga dektina-1 u modulaciji bioloških funkcija humanih DĆ je publikovana u svega nekoliko studija do sada (Skrzypek i sar., 2009; Agrawal i sar., 2010; Higashi i sar., 2010). Pokazano je da mišje i humane DĆ stimulisane kurdlanom povećavaju ekspresiju kostimulatornih molekula i produkuju brojne proinflamatorne (IL-1β, IL-6, IL-12, IL-23) i anti-inflamatorne (IL-10) citokine (LeibundGut-Landmann i sar., 2007; Gringhuis i sar., 2009; Skrzypek i sar., 2009; Agrawal i sar., 2010; Higashi i sar., 2010).

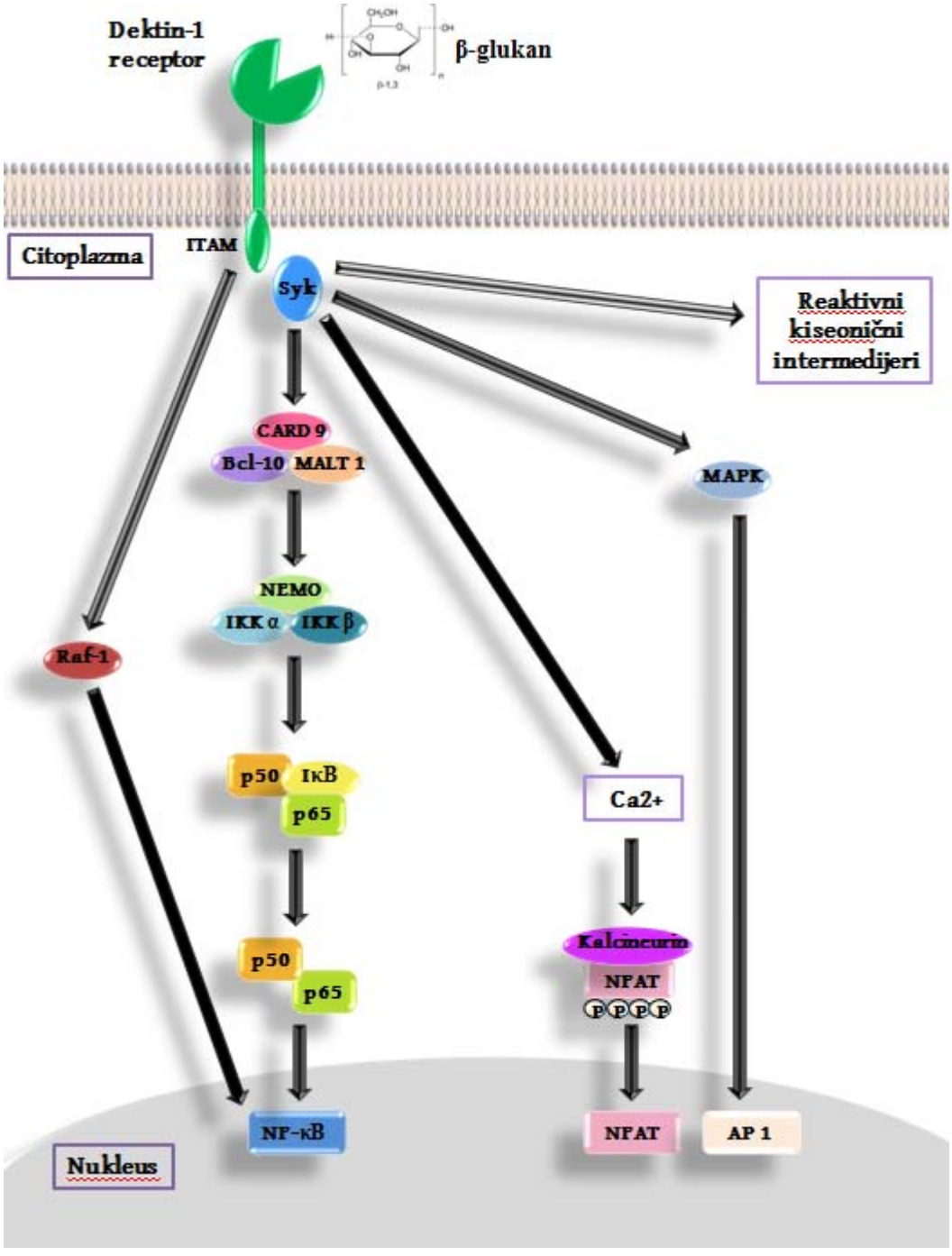
Aktivacija dektin-1 receptora primenom kurdlana dovodi do sazrevanja MoDĆ u ćelije koje produkuju povećane nivoe IL-23 i IL-27 i smanjen nivo IL-10. Razlike između literaturnih i naših rezultata se mogu objasniti primenom različitih koncentracija kurdlana i različitim načinima njegove pripreme, kako od strane proizvođača tako i u laboratoriji (Stopinšek i sar., 2011). Uprkos tome što je nivo IL-12 bio nedetektabilan u kulturama MoDĆ stimulisanih kurdlanom, MoDĆ tretirane na ovaj način usmeravale su imunski odgovor ka Th1 pravcu u kokulturi sa alogenim $CD4^+$ T limfocitima. Ovaj efekat se može objasniti dodatnom kontaktnom stimulacijom produkcije IL-12 usled interakcije CD40 na MoDĆ sa CD40 ligandom na $CD4^+$ T limfocitima u alogenoj kulturi (Snijders i sar., 1998). MoDĆ stimulisane kurdlanom su produkovale značajno veće količine IL-23 i usmeravale imunski odgovor i u Th17 pravcu, što je već opisan fenomen (Agrawal i sar., 2010).

Istovremena aktivacija TLR3 i dektin-1 receptora na MoDĆ u našim istraživanjima je dovela do povećanja produkcije IL-12, IL-23 i IL-10, dok je produkcija IL-27 i IL-6 bila smanjena. MoDĆ stimulisane na ovaj način su indukovale značajno veću produkciju IFN-γ i IL-17 u alogenoj kokulturi, u poređenju sa efektom pojedinačnih agonista. Ovaj efekat je bio izraženiji kada su za kokultivaciju sa MoDĆ korišćeni purifikovani naivni (snažnije produkuju IFN-γ) i memorijski (snažnije produkuju IL-17) $CD4^+$ T limfociti, a nije bio rezultat promene odnosa $CD4^+CD45RA^+$/ $CD4^+CD45RO^+$ podtipova niti je pokazano da ova dva podtipa imaju različite stope proliferacije.

Povećanje produkcije citokina se može objasniti ekspanzijom jednostruko pozitivnih IFN-γ^+ i IL-17^+ $CD4^+$ T ćelija, ali i dvostruko pozitivnih IFN-γ^+/IL-17^+ $CD4^+$ T ćelija. Poznato je da se brojnost IFN-γ^+/IL-17^+ dvostruko pozitivnih ćelija se povećava u tkivima pod inflamacijom ili u krvi pacijenata sa hroničnim inflamatornim bolestima. Ove ćelije su fenotipski nestabilne u kulturi i mogu postati ili IFN-γ ili IL-17 produkujuće $CD4^+$ T ćelije (Boniface i sar., 2010). Na osnovu rezultata naših istraživanja pretpostavili smo da citokini IL-12 familije koje sintetišu MoDĆ nakon istovremene aktivacije TLR3 i dektina-1, utiču na polarizaciju imunskog odgovora, makar jednim delom, stimulacijom diferencijacije dvostruko pozitivnih IFN-γ^+/IL-17^+ $CD4^+$ T ćelija.

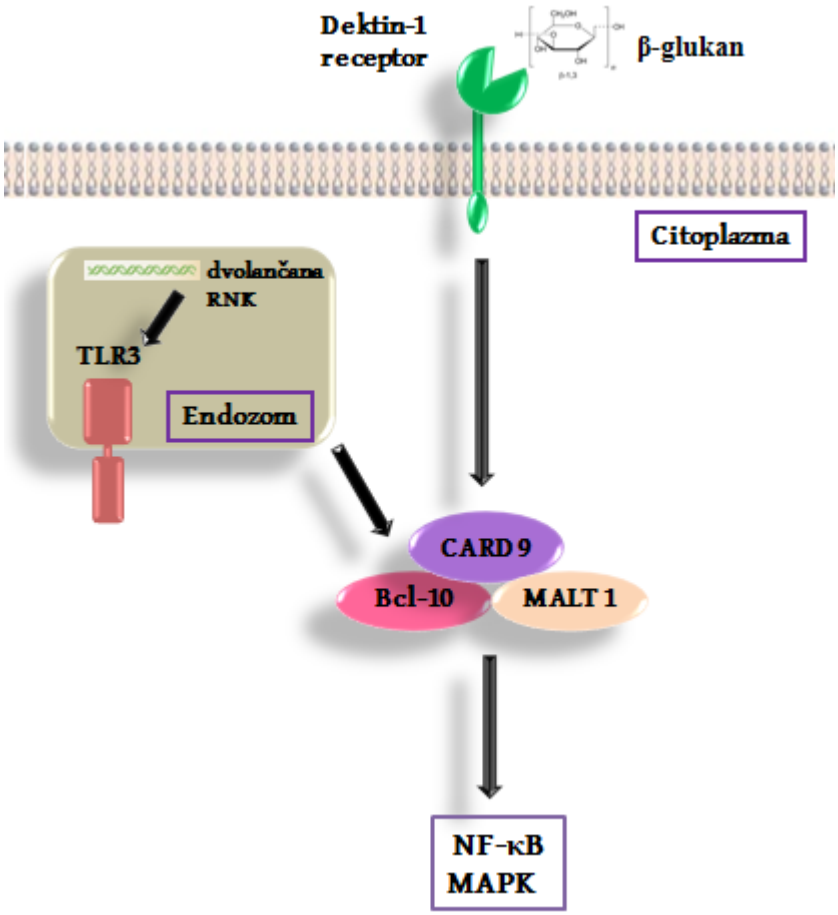
Uticaj kombinovane primene Poly (I:C) i kurdlan na MoDĆ nije još uvek ispitan, ali su brojne dosadašnje studije pokazale da efekti modulacije funkcije MoDĆ primenom liganada dektin-1 receptora u kombinaciji sa TLR agonistima zavise od vrste, tipa receptora modelskog prepoznavanja i uslova kultivacije (Gerosa i sar., 2008; Dennehy i sar., 2009; Gringhuis i sar., 2009; Stopinšek i sar., 2011).

Molekuli uključeni u signalnu kaskadu mogu biti u osnovi uticaja kombinovane primene agonista TLR3 i dektin-1 receptora na povećanje produkcije citokina od strane MoDĆ *(Slika 9).*



Slika 9. Signalna kaskada pokrenuta aktivacijom dektin-1 receptora na DĆ

Jedan od njih, adaptorski protein CARD9 (engl. caspase recruitment domain-containing protein 9) se aktivira nakon stimulacije produktima mikroba, kao i primenom Poly (I:C) (Hsu i sar., 2007) i agonista dektin-1 receptora (Gross i sar., 2006) *(Slika 10).*



Slika 10. Potencijalna uloga adaptornog proteina CARD9 u kooperaciji TLR3 i dektin-1 receptora

Aktivacija MAP kinaze i NF-κB od strane CARD9 modifikuje gensku ekspresiju i fino podešava citokinski profil, uključujući dodatnu produkciju p40 subjedinice (Colonna, 2007) koja je zajednička za IL-12 i IL-23 (McKenzie i sar., 2005). Za sada je nepoznato i ostaje da se ispita da li su još neki mehanizmi uključeni i u kojoj meri signalna kaskada pokrenuta aktivacijom pojedinačnog receptora modifikuje ekspresiju drugog receptora (Weck i sar., 2008).

Stimulacija MoDĆ kombinacijom Poly (I:C) i kudlana dovodi do povećanja produkcije IL-10 i smanjenja produkcije IL-27 od strane MoDĆ. IL-27 podstiče

usmeravanje imunskog odgovora ka Th1 pravcu (Pflanz i sar., 2002). Pojedini rezultati ukazuju na inhibitorni uticaj IL-27 na produkciju IL-23 od strane MoDĆ kao i IL-17 od strane $CD4^+$ T ćelija (Diveu i sar., 2009). Balans u produkciji IL-23 i IL-27 reguliše Th17 imunski odgovor. Smatra se da je odnos IL-23 i IL-27 značajan za diferencijaciju Th17 $CD4^+$ T ćelija u istoj meri u kojoj je Th1/Th2 diferencijacija određena odnosom nivoa IL-12 i IL-4 citokina (Yoshimura i sar., 2006). Stoga, naši rezultati eksperimenata ukazuju da je smanjena produkcija IL-27 značajna za dodatno potenciranje Th17 odgovora.

Sa druge strane, IL-10, snažan anti-inflamatorni i imunosupresivni citokin, ima značajnu ulogu u ograničavanju Th1 i Th17 odgovora u cilju sprečavanja nastanka imunopatoloških stanja kada su prisutni snažni inflamatorni stimulusi (Jankovic i Trinchieri, 2007; Saraiva i sar., 2009). U skladu sa ovim konceptom, povećana produkcija IL-10 u našoj studiji nakon stimulacije MoDĆ kombinacijom agonista TLR3 i dektin-1 receptora se može smatrati značajnim mehanizmom za vraćanje imunskog odgovora u stanje homeostaze. Takođe, smanjenje aloreaktivne sposobnosti MoDĆ se može objasniti povećanom produkcijom IL-10. Naime, uprkos visokoj ekspresiji adhezivnih i kostimulatornih molekula kao i visokoj produkciji stimulatornih citokina (IL-12 i IL-23) od strane MoDĆ, proliferacija $CD4^+$ T ćelija u alogenoj kulturi je bila niska pri višim odnosima MoDĆ/CD^+ T limfocita. Povećanje proliferativnog odgovora pri najnižem odnosu MoDĆ/CD^+ T limfocita (1:80) se može objasniti prevagom stimulatornih solubilnih faktora, imajući u vidu da nije došlo do dodatnog povećanja ekspresije kostimulatornih molekula na MoDĆ. MoDĆ stimulisane kombinacijom agonista TLR3 i dektin-1 receptora su povećavale ekspresiju CCR7 molekula što ukazuje na to da interakcija ova dva PRR stimuliše fenotipsko sazrevanje MoDĆ sa poboljšanim migratornim sposobnostima.

U zaključku, efekat istovremene aktivacije TLR3 i dektin-1 receptora na MoDĆ se ogleda u pospešivanju njihove sposobnosti da stimulišu diferencijaciju Th1 i Th17 ćelija *in vitro*. Predmet budućih istraživanja će biti ispitivanje prisustva ovog efekta i u *in vivo* uslovima i na koji način utiče na imunski odgovor u celini.

5.4. Efekat kombinovane primene Poly (I:C) i TNF-α na diferencijaciju MoDĆ

Za modulaciju funkcija DĆ pored stimulacije PRR značajnu ulogu imaju i citokini prirodnog imuniteta koji se produkuju prilikom infekcije. Kinetika sazrevanja MoDĆ i dužina izlaganja patogenu (Poly (I:C) u našoj studiji) u tkivu zahvaćenom inflamacijom (TNF-α u našoj studiji) su značajni faktori koji određuju finalni ishod imunskog odgovora (Spisek i sar., 2003). Brojne studije su ukazale na uticaj dužine stimulacije na produkciju citokina od strane MoDĆ (Kaliński i sar., 1999; Langenkamp i sar., 2000; Langenkamp i sar., 2002).

MoDĆ izložene stimulusu tokom 24h su nazvane "aktivnim" MoDĆ, koje sintetišu značajne nivoe citokina, dok su MoDĆ stimulisane tokom vremenskog perioda dužeg od 24h nazvane "iscrpljenim" MoDĆ, koje su izgubile kapacitet produkcije citokina (Langenkamp i sar., 2000), ali imaju visoku ekspresiju kostimulatornih molekula (Repnik i sar., 2008). Pretpostavlja se da TNF-α ima ključnu ulogu u uspostavljanju balansa između pro- i anti-inflamatornih citokina produkovanih od strane MoDĆ (Hirata i sar., 2011). Kako je ovaj balans presudan za uspostavljanje homeostaze imunskog odgovora, detaljno ispitivanje regulatorne uloge TNF-α može biti od značaja za terapiju patoloških stanja imunskog sistema.

U cilju razjašnjenja ovih problema, ispitivali smo uticaj kombinovane primene različitih doza agonista TLR3, Poly (I:C) i proinflamatornog citokina TNF-α tokom različitih vremenskih perioda na fenotipska i funkcionalna svojstva MoDĆ. U sledećem koraku smo za stimulaciju MoDĆ koristili solubilni ligand za CD40 molekul i time simulirali interakciju MoDĆ sa T ćelijama posredovanu CD40 molekulom i CD40 ligandom.

U kliničkim studijama kao jedan od prvih agenasa za stimulaciju MoDĆ za stimulaciju DĆ korišćen je TNF-α (Bremers i sar., 2000). Najveći broj literaturnih podataka se odnosi na primenu ovog citokina u protokolima za imunoterapiju. Kasnije je pokazano da ovaj proinflamatorni citokin dovodi do nepotpunog sazrevanja MoDĆ koje se karakteriše povećanjem ekspresije molekula MHC klase II i kostimulatornih molekula, niskom ekspresijom proinflamatornih citokina i slabim

alostimulatornim potencijalom (Lutz i Schuler, 2002; Decker i sar., 2008). Karakteristike MoDĆ kultivisanih u prisustvu TNF-α u našem istraživanju su u saglasnosti sa ovim podacima iz literature. Međutim, uprkos nedetektabilnom nivou IL-12 produkovanom od strane MoDĆ stimulisanih sa TNF-α, ovako tretirane MoDĆ su u kokulturi usmeravale odgovor alogenih $CD4^+$ T limfocita ka Th1 pravcu. Pored toga, MoDĆ tretirane sa TNF-α su sekretovale povećan nivo Th17 polarizujućeg citokina, IL-23 (McKenzie i sar., 2005; Sallusto i Lanzavecchia, 2009; Yu i sar., 2010), i najverovatnije tako stimulisale produkciju IL-17 u kokulturi sa alogenim $CD4^+$ T ćelijama.

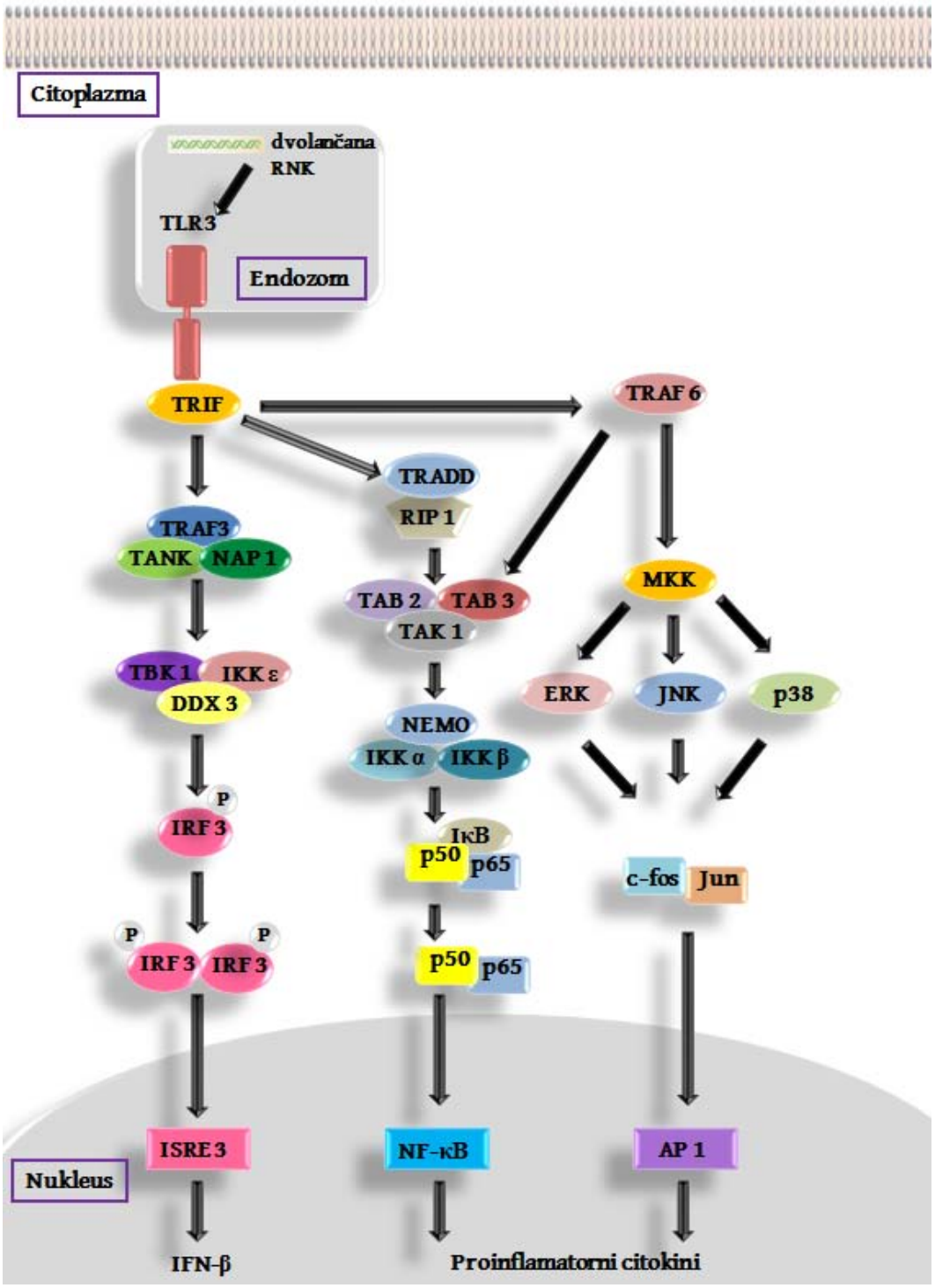
Efekat kombinovane primene Poly (I:C) i TNF-α na fenotipske karakteristike MoDĆ u našoj studiji se ogledao u povećanju ekspresije kostimulatornih i adhezivnih molekula uz statistički značajno povećanje ekspresije CD80, CD86 i CD54 molekula. Povećano ispoljavanje ovih površinskih markera, posredstvom kojih MoDĆ interaguju sa T ćelijama i aktiviraju ih, ukazuje na fenotipsku zrelost MoDĆ što je u skladu sa prethodno publikovanim rezultatima (Spisek i sar., 2001; Spisek i sar., 2003).

Uprkos tome, MoDĆ diferencirane u prisustvu TNF-α i Poly (I:C) su imale smanjen alostimulatorni kapacitet i sniženu produkciju IL-12, u poređenju sa MoDĆ stimulisanim samo sa Poly (I:C). Smanjenje produkcije IL-12 od strane MoDĆ diferenciranih u prisustvu TNF- α i Poly (I:C) se odrazilo na smanjenje produkcije IFN-γ u kokulturi sa alogenim $CD4^+$ T ćelijama, u poređenju sa nivoom ovog citokina u alogenoj kulturi sa MoDĆ tretiranim samo sa Poly (I:C). Nasuprot rezultatima naše stuije, podaci iz literature pokazuju da MoDĆ stimulisane sa Poly (I:C) i TNF-α produkuju značajan nivo IL-12 i efikasno usmeravaju $CD4^+$ T limfocite ka Th1 pravcu (Spisek, i sar., 2001).

Nesklad između rezultata naše studije i literaturnih podataka se može objasniti time što smo u našoj studiji upoređivali produkciju IL-12 između MoDĆ stimulisanih kombinacijom TNF-α i Poly (I:C) i MoDĆ stimulisanih samo sa Poly (I:C), dok je u pomenutoj studiji poređenje vršeno sa produkcijom citokina od strane MoDĆ stimulisanih koktelom citokina. Naime, koktel citokina, tzv. “zlatni

standard", za stimulaciju DĆ za njihovu primenu kao vakcina u lečenju humanih malignih tumora predstavlja koktel citokina koji se sastoji od TNF-α, IL-1β, IL-6 i PGE_2 (Jonuleit i sar., 1997). Nedostatak DĆ dobijenih na ovaj način se ogleda u izuzetno niskoj produkciji IL-12 (Pedersen i sar., 2006), dok je Poly (I:C) snažan stimulator produkcije IL-12 od strane MoDĆ (Cella i sar., 1999b; Verdijk RM, i sar., 1999; Rouas R, i sar., 2004). Rezultati naše studije ukazuju na to da se kombinovani efekat TNF-α i Poly (I:C) tokom diferencijacije MoDĆ ogleda u smanjenju njihove imunogenosti i funkcionalnosti, u poređenju sa efektom samog Poly (I:C).

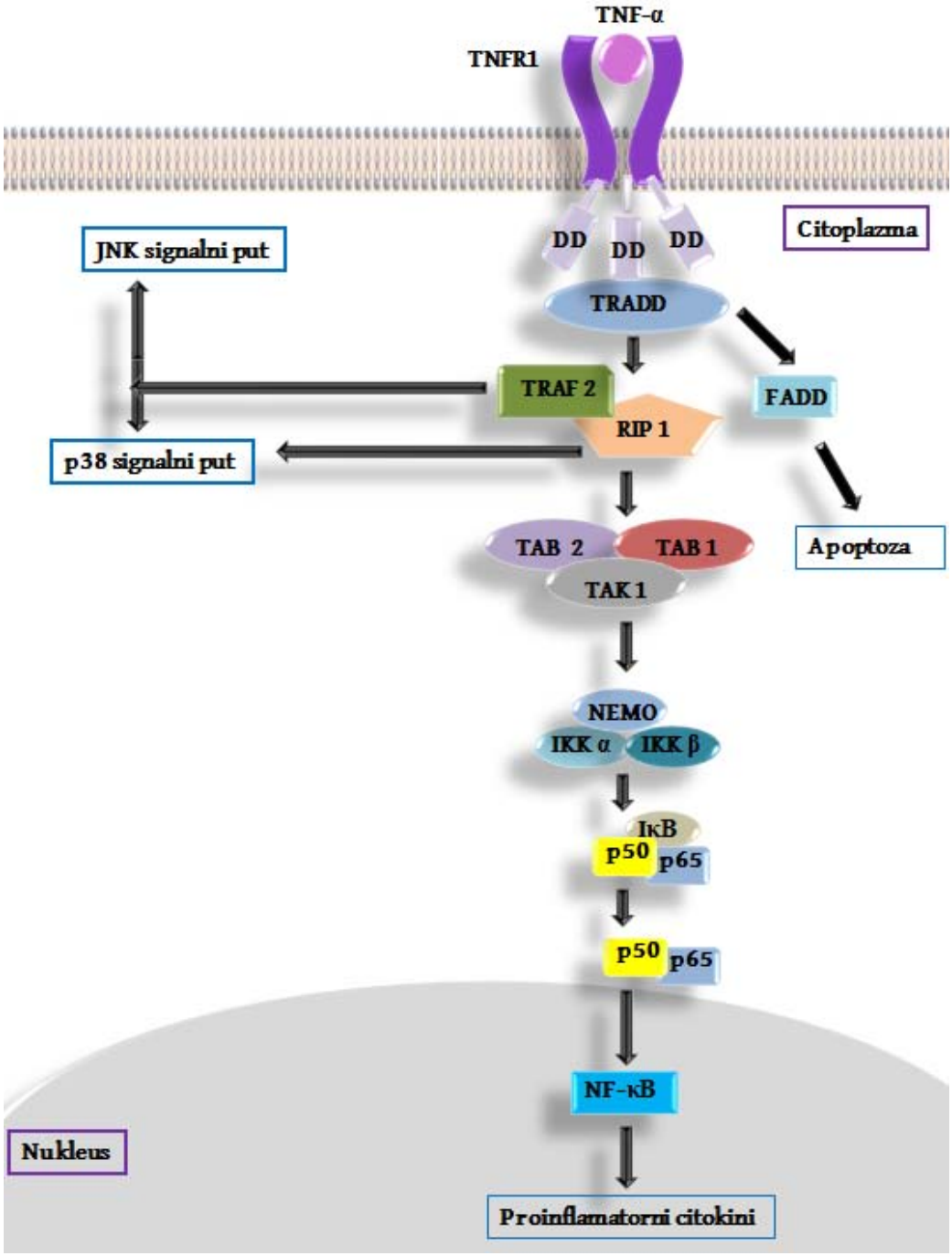
Mehanizmi kooperacije signala pokrenutih aktivacijom Poly (I:C) i TNF-α nisu još uvek razjašnjeni. Aktivacija TLR3, nakon angažovanja TRIF adaptorskog proteina, pokreće signalni put koji se sastoji iz tri glavna modula u okviru kojih dolazi do aktivacije transkripcionih faktora: IRF3, NF-κB i AP-1 *(Slika 11).*



Slika 11. Signalna kaskada pokrenuta aktivacijom TLR3 od strane dvolančane RNK u DĆ

Ovi moduli su međusobno povezani i delimično se preklapaju (Kawai i Akira, 2010). Adaptor TRAF3 predstavlja ključnu vezu između TRIF i kompleksa kinaza koje fosforilišu IRF3. Aktivacija transkripcionih faktora posredovana sa IRF3 dovodi do ekspresije IL-12p35 subjedinice (Goriely i sar., 2005). U aktivaciji NF-κB učestvuju dva nezavisna puta koji se preklapaju na nivou IKK kompleksa: receptor interagujući protein 1 (engl. receptor interacting protein 1, RIP1) i TRAF6. Oba TRIF-RIP1 i TRIF-TRAF6 signalna puta konvergiraju u IKK kompleksu koji se sastoji od katalitičkih subjedinica, IKKα i IKKβ, i regulatorne subjedinice, IKKγ. Protein kinaza transformišućeg faktora rasta 1 (engl. Transforming growth factor activated protein kinase 1, TAK1) biva aktivirana u nakon tretiranja MoDĆ sa Poly (I:C) i fosforiliše IKKα i IKKα nakon čega oni fosforilišu inhibitor NF-κB, IκB. Translokacija NF-κB u nukleus i vezivanje za DNK indukuju ekspresiju IFN-γ i drugih citokina i hemokina (IL-6, TNF-α, CCL3, IL-12). Aktivirani transkripcioni faktor AP-1 indukuje citokine i hemokine, poput IL-6, TNF-α i CCL3 (Cusson-Hermance i sar., 2005; Matsumoto i Seya, 2008).

TNF-α se vezuje za dva različita receptora: TNF receptor tip 1 (TNFR1; CD120a) i TNF receptor tip 2 (TNFR1; CD120b) (Vandenabeele i sar., 1995). Većinu funkcija TNF-α ostvaruje posredstvom TNFR1 *(Slika 12)*, koji se konstituitivno eksprimira u većini tkiva. TNFR1 regrutuje proteine domena smrti asocirane sa TNFR1 (engl. death-domain proteins including TNFR1-associated death domain protein, TRADD) i proteine domena smrti asocirane sa Fas (engl. Fas-associated death domain proteins, FADD) (Baud i Karin, 2001). TRADD aktivira FADD, RIP1 i TRAF2. TRAF 2 i RIP1 aktiviraju MAP kinaze (Wajant i sar., 2001; Wajant i Scheurich, 2001; Balkwill, 2009).



Slika 12. Signalna kaskada pokrenuta aktivacijom TNFR1 solubilnim TNF-α

Dakle, oba signalna puta pokrenuta aktivacijom TNF-α i TLR3 aktiviraju RIP-1. RIP1 je važan za aktivaciju NF-κB u kasnijim fazama imunskog odgovora kada je TNF-α aktivan, ali i u ranim fazama imunskog odgvora kada se pokreće antivirusni imunski odgovor nakon aktivacije TLR3 (Meylan i sar., 2004).

U cilju ispitivanja uticaja kinetike aktivacije MoDĆ na njihov potencijal da usmere efektorske funkcije T limfocita, MoDĆ smo stimulisali sa različitim koncentracijama Poly (I:C), TNF-α i njihovim kombinacijama tokom 24h i 48h. Produžena stimulacija MoDĆ je dovela do razvoja MoDĆ koje su iscrpele potencijal za produkciju IL-12. Naime, poznato je da je produkcija IL-12 ograničena na relativno kratak vremenski period (12-24h) nakon izlaganja MoDĆ kombinaciji Poly (I:C) i TNF-α (Spisek i sar., 2003) i da nakon dostizanja platoa za produkciju IL-12, MoDĆ ulaze u "iscrpljeno" stanje koje se karakteriše izmenjenim potencijalom za stimulaciju T limfocita (Langenkamp i sar., 2000). Produkcija IL-23 je imala isti obrazac kao i produkcija IL-12. Sa druge strane, IL-10 je produkovan kontinualno u skladu sa sopstvenom ulogom u sprečavanju razvoja patološkog imunskog odgovora (Spisek i sar., 2003) i rezultati naše studije su u skladu sa ovim rezultatima. Takođe smo uočili dozno-zavisan uticaj Poly (I:C) na produkciju IL-12 nakon 24h stimulacije koji je bio praćen povećanjem produkcije IL-10. Nasuprot publikovanim rezultatima (Spisek i sar., 2003) produžena stimulacija MoDĆ sa TNF-α u kombinaciji sa svim ispitivanim koncentracijama Poly (I:C) u našoj studiji nije dovela do značajnog porasta produkcije IL-12, kao ni polarizacije imunskog odgovora ka Th1 pravcu. Nasuprot tome, uočeno je smanjenje Th1 i Th17 odgovora pogotovo kada su primenjene više koncentracije Poly (I:C) i TNF-α, u poređenju sa MoDĆ stimulisanih samo sa Poly (I:C).

Sledeći cilj ovog istraživanja bio je ispitivanje modulatornog efekta interakcije CD40 molekula sa CD40 ligandom značajne za finalnu maturaciju MoDĆ (Mackey i sar., 1998) na funkcionalne karakteristike MoDĆ stimulisanih kombinacijom Poly (I:C) i TNF-α. MoDĆ tretirane tokom 24h sa TNF-α, različitim koncentracijama Poly (I:C) i njihovim kombinacijama su stimulisane dodatna 24h solubilnim ligandom za CD40 molekul. Prisustvo CD40L je pospešilo produkciju IL-12 od strane MoDĆ stimulisanih svim ispitivanim koncentracijama Poly (I:C). Stimulatorni efekat CD40L na produkciju IL-12 je bio najizraženiji kada su MoDĆ stimulisane optimalnom koncentracijom Poly (I:C) i u alogenoj kulturi ove MoDĆ su dovele do povećanja produkcije IFN-γ. U kulturama MoDĆ pretretiranih sa TNF-α je došlo do povećanja produkcije IL-12 što je bilo neočekivano imajući u vidu prethodno publikovane rezultate (Decker i sar., 2008), ali ovaj porast nije bio

dovoljan da polarizuje imunski odgovor u Th1 pravcu. Aktivacija CD40 na MoDĆ pretretiranim najnižom koncentracijom Poly (I:C) dovela je do povećanja produkcije IL-23 koja je bila pospešena dodavanjem TNF-α i dovoljna da polarizuje imunski odgovor u Th17 pravcu. Najizraženiji efekat CD40:CD40L interakcije je bilo stimulisanje imunoregulatornog odgovora posredovanog sa IL-10 od strane MoDĆ pretretiranih najvećom koncentracijom Poly (I:C) i TNF-α kada je smanjena produkcija IL-12 i IL-23 indukovala smanjenje Th1 i Th17 odgovora.

Dakle, povećanjem koncentracije Poly (I:C) u kombinaciji sa TNF-α može se modulisati Th polarizaciona sposobnost MoDĆ od Th17 ka imunoregulatornoj.

5.5. Uticaj povezivanja CD40 molekula i IFN-γ na funkcionalne karakteristike MoDĆ diferenciranih u prisustvu Poly (I:C)

Poslednji deo istraživanja se odnosio na ispitivanje uticaja signala stečenog imuniteta koje DĆ dobijaju tokom njihove interakcije sa T ćelijama, uključujući signalizaciju preko CD40 molekula i receptora za IFN-γ.

Aktivacija CD40 molekula na DĆ je važan korak u sazrevanju ovih ćelija. U našim eksperimentima koristili smo ćelijsku liniju transfektovanu za CD40 ligand radi simuliranja CD40:CD40L interakcija između DĆ i T ćelija kojima se ostvaruje recipročna regulacija između T limfocita i DĆ (Mackey i sar., 1998; Rissoan i sar., 1999). Aktivacija CD40 molekula na nezrelim i MoDĆ stimulisanim sa Poly (I:C) u našoj studiji je značajno pospešila njihov alostimulatorni potencijal, najverovatnije posredstvom povećane ekspresije kostimulatornih molekula, poput ICAM-1, HLA-DQ, CD80 i CD86 (Cella i sar., 1996). Poznato je da aktivacija CD40 na nezrelim MoDĆ stimuliše značajnu produkciju IL-12 (Snijders i sar., 1998) koji kasnije indukuje produkciju IFN-γ od strane T limfocita. Rezultati našeg istraživanja su ukazali na fenomen koji nije publikovan do sad. Po prvi put pokazano je da aktivacija CD40 na nezrelim MoDĆ pospešuje produkciju IL-23 što je praćeno povećanjem produkcije IL-17 od strane $CD4^+$ T limfocita u alogenoj kulturi. Produkcija IL-17 je dodatno pospešena aktivacijom CD40 na MoDĆ koje su stimulisane sa Poly (I:C). Stimulacija Th17 odgovora nakon aktivacije CD40 molekula na MoDĆ tretiranim sa Poly (I:C) je

praćena smanjenjem Th1 odgovora. Poznato je da nekontrolisan Th1 odgovor može biti štetan (Liblau i sar., 1995) pa stoga signalizacija preko CD40 molekula može imati protektivnu i imunomodulatornu ulogu.

IFN-γ je klasifikovan kao interferon tipa II IFN u skladu sa specifičnošću receptora i homologijom sekvence (Schroder i sar., 2004). Interferoni su najpre bili opisani kao faktori koji interferiraju sa replikacijom virusa (Isaacs i Lindenmann, 1957). IFN-γ produkuju NK ćelije i postoji pretpostavka da ga produkuju i APĆ u prvim stadijumima infekcije, a glavni izvor ovog citokina su T limfociti u stečenom imunskom odgovoru (Frucht i sar., 2001). IFN-γ pospešuje obradu i prezentaciju antigena, kao i ekspresiju kostimulatornih molekula na APĆ (Schroder i sar., 2004). U našem istraživanju smo pokazali da tretman sa IFN-γ nezrelih i MoDĆ stimulisanih sa Poly (I:C) stimuliše alostimulatorni potencijal MoDĆ u kokulturi sa alogenim $CD4^+$ T ćelijama. Alostimulatorna sposobnost MoDĆ je opadala sa smanjenjem odnosa MoDĆ/$CD4^+$ T ćelija. Pri višim odnosima MoDĆ/$CD4^+$ T ćelija alostimulatorni potencijal MoDĆ je bio niži. Ovaj efekat se može objasniti smanjenim uticajem kostimulatornih i adhezivnih molekula kao i IL-12 usled manjeg broja MoDĆ.

Značajan nalaz ovog istraživanja je dvostruka uloga IFN-γ na produkciju IL-12: stimulacija produkcije IL-12 od strane nezrelih MoDĆ i supresija produkcije IL-12 od strane MoDĆ pretretiranih sa Poly (I:C) nakon kultivisanja sa IFN-γ. Povećanje produkcije IL-12 je stimulisalo povećanje produkcije IFN-γ i smanjenje produkcije IL-5 i IL-17 od strane alogenih $CD4^+$ T ćelija u kokulturi sa nezrelim MoDĆ. Povećanje produkcije IL-12 od strane nezrelih MoDĆ je u skladu sa prethodno publikovanim rezultatima (Boullart i sar., 2008). IL-12 stimuliše T ćelije i NK ćelije da sintetišu IFN-γ (Walzer i sar., 2005) koji, sa druge strane, stimuliše produkciju IL-12 čime se formira pozitivna povratna sprega. Smanjenje produkcije IL-5 je u skladu sa konceptom recipročne regulacije Th1 i Th2 odgovora (Glimcher i Murphy, 2000) i direktnim inhibitornim efektom IFN-γ na razvoj Th2 ćelija (Schroder i sar., 2004). Produkcija IL-23 od strane MoDĆ stimulisanih sa IFN-γ se nije značajno promenila, ali su uprkos tome ove MoDĆ inhibirale produkciju IL-17 u alogenoj

kulturi. Naime, poznato je da IL-23 pretežno deluje na već diferencirane Th17 ćelije (Sallusto i Lanzavecchia, 2009), pa se može pretpostaviti da su MoDĆ tretirane sa IFN-γ inhibirale diferencijaciju $Th17^{+}$ ćelija modulisanjem produkcije citokina koji su neophodni za diferencijaciju Th17 ćelija, poput TGF-β, IL-1β, IL-6 i IL-21 (Korn i sar., 2009). Ova pretpostavka će biti ispitana u predstojećim istraživanjima.

Jedan od ključnih nalaza ovog istraživanja je uticaj kombinovane primene IFN-γ i Poly (I:C) na smanjenje produkcije IL-12 i povećanja produkcije IL-10 od strane MoDĆ koje u alogenoj kulturi smanjuju Th1 odgovor i stimulišu imunoregulatorni milje posredovan IL-10. Nije u potpunosti razjašnjeno da li IFN-γ deluje primarno na smanjenje produkcije IL-12 od strane MoDĆ tretiranih u prisustvu Poly (I:C) ili stimuliše produkciju IL-10. Kao što je poznato, IL-10 je snažni anti-inflamatorni i imunosupresivni citokin koji inhibira produkciju IL-12 od strane MoDĆ (Buelens i sar., 1997) čime se sprečava razvoj patološkog Th1 odgovora u uslovima u kojima je prisutan snažan inflamatorni stimulus (Jankovic i Trinchieri, 2007; Saraiva, i sar., 2009).

Nedavno je pokazano da IFN-γ, pored uloge u stimulaciji produkcije proinflamatornih citokina tokom aktivacije DĆ, takođe aktivira imunosupresivni enzim IDO (engl. indoleamine 2,3-dioxygenase) u DĆ (Jurgens i sar., 2009). MoDĆ koje su IDO^{+} ispoljavaju imunoregulatorni potencijal koji je neophodan za ograničavanje imunskog odgovora (Munn i sar., 2002). Pored toga, IFN-γ stimuliše razvoj adaptivnih regulatornih T ćelija (Jurgens i sar., 2009). Naši rezultati podržavaju koncept da IFN-γ, kao dominantni efektorski citokin Th1 ćelija, pored proinflamatornih karakteristika ispoljava značajnu ulogu u ograničavanju preteranog i potencijalno štetnog imunskog odgovora i održavanju homeostaze, a na taj način iskazuje još veći značaj u imunskom odgovoru.

MoDĆ tretirane sa ligandom za CD40 molekul polarizuju imunski odgovor u Th1 pravcu. Aktivacija CD40 molekula na MoDĆ tretiranim sa Poly (I:C) usmerava imunski odgovor ka Th17 pravcu. Stimulacija nezrelih MoDĆ sa IFN-γ smanjuje Th2 i Th17 odgovore, dok se efekat ovog citokina na MoDĆ stimulisanim sa Poly (I:C) ogledao u smanjenju Th1 odgovora i stimulaciji imunoregulatornih mehanizama

pospešivanjem produkcije IL-10 od strane alogenih $CD4^+$ T limfocita tokom kokultivacije.

5.6. Terapeutski potencijal DĆ diferenciranih u prisustvu agonista endozomnih toll-sličnih receptora, dektin-1 receptora i proinflamatornih citokina

Aktuelni protokoli za stimulaciju DĆ za primenu u imunoterapiji tumora se zasnivaju na dobijanju imunogenih DĆ koje usmeravaju imunski odgovor u Th1 pravcu (Ikeda H, i sar., 2002). Th1 ćelije su smatrane najznačajnijim efektorskim $CD4^+$ T ćelijama u anti-tumorskom imunskom odgovoru zbog svog potencijala da stimulišu citotoksične funkcije $CD8^+$ T limfocita. Sa druge strane, karakteristike Th17 ćelija, poput stimulacije zapaljenskih procesa, pospešivanja antigen-prezentujućih funkcija DĆ, usmeravanja migracije leukocita ka tumorima i olakšavanja aktivacije i diferencijacije $CD8^+$ T ćelija ukazuju na njihovu moguću primenu u imunoterapiji tumora (Muranski P, i sar., 2008; Martin-Orozco N, i sar., 2009). Konkretna uloga Th17 ćelija u anti-tumorskoj imunosti je i dalje predmet istraživanja i debata jer su pokazane i pro-tumorske (Miyahara Y, i sar., 2008; Kryczek I, i sar., 2009) i anti-tumorske funkcije IL-17 (Alshaker HA i Matalka KZ, 2011). Donedavno se smatralo da je, usled konverzije Th17 u Th1 ćelije, uticaj Th17 ćelija zavisan od IFN-γ (Muranski P, i sar., 2008), ali je potvrđena protektivna uloga Th17 ćelija neizmenjenog citokinskog profila u anti-tumorskom imunskom odgovoru (Martin-Orozco N, i sar., 2009).

Cilj našeg istraživanja je bio ispitivanje modulacije funkcije humanih MoDĆ kombinovanom primenom agonista endozomnih TLR, dektin-1 receptora i proinflamatornih citokina radi razvoja protokola za dobijanje zrelih DĆ koje dovode do proliferacije T limfocita i stimulacije optimalnih Th1 i Th17 odgovora, a samim tim i efikasnog anti-tumorskog odgovora. Opisane karakteristike su u našem istraživanju ispoljile MoDĆ stimulisane optimalnom koncentracijom Poly (I:C), kombinacijom suboptimalnih koncentracija Poly (I:C) i loksoribina i kombinacijom

optimalnih koncentracija Poly (I:C) i kurdlana. Cilj predstojećih istraživanja će biti ispitivanje ovih efekata u *in vivo* uslovima.

6. ZAKLJUČCI

Na osnovu obavljenih istraživanja i dobijenih rezultata mogu se se izvesti sledeći zaključci:

- Optimalna koncentracija Poly (I:C) stimuliše sazrevanje MoDĆ koje pokazuju značajni alostimulatorni kapacitet, stimulišu Th1 i Th17 imunski odgovor, a inhibiraju Th2 odgovor tokom kokultivacije sa alogenim $CD4^+$ T limfocitima;

- Stimulacija MoDĆ suboptimalnim koncentracijama Poly (I:C) i loksoribina dovodi do blagog povećanja ekspresije HLA-DR, CD86, CD83, CD54 i CD40 u poređenju sa ekspresijom na MoDĆ tretiranim pojedinačnim agonistima. MoDĆ pripremljene na ovaj način indukuju Th1 i Th17 odgovor $CD4^+$ T limfocita. Kultivacija MoDĆ u prisustvu optimalnih koncentracija Poly (I:C) i loksoribina dovodi do smanjenja ekspresije HLA-DR i kostimulatornih molekula, u poređenju sa MoDĆ stimulisanim pojedinačnim agonistima. MoDĆ stimulisane optimalnim koncentracija oba agonista usmeravaju imunski odgovor ka Th1 pravcu. Primena suboptimalne koncentracije Poly (I:C) i optimalne koncentracije loksoribina dovodi do povećanja ekspresije CD86 i smanjenja HLA-DR molekula na MoDĆ. MoDĆ pripremljene na ovaj način pokazuju najizraženiji potencijal za usmeravanje odgovora $CD4^+$ T limfocita ka Th1 pravcu;

- Kooperacija TLR3 i dektin-1 receptora stimuliše fenotipsko sazrevanje MoDĆ i ekspresiju CCR7 molekula koji im omogućava bolji migratorni potencijal ka perifernim limfoidnim organima. Ovaj način stimulacije MoDĆ poboljšava njihovu sposobnost indukcije Th1 i Th17 odgovora alogenih $CD4^+$ T limfocita;

- Diferencijacija MoDĆ u prisustvu TNF-α i optimalne koncentracije Poly (I:C) povećava ekspresiju CD80, CD86 i CD54 molekula, u poređenju sa efekatom samog Poly (I:C). Sa druge strane, MoDĆ pripremljene na ovaj način imaju manji alostimulatorni potencijal i

smanjenu sposobnost indukcije Th1 odgovora T limfocita, u poređenju sa MoDĆ diferenciranim u prisustvu Poly (I:C). Ispitivanjem dozno- i vremenski- zavisnog efekta kombinovane primene TNF-α i Poly (I:C) ustanovljeno je da dozno-zavisni efekti izraženi nakon 24h nisu prisutni nakon 48h stimulacije. Dozno-zavisni efekti nakon 24h stimulacije su se ogledali u smanjenju Th1 i Th17 polarizacione sposobnosti MoDĆ, posebno kada je u kombinaciji sa TNF-α primenjena najviša koncentracija Poly (I:C). Ispitivanjem uticaja interakcije CD40:CD40L na funkcionalne karakteristike MoDĆ diferenciranih u prisustvu TNF-α i različitih koncentracija Poly (I:C) ustanovljen je najizraženiji efekat tokom primene najviše koncentracije Poly (I:C). Ovako stimulisane MoDĆ ispoljile su smanjen Th1 i Th17 polarizujući kapacitet u korist imunoregulatornog u kokulturi sa alogenim $CD4^+$ T limfocitima;

- Nezrele MoDĆ na kojima je aktiviran CD40 molekul imaju značajan potencijal za usmeravanje imunskog odgovora ka Th1 pravcu, dok MoDĆ pretretirane sa Poly (I:C) kojima je potom aktiviran CD40 molekul polarizuju imunski odgovor ka Th17 pravcu. Nezrele MoDĆ stimulisane IFN-γ imaju manji potencijal za usmeravanje ka Th2 i Th17 odgovoru, dok se efekat ovog citokina na MoDĆ pretretiranim sa Poly (I:C) ogleda u smanjenju sposobnosti MoDĆ da indukuju Th1 odgovora i povećanju produkcije IL-10 (imunoregulatorni mehanizmi) od strane alogenih $CD4^+$ T limfocita.

Svi dobijeni rezultati pokazuju veliku plastičnost u funkcionalnom odgovoru MoDĆ. Značajna imunogena svojstva Poly (I:C) koja su do sada iskorišćena u pripremi MoDĆ kao tumorskih vakcina se mogu dodatno pospešiti primenom stimulatora dektinskih receptora i TNF-α. Ipak neki od povoljnih efekata mogu biti umanjeni ili usmereni u suprotnom pravcu dodavanjem solubilnog CD40L i IFN-γ. Ovi rezultati mogu pomoći u boljem razumevanju biološkog ponašanja MoDĆ nakon transfera *in vivo*.

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8. PRILOZI

Rezultati prikazani u disertaciji su objavljeni u sledećim člancima:

1. **Ana Dragičević**, Tanja Džopalić, Saša Vasilijić, Dragana Vučević, Biljana Božić, Ivana Majstorović, Bela Balint, Miodrag Čolić. The influence of CD40 ligation and interferon-γ on functional properties of human monocyte derived dendritic cells matured with polyinosinic-polycytidylic acid. Vojnosanitetski pregled 2011;68(4): 301-308.

2. Tanja Džopalić, Ivan Rajković, **Ana Dragičević**, Miodrag Čolić. The response of human dendritic cells to co-ligation of pattern-recognition receptors. Immunol Res. 2012; DOI: 10.1007/s12026-012-8279-5.

3. **Ana Dragičević**, Tanja Džopalić, Saša Vasilijić, Dragana Vučević, Sergej Tomić, Biljana Božić, Miodrag Čolić. Signaling through Toll-like receptor 3 and Dectin-1 potentiates the capability of human monocyte-derived dendritic cells to promote T-helper 1 and T-helper 17 immune responses. Cytotherapy. 2012; DOI:10.3109/14653249.2012.667873.

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ORIGINAL ARTICLES

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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, 14]. 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 1.8×10^6/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 (5×10^5 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

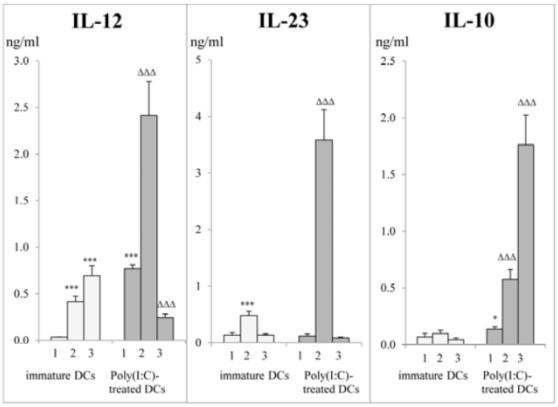

Fig. 1 - Cytokine production by immature human monocyte-derived dendritic cells (MoDCs) and polynosinic-polycytidylic acid – Poly(I:C)-treated MoDCs activated by CD40 ligation and interferon (IFN)-γ

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.

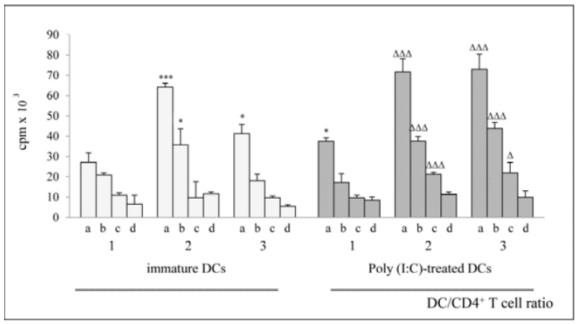

Fig. 2 - Allostimulatory activity of immature human monocyte-derived dendritic cells (MoDCs) and polynosinic-polycytidylic acid – Poly(I:C)-treated MoDCs stimulated with CD40L-transfected J558 cells and interferon (IFN)-γ

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 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].

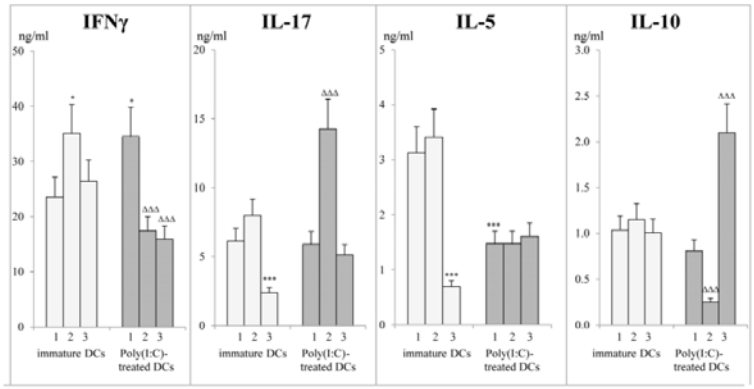

Fig. 3 - Polarization of Th immune response by immature human monocyte-derived dendritic cells (MoDCs) and polynosinic-polycytidylic acid – Poly(I:C)-treated MoDCs activated with CD40L-transfected cells and interferon (IFN)-γ
Production of cytokines in MoDC/CD4$^+$ T-cell cocultures was measured using 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

(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.

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

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.

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The response of human dendritic cells to co-ligation of pattern-recognition receptors

Tanja Dzopalic, Ivan Rajkovic, Ana Dragicevic and Miodrag Colic

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Keywords Monocyte-derived dendritic cells - Pattern-recognition receptors - Toll-like receptors - C-type lectins - Immunotherapy

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Introduction

Dendritic cells (DCs) are bone marrow-derived antigen-presenting cells (APCs) that represent a key bridge between innate and adaptive immunity [1]. These cells have a unique capability to activate naïve T cells and to initiate $CD4^+$ T helper (Th), $CD8^+$ cytotoxic T lymphocytes (CTLs) and T-cell dependent antibody responses [1]. The antigen-presenting function of DCs is manifested by capturing and processing antigens into peptides that are displayed on their surface for presentation to T cells after migration to secondary lymphoid organs [2]. DCs capture antigens based on the expression of a wide variety of pattern-recognition receptors (PRRs), which discriminate self tissues from infectious nonself through pathogen-associated molecular patterns (PAMPs) recognition. There are several described PRRs, including Toll-like receptors (TLRs), C-type lectins (CLRs), cytoplasmic retinoic acid–inducible gene-I-like receptors (RLRs), nucleotide oligomerization domain-like receptors (NLRs) and many others [3–5]. Activation of PRRs triggers signaling pathways, which is followed by phenotypic changes and functional maturation of DCs.

Mature MoDCs acquire migratory properties by modulation of the expression pattern of receptors for chemokines, down-regulate phagocytosis and endocytosis, and up-regulate expression of HLA class I and class II molecules, adhesion molecules (CD54), co-stimulatory molecules (CD80 and CD86) and maturation markers, the properties necessary for their optimal T-cell polarization capacity [6]. T-cell polarization to either Th1, Th2 or Th17 profile depends on the type of co-stimulatory molecules and cytokines expressed by the DCs, which is in turn determined by the TLR signaling profile [7].

DCs are a heterogeneous group of cells comprising functionally distinct subtypes. Based on their differences in function, localization and phenotype, two main populations

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of DCs in human peripheral blood have been described: CD11c$^+$ myeloid DCs (mDCs), which include Langerhans cells, dermal and interstitial DCs, and CD11c$^-$/CD123$^+$/CD4$^+$ plasmacytoid DCs (pDCs) [8]. Due to the expression of distinct PRRs capable for responding to various pathogens, both of them are able to initiate characteristic T-cell responses [6]. The functional plasticity and diversity of these DC subsets make them potentially very useful in designing effective cancer vaccines [9].

At least two mDC subtypes that specifically populate epithelial tissues can be described: Langerhans cells (LCs) and interstitial dendritic cells (IDCs). LCs resident in epidermis and mucosal epithelia are characterized by high expression of CD1a, Langerin molecules and E-cadherin. CD1a family of proteins binds and presents lipids while Langerin (CD207) receptors make contact with mannose and related sugars. After establishing contact with soluble or cell-surface antigens, Langerin molecules form Birbeck granules (characteristic tennis-racquet shape structures), which are involved in the process of alternative antigen presentation to T cells and/or their further endosomal processing. E-cadherin is an adhesion molecule that anchors epidermal LCs to keratinocytes and epithelial T cells [10–12].

As APCs of myeloid origin, both cell populations share some basic functional features with other DC populations [13]. After the uptake and processing of foreign pathogens that penetrate epithelia, resident LCs and IDCs mature during migration toward the local lymph nodes thus acquiring the capability to prime T cells. It was shown in ex vivo experiments that during this process LCs up-regulate MHC II molecules, co-stimulatory molecules and CC-chemokine receptor 7 (CCR-7) while down-regulating CD1a, Langerin, E-cadherin and CD54 [12, 14]. It is believed that migration of these populations can be initiated independently of maturation after the exposition to self-antigens [15, 16]. Immature LCs and IDCs can probably promote peripheral tolerance in the nearby lymph node either by inducing anergy or apoptosis of self-reactive naïve T-cell clones or by induction of T regulatory cells (Tregs) [17]. The roles of LCs and IDCs to prime naïve T cells or to induce anergy of T cells can be explained by specific TLR profiles and other PRRs of these cells.

Since the extent of circulating DCs in human blood is very low, the most commonly used approach exploit in vitro-generated monocyte-derived DCs (MoDCs) [18]. It includes cultivation of monocytes, obtained from peripheral blood mononuclear cells (PBMCs), with recombinant granulocyte macrophage-colony-stimulating factor (GM-CSF) and interleukin (IL)-4, which are able to differentiate into immature MoDCs after 5–6 days [19]. The addition of transforming growth factor β (TGF-β) to this cytokine cocktail results in the generation of Langerhans-like DCs (MoLCs) [20].

The aim of this review is to provide insights into the response of in vitro-generated MoDCs and MoLCs to the co-ligation of PRRs, as a basis for new immunotherapy perspectives.

TLRs

The evolutionary conserved TLRs are best-characterized PRRs, which discriminate self from nonself, providing the first line of defense against invading pathogens. Although a part of the innate immunity, TLRs initiate long-standing and protective immunity by presenting antigen motifs to the cells of adaptive immune system [21]. To date, 10 and 12 functionally active TLRs have been identified in humans and mice, respectively. They are most commonly divided into two groups depending on their cellular localization and PAMP ligands. One group refers to TLRs expressed on cell surfaces and recognize mainly microbial membrane components (TLR1, TLR2, TLR4, TLR5, TLR6). The other group consisting of TLR3, TLR7, TLR8 and TLR9 is expressed in intracellular vesicles (endosomes, lysosomes, endoplasmic reticulum) where they recognize microbial nucleic acids [22]. The TLRs are expressed by various immunologically relevant cell types, including DCs, macrophages and B cells [23], but DCs are particularly important for all aspects of TLR function.

TLRs are a family of transmembrane receptors consisting of ectodomains with multiple leucine-rich repeats (LRRs), linked by a transmembrane domain to a conserved cytosolic domain called the Toll/IL-1 receptor homology (TIR) domain [21]. The super-family of TIR domain-containing receptors also includes the IL-1R/IL-18R family, which act by recruiting and homodimerizing with TIR domain-containing adaptors proteins. Myeloid differentiation protein 88 (MyD88), as a major adaptor protein linking these receptors, is required for signaling through all TLRs, except TLR3, which acts through the adaptor molecule Toll/IL-1R domain-containing adaptor-inducing interferon (IFN)-β (TRIF), while TLR4 signals through both MyD88- and TRIF-dependent pathways. Recruitment of MyD88 leads to the activation of IL-1 receptor-associated kinases (IRAKs) and TNF receptor-associated factor 6 (TRAF6), consecutively activating transcription factors, such as nuclear factor-κB (NF-κB) [24]. TLRs are widely expressed on immune cells and possess distinctive functions depending on the cell type and signaling pathways [25]. TLR agonists, as maturation agents for DCs, have been tested as an attractive agent for initiating or boosting the anti-tumor response [26].

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TLR2, expressed by both MoDCs and mDCs [27], is involved in the recognition of a wide spectrum of microbial molecules, such as Gram-positive and Gram-negative bacteria, fungi, parasites and viruses [28]. Due to the formation of heterodimers with TLR1 and TLR6, TLR2 has a capacity to bind a variety of ligands [29]. TLR2/TLR1 and TLR2/TLR6 activation leads to DC maturation and NF-κB activation through recruitment of MyD88, and subsequent production of numerous cytokines [30]. Recent study has shown that, through activation of specific pathways involving TLR2, zymosan can activate DCs [31]. Zymosan is a crude cell-wall component mixture of the baker's yeast extracts from *Saccharomyces cerevisiae*, composed mainly of β-glucans, mannans and chitins [32]. Investigating differential regulation of cytokine production in human DCs, Gerosa et al. [33] have shown that zymosan stimulated IL-23 rather than IL-12p70 production in MoDCs, suggesting its role in Th17 immune response. The same study reported that low levels of IL-12p70 did not affect Th1 response, induced by zymosan-stimulated MoDCs. Wei et al. [32] have shown that this TLR2 ligand can promote phenotypic maturation of human MoDCs, followed by an increased allostimulatory capacity and production of both pro- and anti-inflammatory cytokines.

It has been shown that freshly purified LCs and MoLCs display TLR2 and its associated receptors TLR1 and TLR6, but no TLR4 [34]. This expression pattern was also similar to that of the freshly isolated LCs from human skin [35]. After stimulation with a TLR2 agonist, peptidoglycan (PGN), MoLCs weakly up-regulated maturation markers CD80, CD86 and HLA-DR and produced smaller amounts of IL-12p70 and IL-10 when compared to MoDCs. Stimulation with a TLR4 agonist, lipopolysaccharide (LPS), did not show any measurable production of these cytokines, nor changes in the expression of maturation markers, when compared to control [36]. Since Gram-positive bacterial species are the most important commensal bacteria in skin that can also cause severe systemic infections, it is most likely that production of IL-10 after TLR2 ligation with PGN can be required for avoiding reaction against commensals. [34]. Experiments on murine DCs supported this hypothesis because TLR2$^{-/-}$ mice showed impaired immunity against *Staphilococcus aureus* [37]. Recent studies imply that LCs activated by TLR2 ligands can also induce Th17 polarization of allogeneic CD4^{+}T cells [35].

TLR3 is a PRR highly expressed in immature MoDCs [29]. Ligation of TLR3 by its specific agonist double-stranded (ds)RNA, that signals the presence of infectious agent, is followed by activation of DCs and induction of protection to viral cytopathic effect [38]. Polyinosinic-polycytidylic acid (Poly (I:C)) is a synthetic analogue of dsRNA and a TLR3 agonist. Studies so far have shown that Poly (I:C) induces phenotypic maturation of MoDCs [39]. Therefore, Poly (I:C) is an appropriate maturation agent for MoDCs. Poly (I:C)-treated MoDCs are in vitro and in vivo potent producers of bioactive IL-12, a crucial initiator of the Th1 response [39] and weak producers of IL-10, an anti-inflammatory cytokine that drive T cells toward Th2 and T regulatory cell (Treg) response [40, 41]. We have found that stimulation of TLR3 increases the production of IL-17, a signature cytokine of the Th17 subset of CD4^{+} T cells whose expansion and maturation are promoted by IL-23, which is in consistence with results published by Sallusto et al. [42]. Collectively, stimulation of MoDCs with Poly (I:C) polarizes the immune response toward strong Th1 and moderate Th17 responses, respectively.

LC-like DCs derived from human cord blood express TLR3 receptors [43]. Recent study with bafilomycin, an inhibitor of endosomal acidification, on LCs isolated from human skin, showed that TLR3 is responsible for the effects of Poly(I:C) on the induction of maturation and allostimulatory properties and Th1 polarizing capability of LCs [44]. Our results on MoLCs mostly corroborated these results, but an interesting finding was that Poly (I:C) did not modulate Th2 or Th17 polarization compared to control (unpublished data).

TLR4, as a founding member of the human TLR family, was described as essential for recognition of bacterial LPS, a component of the outer membrane of Gram-negative bacteria [21]. TLR4 interacts with three different extracellular proteins—LPS binding protein (LBP), CD14 and myeloid differentiation protein 2 (MD-2)—to induce a signaling cascade leading to the activation of both the MyD88- and TRIF-dependent pathways. This activation is necessary for the induction of inflammatory cytokines. LPS is one of the most commonly used compounds for the induction of DC maturation, which in turn produce more cytokines and express co-stimulatory molecules to activate T cells [45, 46]. LPS also stimulates macrophages to produce proinflammatory cytokines [47]. Such treated MoDCs produce moderate levels of IL-12, but also IL-10 [46]. TLR4 signaling pathway can mediate a signal from dying tumor cells that causes DCs to mature and elicit phagocytosis [48]. Since the immune response activated from dying tumor cells is essential for a successful cancer therapy, TLR4 agonists could be tested for the enhancement of current chemotherapeutic regimens [49].

TLR5 is distinctive for its ability in recognizing the flagellin protein component of bacterial flagella [28]. Intestinal epithelial cells, as well as lamina propria DCs, express TLR5 in a wide range, suggesting its role in the detection of flagellated bacteria in the gut [24]. The expression of TLR5 on MoDCs and mDCs has been described as well [50]. Study by Means et al. [51] have shown that ligation of TLR5 by flagellin could induce phenotypic maturation of MoDCs, as judged by increased

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expression of maturation and co-stimulatory markers. The same group reported that flagellin-matured DCs displayed an enhanced T-cell stimulatory capacity with a concomitant decrease in the endocytic activity. Recent study showed that stimulated DCs from lamina propria by flagellin are good stimulators of Th1 and Th17 immune responses [52].

TLR7 and TLR8 are natural receptors for single-stranded (ss) RNA and, together with TLR3 and TLR9, act as powerful activators of the innate immune response upon viral infections [53]. Besides ssRNA, several chemical compounds have been described as TLR7/8 agonists, such as resiquimod, CL075 and CL097 [54]. Stimulation of MoDCs with these compounds resulted in their maturation with the capability for polarization of the immune response toward Th1 [55]. Therefore, in order to understand the essence of MoDCs activation by common TLR7/8 ligands, it is important to evaluate the effects of selective TLR7 and TLR8 agonists. There are two well-known groups of selective TLR7 agonists, imidazoquinolines (imiquimod and gardiquimod) and guanosine derivatives (7-allyl-7,8-dihydro-8-oxo-guanosine (loxoribine) and 7-thia-8-oxo-guanosine (7-TOG)) [54]. The data regarding their effect on MoDCs are controversial. Namely, a number of studies showed that selective TLR7 ligands could stimulate only preactivated MoDCs [56, 57]. The other group of authors described that imidazoquinolines inhibited differentiation, but enhanced maturation of human MoDCs [58]. Our group studied selective TLR7 agonists (loxoribine and 7-TOG) and showed that loxoribine, at relatively high concentrations (250 μM), was able to induce maturation of MoDCs without additional stimuli and the effect was partly dependent on the up-regulation of TLR7 expression. In addition, loxoribine-treated MoDCs were potent inducers of Th1 and Th17 immune responses [59].

In our previous in vitro studies in rodents, we described 7-TOG as a strong modulator of the immune response [60, 61]. Later investigations into human monocytes demonstrated significantly augmented stimulatory potential of 7-TOG-treated monocytes on T-cell proliferation, which was partially dependent on IL-12 production. Different TLR7 expression in rodent and human, as well as possible dosage-dependance and characteristic chemical structure, led us to further investigate the modulatory activity of 7-TOG on human MoDCs. We showed that this guanosine analogue was able to activate immature MoDCs in a strong dose-dependent manner. Namely, the highest applied concentration of 7-TOG (250 μM) was a potent inducer of MoDCs maturation, which directed the immune response toward Th1 and Th17. The intermediate concentration of 7-TOG (100 μM) was, however, a weak stimulator of MoDCs maturation, and such treated MoDCs stimulated the Th17 response. On the contrary, the lowest applied concentration of 7-TOG (25 μM) did not trigger maturation of MoDCs, but primed these cells to polarize the immune response toward Th2 (unpublished data). Due to the endosomal localization of TLR7, delivery of agonists to endosome is significantly facilitated through the process of phagocytosis or endocytosis. This could be advantageous in increasing the intracellular concentrations of the compounds and subsequent better DCs activation. Therefore, our research group was studying multi-walled carbon nanotubes (MWCNTs) as a delivery system of 7-TOG to endosomal TLR7 in human MoDCs, when different concentrations of 7-TOG were covalently attached to MWCNTs by the original method [62]. Light, confocal and transmission electron microscopic observations confirmed efficient phagocytosis of 7-TOG-MWCNT nanoparticles (Fig. 1). This delivery system showed that low concentrations of 7-TOG attached to MWCNTs was able to promote both Th1 and Th17 immune responses, as efficiently as ten times higher concentrations of soluble 7-TOG (unpublished data). These findings support the hypothesis that functionalized MWCNTs may be a challenging system for delivery of 7-TOG to MoDCs, in order to improve the protocols for preparation of DC vaccines.

TLR9 recognizes specific unmethylated 2′-deoxyribo (cytidine-phosphateguanosine) (CpG) DNA motifs that distinguish microbial DNA from mammalian DNA [63]. CpG-containing oligonucleotides (ODNs) can mimic natural CpG sequences and are used as TLR9 ligands [64]. In humans, TLR9 can be found on pDCs, activated monocytes, NK cells and B-lymphocytes [64].Until recently, it was believed that MoDCs and mDCs do not express TLR9 [65]. However, using synthetic CpG-A ODN D19, Hoene et al. [66] proved the existence of TLR9 in MoDCs, in amounts comparable with pDCs. It has been shown that ligation of TLR9 induces maturation of MoDCs, with a subsequent increased T-cell proliferation and Th1 differentiation. Data about the modulatory effects of TLR9 agonists on MoLCs are scarce.

Co-ligation of TLRs

Although triggering of single TLRs results in phenotypic and functional changes in DCs, cooperation between multiple TLRs is needed in order to achieve an effective immune response. Therefore, numerous studies using simultaneous engagement of multiple TLRs were performed.

The co-ligation of TLR2 and TLR4 caused greater production of TNF-α and IL-6 by human monocytes than ligation of single receptors did [67, 68]. Further results indicated the secretion of additional levels of TNF-α, IL-6 and IL-12p40 if Poly (I:C) and CpG DNA simultaneously

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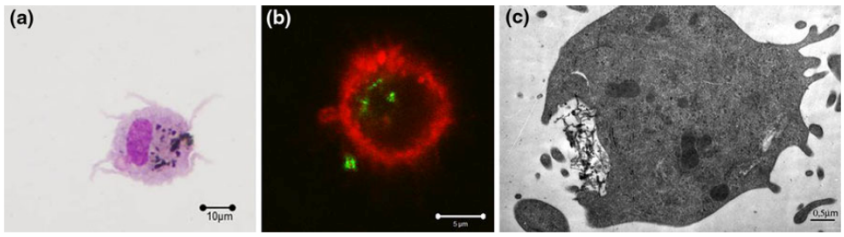

Fig. 1 Internalization of 7-TOG attached to MWCNTs by MoDCs. MoDCs were generated by cultivation of human monocytes in the presence of GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) for 6 days and functionalized MWCNTs with 7-TOG for another 48 h. **a** In order to evaluate ingestion of MWCNTs by MoDCs, cytospins were prepared and observed by light microscopy. *Black dots* inside the cell are presumed to be ingested nanoparticles; **b** For confirmation, cytospins were processed for confocal microscopy. Fluorescent 7-TOG-MWCNTs, coupled with FITC, are seen inside the HLA-DR/Alexa 548^{+} cell; **c** Finally, MoDCs cultivated with 7-TOG-MWNCTs were evaluated by transmission electron microscopy (TEM) after appropriate processing. TEM micrograph shows protrusion of cell membrane, forming coated pit with ingested nanoparticles

activated mouse macrophages, suggesting, for the first time, that multiple TLR ligands increase the host resistance [69]. Napolitani et al. [70] are among the first describing the strong synergistic effects of TLR ligands on cytokine production by human DCs. Namely, this group showed that TLR3 or TLR4 potently acted in synergy with TLR7/8 in the enhancement of IL-12 and IL-23 production by human MoDCs, which resulted in additional potentiation of Th1 polarizing capacity. These findings were explained by the fact that simultaneous activation of NF-κB, interferon regulatory factors (IRFs), mitogen-activated protein (MAP) kinases (MAPK), phosphoinositide 3-kinase (PI-3 K) and Signal Transducer and Activator of Transcription (STAT) signaling pathways are involved in this process, suggesting a crucial role for the synergistic gene expression during the combined TLR3 or TLR4 and TLR7/8 ligand stimulation [71].

Similar effects on cytokine production by MoDCs have also been reported for co-ligation of TLR3 or TLR4 with TLR2 (PGN), TLR2/TLR6 (zymosan) and TLR5 (flagellin) [72]. Namely, it has been shown that MoDCs combine and integrate signals received via the IFN-dependent pathway by engagement of TLR3 and activation of TRIF with the MyD88-dependent pathway by ligation of TLR2 (PGN), TLR2/TLR6 and TLR5.

The other group of authors reported that TLR2 is able to restrain the TLR4- and/or TLR7/8-induced production of proinflammatory cytokines, while leaving the cytokines induced by TLR3 or TLR5 unchanged [73]. They also revealed that abrogating the IL-12p70 production by DCs, TLR2 exerts its suppressive function. This inhibition of IL-12p70 release by TLR2 led to an increased DC-mediated T-cell differentiation into Th2 and Th17 cells. Therefore, it can be assumed that the modulation of TLR2-mediated signaling in cooperation with other TLR ligands might provide novel insights for interventions within immune diseases.

Based on our previous papers, where the effects of single TLR3 and TLR7 agonists on MoDCs were investigated [59, 74], we tried to determine whether these agonists, triggering different pathways, may cooperate in MoDC activation. Therefore, we combined suboptimal (10 μg/ml) or optimal (25 μg/ml) concentrations of Poly (I:C) with suboptimal (100 μM) and optimal (250 μM) concentrations of loxoribine. The effect of each combination on phenotypic properties and cytokine production by MoDCs and their Th polarizing capability was studied, and some of these results are presented in Fig. 2.

The first combination of suboptimal concentrations of Poly (I:C) and loxoribine induced maturation of MoDCs, directing the immune response toward Th1, as documented on the basis of levels of IFN-γ in co-culture with allogeneic $CD4^{+}$T cells. Higher production of IFN-γ correlated with the levels of IL-27 and IL-12, which is in agreement with previous study where IL-27, by synergizing with IL-12p70, participates in the expansion and survival of Th1 cells [75]. Such treated MoDCs also potentiated the production of IL-17 by $CD4^{+}$T cells, which correlated with significantly higher amounts of IL-23 in MoDC cultures, compared to the levels of the cytokines induced by suboptimal concentrations of single agonists. This Th17 profile was somewhat expected, knowing that final differentiation of Th17 cells partially depends on IL-23 [76].

Cultivation of MoDCs with a combination of both optimal concentrations of TLR agonists primed them to polarize the immune response toward Th1 with the significant production of IFN-γ by allogeneic $CD4^{+}$T cells. This finding also correlated with the increased levels of IL-12 and IL-27 [77]. This induction of Th1-polarizing signals was not followed by up-regulation of HLA-DR and

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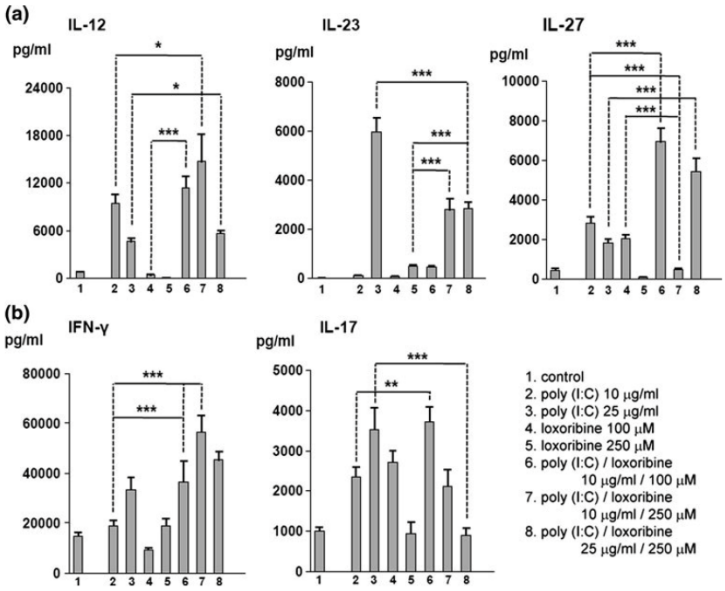

Fig. 2 The effect of TLR3 and TLR7 co-ligation on cytokine production and Th polarization capability of MoDCs. MoDCs were obtained by cultivation of human monocytes for 6 days with GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) and then stimulated with Poly (I:C) and loxoribine in different concentrations for an additional 48 h. **a** Levels of secreted IL-12, IL-23 and IL-27 were determined in cell-free supernatants by ELISA; **b** Allogeneic $CD4^+$T cells were co-cultured with MoDCs (1×10^4 cells/well) at a concentration of 1×10^5 cells/well. Co-cultivation lasted 5 days, when PMA (20 ng/ml) and ionomycin (500 ng/ml) were added for an additional 8 h. Levels of IFN-γ and IL-17 were measured in $CD4^+$T-cell/DC co-culture supernatants using sandwich ELISA kits following the manufacturer's instructions. Cytokine levels are given as $x \pm$ S.D. ($n = 3$ donors). *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ compared to relevant controls

co-stimulatory molecules. On the contrary, their expression was down-regulated, compared to the effect of single agonists. A similar finding was described by Napolitani et al. [70] who suggested an existence of different induction thresholds between these two "programs" and indicated that such co-ligation may represent a so-called "security code," which ensures powerful effectors (Th1 response) to be generated only in response to invading pathogens. In addition, distinct cellular compartment of TLR3 and TLR7 could be of importance, when dsRNA can initiate TLR3 and prime DCs to a subsequent activation of TLR7 in the endosomal compartment. On the other hand, such treated MoDCs decreased the production of IL-23, compared to the effect of single Poly (I:C), and the effect was followed by decreased production of IL-17 in co-culture with $CD4^+$T cells. The enhancement of IL-27 production could be responsible for down-regulation of IL-23, according to literature data [78]. High amounts of IL-27 could be also explained by suppression of IL-4 production and subsequent decrease in the Th2 cell differentiation [79]. When a suboptimal concentration of poly (I:C) was combined with an optimal loxoribine concentration to activate MoDCs, the Th1 immune response was mostly pronounced and followed by increased levels of IL-12 in MoDC cultures. These findings confirmed again the fact that DCs have very large capacity to produce IL-12 that is "deployed," only in the response to multiple stimuli acting simultaneously [70].

Ligation of TLR3 and TLR7 by their specific ligands results in conformational changes in the receptors, leading to downstream signal transduction, which primarily involves MyD88- and TRIF-dependent pathways [80]. Namely, it is known that signaling through TLR3 and TLR7 differs. Activation of TLR3 leads to the induction of IRF3, NF-κB and MAPK through the engagement of TRIF adapter protein, whereas TLR7 signaling activate NF-κB via a MyD88-dependent pathway and activation of IRF7 [28]. Furthermore, it has been shown that ongoing stimulation through the TRIF-dependent pathway is required for the enhancement of the MyD88-dependent DC function

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and for the T-cell activation [81]. Based on these findings, we postulated that maybe combination of these signaling pathways could modulate the final outcome of the immune response. Namely, the MyD88-independent pathway corresponding to signal 2 increases the T-cell priming, but the MyD88-dependent pathway, as signal 3, determines a T-cell response [81]. This crosstalk may be established to avoid induction of unnecessary activation of immune responses while allowing an immune response against tumors.

C-type lectin receptors (CLRs)

C-type lectin receptors are family of both soluble and transmembrane proteins that possess at least one carbohydrate recognition domain (CRD) with unique specificity. They provide DCs with the ability to recognize mannose, fucose and glucan carbohydrate structures that are part of the most pathogens [82]. CLRs can be divided into two groups according to their molecular structures. Group I has its amino (N) terminus pointing outwards of the cell and has several CRDs at the carboxyl (C) terminus. It is named the mannose receptor (MR) family, which includes mannose receptor (CD206) and DEC-205 (CD205) [4]. Group II has its N terminus pointing into the cytoplasm of the cell and has single CRD. It is called the asialoglycoprotein receptor family and comprises the DC-specific ICAM3-grabbing nonintegrin (DC-SIGN, CD209), Langerin (CD207) and the members of Dectin-1 and DC immunoreceptor (DCIR) families. The Dectin-1 family includes Dectin-1, lectin-like oxidized low-density lipoprotein receptor (LOX-1), C-type lectin receptors 1 and 2 (CLEC-1 and 2) and myeloid inhibitory C-type lectin-like receptor (MICL), while DCIR family includes DCIR, DC immunoactivating receptor (DCAR), Dectin-2 and blood DC antigen protein 2 (BDCA-2) [83, 84].

Triggering of CLR by different pathogens can induce gene expression independently of other PRR signaling (Dectin-1, Langerin) or, by contrast, induce signaling pathways that can only modulate the TLR-induced gene expression (DC-SIGN, BDCA2, DCIR and MICL). Common feature of both signaling pathways is the induction of NF-κB, a key mediator of inducible gene expression in the immune system, although other transcription factors contribute to the CLR-induced signal transduction as well [28, 85].

Dectin-1 is one of many PRRs that mediate their own signaling and can synergize with TLRs to initiate specific responses to pathogens [86]. Dectin-1 was first identified as a DC-associated C-type lectin, containing a single extracellular CRD motif at the COOH-terminal end that recognizes ß-1,3-glucans found mainly in the cell walls of fungi and an immunoreceptor tyrosine-based activation motif-like sequence (hemITAM) in the cytoplasmic domain [87], necessary for intracellular signal generation [88]. Subsequent studies have shown that mouse Dectin-1 is highly expressed on neutrophils, alveolar macrophages and inflammatory macrophages with lower expression on resident macrophages, DCs and some T cells [89]. Human Dectin-1 is expressed additionally on B cells. Dectin-1 recognizes soluble and particulate β-glucan [87, 89] that makes up to 50% of the fungal cell wall. Activation of the Dectin-1 receptor is followed by the recruitment of the tyrosine kinase Syk by hemiITAM sequence in the cytoplasmic domain. This initiates downstream signaling cascade that culminates with the activation of NF-κB, the transcription factor NFAT and MAPK [90]. Upon ligand recognition, Dectin-1 can induce diverse responses such as an oxidative burst, inflammatory cytokine production and phagocytosis [88].

The role of Dectin-1 in modulating the biological functions, of human DCs, is reported in only few studies. It has been shown that mouse DCs stimulated with curdlan, a Dectin-1 agonist, increased co-stimulatory molecules (CD80, CD86 and CD40) [90]. These results are similar to those showing an up-regulation of CD40 and CD86 by human DCs treated with *Candida albicans*. It is known that the response of DCs to *C. albicans* is largely dependent on Dectin-1 recognition [91]. Our results (unpublished data) support these findings and additionally showed an up-regulation of HLA-DR and CD83 expression by curdlan-treated MoDCs. Collectively, all these data demonstrated that Dectin-1 could be instrumental in promoting an efficient antigen presentation and that curdlan, through activation of Dectin-1, induce phenotypic maturation of human MoDCs. Previous studies on human MoDCs have shown that stimulation of Dectin-1 signaling pathway with curdlan increases the production of IL-6, TNF-α, IL-1β, IL-10, IL-12p35, IL-23p19 and IL-12p40 [92]. Activation of the Dectin-1 pathway in our study increased the production of IL-27 and lowered the production of IL-10 by MoDCs, while the levels of IL-6, TNF-α and IL-23 were unchanged, compared to control. Although the levels of both IL-12 and IL-23 were very low or undetectable, an intriguing finding of our study and a previous publication [90] was the ability of curdlan to promote Th1 and Th17 polarizing capabilities of MoDCs.

Dectin-2 is mostly expressed on immature MoLCs and to a lesser extent on immature MoDCs. Maturation of MoLCs is followed by reduced expression of Dectin-2, while the opposite effect was observed on MoDCs [93]. This receptor is also detected in monocytes, macrophages, B cells and neutrophils [94]. Triggering the Dectin-2 receptor with fungal hyphae from *Candida*, *Trichophyton* and *Microsporum species* leads to the production of TNF-α and IL-6, independently on other TLRs by DCs [95].

DC-SIGN (CD209) was first identified as an intercellular binding molecule, necessary for establishing the DC/

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T-cell contact, which influences the T-cell proliferation and for binding and presentation of unprocessed HIV-1 virions to $CD4^+$ T cells [4, 96]. DC-SIGN recognizes mannose- and fucose-containing pathogens and modulates TLR3-, TLR4- and TLR5-induced immune responses by DCs. Activation of this receptor induces an up-regulation of the TLR-induced IL-10 production [97].

Recent studies have shown that Langerin has a similar role on LCs as Dectin-1 receptor and DC-SIGN have on DCs. Namely, although LCs also express Dectin-1 it was found that Langerin is the main CLR for fungi [98]. Langerin displays extracellular C-terminal CRD and transmembrane anchor segment that are similar to Dectin-1. Oligomerization of three Langerin molecules via the α-helicoidal neck region that connects aforementioned segments is responsible for binding specificity of this receptor to glycoprotein ligands. In addition, this extracellular domains promote folding and superimposition of plasma membrane that lead to the formation of ultramicroscopically characteristic Birbeck granules [99]. The granules can play a possible role as the subsequent intracellular storage that shelter material bound to Langerin or CD1a molecules from degradation until LCs receive exact activation signal. It was shown in vitro that Langerin and Birbeck granules are simultaneously down-regulated while MHC class II and CD86 are up-regulated after maturation of LC maturation influenced by CD40 ligation. Since proinflammatory signals, such as TNF-α, increase the expression of Langerin, it can be postulated that migration and maturation of LCs in lymph nodes can be independently regulated. This finding may also explain the presence of Langrin + cells outside of skin or mucosa [11]. Langerin lacks an intracellular tyrosine-based motif that acts as a potential signal transduction site. It contains a sequence of proline-rich repeats, which is known to interact with SH3 (src homology 3) domain proteins that regulate cytoskeleton movements connected with vesicular transport [99]. Similarly as DC-SIGN, Langerin can introduce HIV-1 virion to T cells and at the same time prevent their infection [100].

Recent ex vivo experiments on human LCs confirmed that not only Dectin-1 but also Langerin can interact with β-glucans [101]. Curdlan was shown to strongly bind to human LCs. Since LCs isolated from epidermis expressed both Langerin and Dectin-1 receptors, but no DC-SIGN, it is possible that the main CLR for fungi on LCs could be Langerin [98]. Our study showed that MoLCs stimulated with curdlan induced stronger Th1, Th2 and Th17 responses compared to control, nonstimulated MoLCs (unpublished data). This result is very similar to the effect that we have found in our previous experiment when MoLCs were matured with classic cocktail of proinflammatory cytokines and modulators [15].

BDCA2 is considered as a specific marker of pDCs, known for their ability to produce large amount of type I IFNs upon viral infection [102]. One of the proposed mechanisms of the immune escape by viruses is triggering the BDCA2 molecule. BDCA2 ligation following TLR3 or TLR9 activation leads to up-regulation of IL-10 production and suppression of IL-6 and TNF-α [102].

DCIR and MICL are the only known CLRs that contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domain [85]. Studies based on the use of antibodies for this ITIM-bearing CLRs have shown that activation of DCIR inhibits TLR8-mediated production of IL-12 and TNF-α by myeloid DCs and TLR9-induced production of IFN-α and TNF-α by pDCs. The activation of MICL suppresses TLR4-induced IL-12 production [103, 104]. DCIR is more expressed on $CD14^+/CD1a^-$ MoDCs (correspond to IDDCs) than on $CD1a^+CD14^-$ MoLCs (correspond to LCs). DCIR surface level does not change during differentiation from monocytes to immature MoDCs, but it is down-regulated after stimulation with LPS or CD40L [105]. On the other hand, triggering of MICL augmented CD40L-mediated Th1 response, but inhibited the LPS-mediated response [104].

Co-ligation of TLRs and CLRs

Recent studies have shown that the treatment of MoDCs with a TLR3 ligand, Poly (I:C), or Dectin-1 agonist, curdlan, elicits their maturation into the cells competent to prime effective immune responses. Since individual agonists exert different outcome on immunomodulation, we have performed a study in order to examine the response of MoDCs to the combined effect of Poly (I:C) and curdlan. Our results showed that co-ligation of TLR3 and Dectin-1 leads to the phenotypical maturation of MoDCs, which was similar to the effect of Poly (I:C) alone, except an additional increase in the expression of CCR7. This chemokine receptor could be relevant to improved migratory properties of DCs. MoDCs treated with both ligands up-regulated the production of interleukin IL-12, IL-23 and IL-10, compared to the effect of Poly (I:C), alone. Curdlan-treated MoDCs stimulated the production of IL-17 by alloreactive $CD4^+$ T cells more strongly than Poly (I:C)-treated MoDCs. The opposite effect was observed for IFN-γ production. When combined, these agonists primed MoDCs to further increase the production of IFN-γ by $CD4^+$ T cells in co-culture, especially those of naïve ($CD45RA^+$) phenotype, and IL-17 by memory ($CD45RO^+$) $CD4^+$ T cells (unpublished results). Although no microbe or fungal pathogen that expresses or generates ligands for both TLR3 and Dectin-1 has been found so far, our findings can be

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taken into consideration for development of new strategies in the field of immunotherapy (Fig. 3).

To our knowledge, there is no available data considering the effect of simultaneous activation of TLR3 and Langerin either on LCs or on MoLCs. We have shown in our experiments on MoLCs that this combination diminished allostimulatory potential of MoLCs and at the same time reduced their Th1 and Th17 polarizing capability (unpublished data). This finding still has to be thoroughly analyzed and reevaluated in the next experiments.

NLR and RLR family of PRRs

The other group of non-TLR PRRs (NLR and RLR family) refers to the cytosolic receptors involved in the recognition of microbial components as well. The NLR family includes 22 proteins divided into the group of NACHT, LRR and PYD containing proteins (NALPs), IL-1β-converting enzyme protease-activating factor (IPAF) and nucleotide oligomerization domain (NOD). Signaling through the members of this family is very complex. Generally,

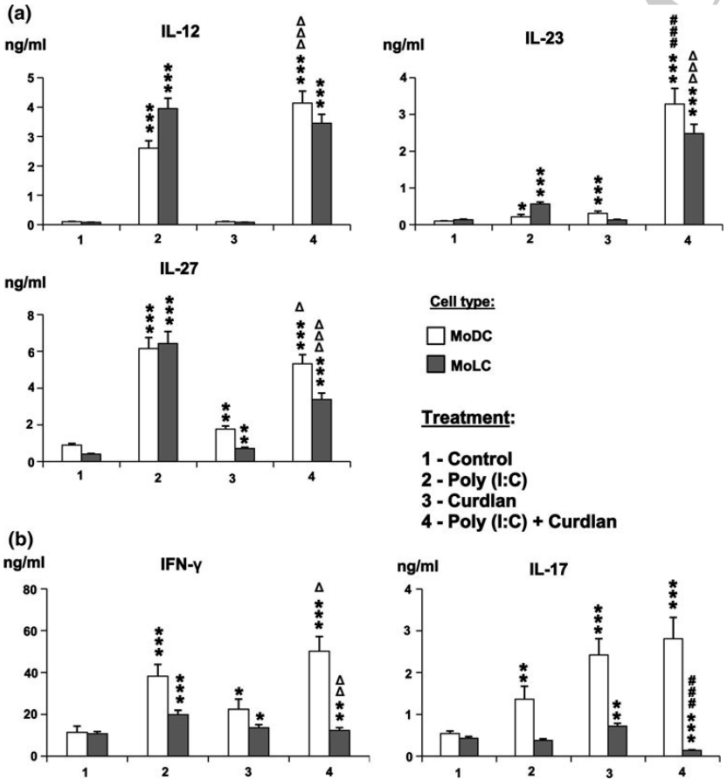

Fig. 3 The combined effect of Poly (I:C) and curdlan on the cytokine production and Th polarization capability of MoDCs and MoLCs. **a** Cultivation of human monocytes for 6 days with GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) generated immature MoDCs, while addition of TGF-β (10 ng/ml) to this cytokine cocktail generated immature MoLCs. Immature MoDCs and MoLCs were stimulated with Poly (I:C) (25 μg/ml), curdlan (100 μg/ml) or their combination for an additional 48 h. Levels of secreted IL-12, IL-23 and IL-27 were determined in cell-free supernatants by ELISA. Values are given as $x \pm$ S.D. ($n = 3$ donors). **b** Purified CD4$^+$ (1×10^5 cells/well) were co-cultivated with MoDCs or MoLCs (1×10^4 cells/well) for 5 days, when PMA (20 ng/ml) and ionomycin (500 ng/ml) were added for an additional 8 h. After that, cell supernatants were collected and used for IFN-γ and IL-17 detection by ELISA and FlowCytomix Human Th1/Th2 11 plex. Cytokine levels are given as $x \pm$ S.D. ($n = 3$ donors). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$ compared to control MoDCs/MoLCs. $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.005$ compared to Poly (I:C)-treated MoDCs/MoLCs. $^{\#\#\#}p < 0.005$ compared to curdlan-treated MoDCs/MoLCs

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activation of NALPs and IPAF leads to the activation of caspase-1, whereas NOD proteins activate NF-κB [110]. Main members of the RLR family are retinoic acid–inducible gene-I (RIG-I), melanoma differentiation–associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). All these cytoplasmic PRRs recognize viral dsRNA [24]. Activation of RLRs goes through recruitment of the kinase TANK-binding kinase 1 (TBK1) and activation of NF-κB and IRF7 and/or IRF3 with culmination in type I IFN production [106].

A number of papers have described synergistic production of cytokines upon co-ligation of NOD1 and NOD2 with many TLR ligands [107]. As shown, the stimulation of DCs with NOD2 and NOD1 agonists in combination with TLR3, TLR4 and TLR9 ligands synergistically induce IL-12p70. DCs stimulated with TLR4 together with either NOD1 or NOD2 activate human T cells to produce high levels of IFN-γ, suggesting that NOD1 as well as NOD2 signals augment the Th1 polarization induced by various TLR signals [108]. The essence of these interactions involves cooperation of signaling pathways, characteristic for activated PRRs. For example, the combination of NOD2 and TLR2 agonists decreases the production of IL-12p70 by DCs. In contrast, co-ligation of NOD2 and TLR7/8 is followed by increased production of this cytokine. These findings suggest that signal transduction pathways upon the NOD1 and NOD2 engagement are different [109]. Simultaneous engagement of TLRs and NALPs also promote characteristic activating pathways. Thus, NALP3 activation raises caspase-1 with the cleavage of pro-IL-1β and pro-IL-18. Nevertheless, co-ligation between TLR7/8 and NALP3 seems to be necessary for full production of inflammatory cytokines in response to *influenza virus* [110]. It is not quite clear how NODs and TLRs activate synergistically the production of inflammatory cytokines. Since both receptors activate NF-κB, it can be postulated that simultaneous engagement of the receptors triggers stronger activation of NF-κB. However, there are other reports suggesting that the enhanced NF-κB activation is only an additive mechanism and as such unable to explain the synergy in cytokine production. It is assumed that the receptors induce different NF-κB isoforms or alter the kinetics of NF-κB activation, sufficient for synergistic results [5].

To date, there are not many studies facing the pathways of TLRs and RLRs co-ligation. Interactions between RLRs (recognizing viral RNA) and TLR3, TLR7 and TLR9 (recognizing viral dsRNA, ssRNA and DNA, respectively) expressed by human MoDCs are best studied [24]. Activation of either TLR or RLR signaling, or both, is required for adequate immune response against viruses. This largely depends on the type of viruses and probably the route of infection, but also of activated signaling pathway. It has been shown that upon recognition of viral nucleic acid sequences, TLRs and RLRs recruit specific adaptor proteins to activate signaling pathways evolving NF-κB, MAP kinases and IRFs that control the transcription of genes encoding type I IFN and other inflammatory cytokines, important for eliminating viruses [24].

Co-ligation strategy to improve immunogenicity of DCs for therapy

The considerable progress made in the knowledge of DC biology and the outcomes of signaling pathways triggered by different PRRs opens the new opportunities for design of improved clinical protocols. Owing to their capacity to regulate immunity and tolerance, DCs are the attractive candidates to be used as cancer vaccines [111]. The aim of cancer immunotherapy is the elicitation of the immune responses that will overcome the suppression caused by cancer cells, which develop various mechanisms to escape immune surveillance. It should be stated that the immune response relies heavily on the immune-stimulatory capacity of DC subsets being used and the choice of maturation stimuli [112].

A key challenge in the immunotherapeutic strategy is to optimize a maturation cocktail that will combine high yield of DCs, potent expression of maturation markers, co-stimulatory and adhesion molecules together with high production of Th cytokines crucial for the anti-tumor immune response. Active specific immunotherapy based on the injection of DCs generated and maturated ex vivo is the most promising approach for developing the potent and long-lasting patient's immune responses against tumors. Many protocols have been developed so far, but immune tolerance to an established tumor is often the crucial factor that limits their clinical efficacy. Therefore, incomplete or incorrect maturation of DCs with tolerogenic properties can even enhance the tolerance to tumor and exacerbation of the disease [113].

Desirable anti-tumor immune response involves CTLs directed against transformed cells and key anti-tumor mediators, such as chemokines and cytokines. These include IFN-γ whose crucial role in the immune response to tumors has been confirmed [114]. Th1 cells were considered as the most important $CD4^+$ T-cell subset for generating anti-tumor immunity because of their potential to potentiate the IFN-γ- dependent cytotoxic function of $CD8^+$ T cells. This is the reason why studies so far have focused on the applicability of Poly (I:C)-treated MoDCs in cancer immunotherapy [115]. The role of Th17 cells in tumor immunity is still highly controversial, since a series of reports have suggested pro-tumor [116] and anti-tumor functions for IL-17 [117, 118]. Anti-tumor effects of Th17

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cells are attributed to their capability to enhance inflammatory responses and to increase antigen presentation by DCs, promote leukocyte homing to tumors and facilitate the $CD8^+$ T-cell priming and differentiation of these cells into effector CTLs [118].

In order to achieve these goals, we co-stimulated MoDCs with different combinations of PRR agonists, including TLR3/TLR7 and TLR3/Dectin-1, and used nanoparticles as carriers for PRR agonists. We showed that all these approaches were superior in triggering Th1 and Th17 responses, compared to the effect of individual agonists or agonist without carriers. In this context, the engagement of TLR7 by using guanosine analogues, which are nontoxic even at very high concentrations [61], could be a new strategy to improve DC immunogenicity and tumor immunotherapy.

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Signaling through Toll-like receptor 3 and Dectin-1 potentiates the capability of human monocyte-derived dendritic cells to promote T-helper 1 and T-helper 17 immune responses

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Background aims. Recent studies have shown that the ligation of Toll-like receptor 3 (TLR3) or Dectin-1 on human monocyte-derived dendritic cells (MoDC) elicits their maturation, but with a different outcome on immunomodulation. Therefore the aim of this work was to study the response of MoDC to the combined effect of polyinosinic:polycytydilic acid [Poly (I:C)] and curdlan, selective TLR3 and Dectin-1 agonists, respectively. *Methods.* Immature MoDC, generated from human monocytes, were treated with Poly (I:C), curdlan or their combination for 2 days. Phenotypic characteristics of MoDC were determined by flow cytometry, and cytokine production was measured by enzyme-linked immunosorbent assay (ELISA) and FlowCytomix, while the stimulatory capability of MoDC was tested using a mixed leukocyte reaction assay. *Results.* The combination of Poly (I:C) and curdlan induced phenotypic maturation of MoDC with the capability to stimulate an alloreactive response. Such treated MoDC up-regulated the production of interleukin (IL)-12, IL-23 and IL-10, compared with the effect of Poly (I:C) alone. Curdlan-treated MoDC stimulated the production of IL-17 by alloreactive $CD4^+$ T cells more strongly than Poly (I:C)-treated MoDC. The opposite effect was observed for interferon(IFN)-γ production. When combined, these agonists primed MoDC to increase further the production of IFN-γ by $CD4^+$ T cells in co-culture, especially those of naive ($CD45RA^+$) phenotype, and IL-17 by memory ($CD45RO^+$) $CD4^+$ T cells. *Conclusions.* Ligation of TLR3 and Dectin-1 receptor up-regulates T-helper (Th) 1 and Th17 immune responses compared with single agonists. These findings may have therapeutic implications for the use of MoDC in immunotherapy.

Keywords

Dectin-1 receptor, human monocyte-derived dendritic cells, T-helper immune response, Toll-like receptor 3

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Signaling through Toll-like receptor 3 and Dectin-1 potentiates the capability of human monocyte-derived dendritic cells to promote T-helper 1 and T-helper 17 immune responses

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Abstract

Background aims. Recent studies have shown that the ligation of Toll-like receptor 3 (TLR3) or Dectin-1 on human monocyte-derived dendritic cells (MoDC) elicits their maturation, but with a different outcome on immunomodulation. Therefore the aim of this work was to study the response of MoDC to the combined effect of polyinosinic:polycytydilic acid [Poly (I:C)] and curdlan, selective TLR3 and Dectin-1 agonists, respectively. *Methods.* Immature MoDC, generated from human monocytes, were treated with Poly (I:C), curdlan or their combination for 2 days. Phenotypic characteristics of MoDC were determined by flow cytometry, and cytokine production was measured by enzyme-linked immunosorbent assay (ELISA) and FlowCytomix, while the stimulatory capability of MoDC was tested using a mixed leukocyte reaction assay. *Results.* The combination of Poly (I:C) and curdlan induced phenotypic maturation of MoDC with the capability to stimulate an alloreactive response. Such treated MoDC up-regulated the production of interleukin (IL)-12, IL-23 and IL-10, compared with the effect of Poly (I:C) alone. Curdlan-treated MoDC stimulated the production of IL-17 by alloreactive CD4+ T cells more strongly than Poly (I:C)-treated MoDC. The opposite effect was observed for interferon (IFN)-γ production. When combined, these agonists primed MoDC to increase further the production of IFN-γ by CD4+ T cells in co-culture, especially those of naive (CD45RA+) phenotype, and IL-17 by memory (CD45RO+) CD4+ T cells. *Conclusions.* Ligation of TLR3 and Dectin-1 receptor up-regulates T-helper (Th) 1 and Th17 immune responses compared with single agonists. These findings may have therapeutic implications for the use of MoDC in immunotherapy.

Key Words: *Dectin-1 receptor, human monocyte-derived dendritic cells, T-helper immune response, Toll-like receptor 3*

Introduction

Dendritic cells (DC), when activated by antigens of infectious micro-organisms and inflammatory products, are mobile sentinels that capture, process and bring antigens to T cells. DC migrate from the periphery to lymphoid organs, express co-stimulatory molecules, and secrete cytokines for the induction of the immune response (1). These potent antigen-presenting cells (APC) express a wide variety of pattern recognition receptors (PRR) by which they recognize a conserved groups of molecules collectively known as pathogen-associated molecular patterns (PAMP). Although triggering of a single PRR, especially Toll-like receptors (TLR) or C-type lectins, results in phenotypic changes in DC, for functional maturation co-operation between multiple PRR is needed in order to achieve an effective immune response (2).

DC-based cancer vaccines have provided encouraging results (3). Human monocyte-derived DC (MoDC) can be prepared easily by plastic adherence of monocytes from peripheral blood mononuclear cells (PBMC) followed by incubation for 5–7 days in granulocyte–monocyte colony-stimulating factor (GM-CSF) and interleukin (IL)-4 containing medium (4). It has been established that maturation of DC is essential because mature DC have a more stable phenotype and are superior stimulators of T-cell responses (5). Therefore a major effort has been made to identify an optimal protocol for DC maturation.

The current 'gold standard' for the generation of DC used in DC-based cancer vaccine studies is maturation of MoDC with tumor necrosis factor (TNF)-α, IL-1β, IL-6 and prostaglandin E_2 (PGE_2)

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(6). One weakness with DC generated in this way is the absence of IL-12 secretion, a key cytokine for induction of effective T-helper (Th) 1 and cytotoxic T-lymphocyte (CTL) responses that are assumed to be essential for cancer vaccination therapy (7).

The combined activation of different PRR can result in complementary, synergistic or antagonistic effects that modulate innate and adaptive immunity (8). Polyinosinic:polycytidylic acid [Poly (I:C)], a synthetic analog of dsRNA and a Toll-like receptor 3 (TLR3) agonist, has been reported to induce stable mature Th1 responses, promoting clinically applicable DC that produce large amounts of IL-12 (9). Dectin-1, a DC-associated C-type lectin, is the first of many PRR that mediate their own signaling and induce the maturation of DC capable of eliciting the generation of different Th effectors. The treatment of DC with Dectin-1 agonists results in their maturation with the capability of eliciting differentiation of Th1 and Th17 cells (10–12).

In order to identify a maturation cocktail combining a high yield of DC, potent expression of maturation markers, co-stimulatory and adhesion molecules together with a high production of Th cytokines crucial for the anti-tumor immune response, we co-stimulated MoDC with a combination of Poly (I:C) and curdlan, a ligand for Dectin-1 receptor. We show that the combination is superior in triggering Th1 and Th17 responses compared with the effect of individual agonists.

Methods

Medium and reagents

Human MoDC were cultured in complete RPMI-1640 medium (ICN, Costa Mesa, CA, USA), composed of 2 mM L-glutamine, 20 μg/mL gentamicin, 50 μM 2-mercaptoethanol (2-ME; Sigma-Aldrich, Munich, Germany) and 10% heat-inactivated fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA). Recombinant human IL-4 was purchased from Roche Diagnostics GmbH (Mannheim, Germany). Recombinant human GM-CSF (Leucomax, specific activity 4.44×10^6 UI) was obtained from Schering-Plough (Basel, Switzerland). Curdlan from *Alcaligenes faecalis* and Poly (I:C) were from Sigma-Aldrich. Monensin sodium was obtained from Sigma-Aldrich.

Cell preparation and MoDC cultures

MoDC were generated from PBMC. Briefly, PBMC from buffy coats of six healthy volunteers (upon written informed consent) were isolated by density centrifugation on Lymphoprep (Nycomed, Oslo, Norway), resuspended in 5 mL 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 complete RPMI medium containing GM-CSF (100 ng/mL) and IL-4 (20 ng/mL). At day 3, a half-of-medium volume was removed and replaced by the same volume of fresh medium containing GM-CSF and IL-4. After 6 days MoDC were replated (5×10^5 cells/mL) in medium with GM-CSF/IL-4 and the addition of different concentrations of curdlan, Poly (I:C) or their combination for an additional 2 days. After 8 days cell-free supernatants were collected and stored at −20°C for subsequent determination of cytokine levels and the cells were used for immunologic studies.

Allogeneic T-cell activation

The ability of $CD4^+$ T cells or their subsets to proliferate was tested in an allogeneic mixed leukocyte reaction (MLR). Allogeneic $CD4^+$ T cells were isolated from PBMC using negative immunomagnetic sorting with $CD4^+$ isolation kits (MACS technology; Myltenyi Biotec, Bergish Gladbach, Germany) following the instructions of the manufacturer. The sorted $CD4^+$ T cells were separated further using a $CD4^+$ CD45RA kit (MACS technology; Myltenyi Biotec). $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ cells were recovered in positive and negative fractions, respectively. Their purity was higher than 95% as checked by using specific fluorochrome-labeled monoclonal antibodies (MAb) and flow cytometry.

Purified $CD4^+$ T cells, $CD4^+$ $CD45RO^+$ T cells or $CD4^+$ $CD45RA^+$ T cells (1×10^5 cells/well) were cultivated with different numbers of allogeneic MoDC in complete RPMI medium with 10% FCS in 96-well round-bottomed cell culture plates. Different MoDC:$CD4^+$ T-cells ratios were used. After 5 days of culture, the cells were pulsed with [^{3}H]thymidine for the last 18 h (1 μCi/well; Amersham, Amersham, UK). Labeled cells were harvested onto glass fiber filters and the incorporation of the radionuclide into DNA was measured further by β-scintillation counting (LKB-1219; Rackbeta, Finland). Results were expressed as counts per minute (c.p.m.) ± standard deviation (SD) of triplicates. [AQ5]

Flow cytometry

Control and stimulated MoDC (2×10^5 cells/tube) were washed in phosphate-buffered saline (PBS) supplemented with 2% FCS and 0.1% NaN_3, and incubated for 45 min at 4°C with one of the following MAb: anti-HLA-DR coupled with phycoerythrin (PE), CD80 conjugated with fluorescein isothyocianate (FITC), CD83–FITC, CD86–PE, CD40–FITC (Serotec, Oxford, UK), CD54–PE (Serotec) and CCR7–FITC (R&D Systems, Minneapolis, MN,

USA). Controls consisted of samples with irrelevant mouse MAb reactive with rat antigens. Cell fluorescence was analyzed using an EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany). At least 5000 cells per sample were analyzed.

The phenotype of $CD4^+$ T cells in co-culture with MoDC was evaluated by using anti-CD45RA–FITC and anti-CD45RO–FITC MAb (Serotec) by the method described for MoDC. For comparison, the phenotype of these cells was also determined before cultivation.

Cytokine assays

The levels of IL-12, IL-23, IL-27, IL-6 and IL-10 were measured in the cell-free supernatants of control or stimulated MoDC cultures (4×10^5 cells/mL culture) by sandwich ELISA assays obtained from R&D Systems, whereas TNF-α was determined using an ELISA kit purchased from Bender MedSystems (Vienna, Austria) following the manufacturer's instructions.

The levels of Th cytokines were evaluated in MoDC:$CD4^+$ T-cell co-culture supernatants. In these experiments, purified $CD4^+$ T cells or $CD4^+$ T-cell subsets (1×10^5 cells/well) were cultivated with allogeneic MoDC (1×10^4 cells/well) in complete RPMI medium with 10% FCS in 96-well round-bottomed cell culture plates. After 5 days phorbol myristate acetate (PMA; 20 ng/mL; Sigma-Aldrich) and ionomycin (500 ng/mL; Merck, Vienna, Austria) were added to the wells. The cells were incubated for an additional 8 h and then harvested, centrifuged, and cell-free supernatants collected and stored at −20°C for the subsequent determination of cytokine levels using a FlowCytomix Human Th1/Th2 11plex Kit from Bender MedSystems (Vienna, Austria) and an IL-17 sandwich ELISA assay (R&D Systems). The FlowCytomix Human Th1/Th2 11plex Kit is a fluorescent bead immunoassay based on the use of beads coated with antibodies specifically reacting with each of the cytokines to be detected in the supernatants. A biotin-conjugated secondary antibody mixture binds to the cytokines captured by the first antibodies. After that, streptavidin–PE binds to the biotin conjugate and emits fluorescent signals. The emitted fluorescence was analyzed using an EPICS XL-MCL flow cytometer (Coulter, Krefeld, Germany).

The Th-polarizing capabilities of MoDC were assessed additionally in the co-culture with purified allogeneic $CD4^+$ T cells, by measuring the production of IL-17 and IFN-γ intracellularly. Briefly, the purified $CD4^+$ T cells (1×10^5 cells/well) were cultivated with allogeneic MoDC (1×10^4 cells/well) for 5 days in complete RPMI medium in 96-well plates. Cells were harvested and incubated with monensin sodium (3 μM) for 6 h. After that, cells were stained with anti-IFN-γ–FITC (R&D Systems) and anti-IL-17–PE (BD Biosciences Pharmingen, San Diego, CA, USA) by using a FIX & PERM® Cell Fixation & Cell Permeabilization Kit (Invitrogen) following the manufacturer's recommendation. Cell fluorescence was analyzed using an EPICS XL-MCL flow cytometer (Coulter). The cells were gated according to cell-specific forward scatter/side scatter (FS/SS) parameters. The results were presented as dot-plots and the percentages of single- and double-positive cells were indicated.

Statistical analysis

Differences in parameters between various groups were evaluated using the Student's *t*-test or one-way ANOVA. Values of $P < 0.05$ or less were considered as statistically significant.

Results

The effect of Poly (I:C) and curdlan on the maturation of human MoDC

In preliminary experiments, immature MoDC were treated with Poly (I:C) (5 μg/mL, 25 μg/mL and 50 μg/mL) or curdlan (10 μg/mL, 50 μg/mL, 100 μg/mL and 200 μg/mL). Based on phenotypic characteristics and the functional capabilities of MoDC, some concentrations of Poly (I:C) (25 μg/mL) and curdlan (100 μg/mL) were found to be suboptimal for activation of MoDC. Therefore, in the following experiments we used these concentrations for the agonists, alone or in combination, for stimulation of immature MoDC for 2 days. After that, the phenotypic and functional characteristics of MoDC were examined. **[AQ6]**

Poly (I:C) stimulated the maturation of MoDC as judged by the up-regulation of HLA-DR, CD86, CD40, CD54, CD83 and CCR7 expression. Curdlan up-regulated the expression of HLA-DR, CD86, CD83, CD40 and CCR7 but its effect was weaker compared with Poly (I:C). Simultaneous treatment of MoDC with both compounds only potentiated the expression of CCR7, whereas other phenotypic characteristics of MoDC did not differ significantly compared with Poly (I:C)-treated MoDC (Table I and Figure 1).

The combination of Poly (I:C) and curdlan potentiates the allostimulatory activity of MoDC

The allostimulatory activity of MoDC was evaluated using an MLR assay. Poly (I:C)- or curdlan-treated

Table I. Effect of Poly (I:C), curdlan and their combination on phenotypic characteristics of human MoDC.

		Control	Poly (I:C)	Curdlan	Poly (I:C)$^+$ curdlan
HLA-DR	%	98.0 ± 1.6	99.0 ± 0.6	99.1 ± 0.8	99.6 ± 0.2
	MFI	28.8 ± 4.3	62.9 ± 9.5[3]	61.2 ± 9.2[3]	75.7 ± 11.4[3]
CD86	%	75.4 ± 10.9	96.5 ± 1.4[3]	95.4 ± 2.0[3]	92.4 ± 3.0[3]
	MFI	13.6 ± 2.0	37.6 ± 5.6[3]	21.6 ± 3.2[1]	36.5 ± 5.5[3]
CD40	%	92.8 ± 5.3	97.5 ± 1.1	98.6 ± 0.9	97.8 ± 1.4
	MFI	15.0 ± 1.8	41.6 ± 5.0[3]	22.6 ± 2.7[2]	44.0 ± 5.3[3]
CD54	%	83.9 ± 13.2	94.2 ± 3.3	83.2 ± 8.4	93.6 ± 3.4
	MFI	23.5 ± 3.5	55.9 ± 8.4[3]	24.5 ± 3.7	59.5 ± 9.0[3]
CD83	%	33.2 ± 5.0	75.9 ± 11.4[3]	56.9 ± 8.5[3]	70.9 ± 10.6[3]
	MFI	3.4 ± 0.5	6.4 ± 1.0[1]	4.7 ± 0.7	6.2 ± 0.8[2]
CCR7	%	2.7 ± 0.4	11.3 ± 1.6[3]	4.0 ± 0.6[1]	18.5 ± 2.9[3,4]
	MFI	2.6 ± 0.4	2.9 ± 0.5	2.2 ± 0.4	5.2 ± 0.6[3,5]

Immature MoDC (control) were treated with Poly (I:C), curdlan and their combination for an additional 2 days, as described in the Methods. After that, the phenotypic characteristics of control and treated-MoDC were determined by flow cytometry. Results are given as mean ± SD ($n = 6$ different donors). %, percentage of positive cells; MFI, mean fluorescence intensity.
[1]$P < 0.05$; [2]$P < 0.01$, [3]$P < 0.005$ compared with the control. [4]$P < 0.05$; [5]$P < 0.01$ compared with Poly (I:C)-treated MoDC.

MoDC stimulated the proliferation of allogeneic CD4$^+$ T cells at lower DC:CD4$^+$ T-cell ratios (1:40 and 1:80). Simultaneous treatment of the cells with both compounds inhibited cellular proliferation at higher (1:10) DC:CD4$^+$ T-cell ratios but enhanced the proliferative response at lower (1:80) DC:CD4$^+$ T-cell ratios, compared with the effect of Poly (I:C)-treated MoDC (Figure 2).

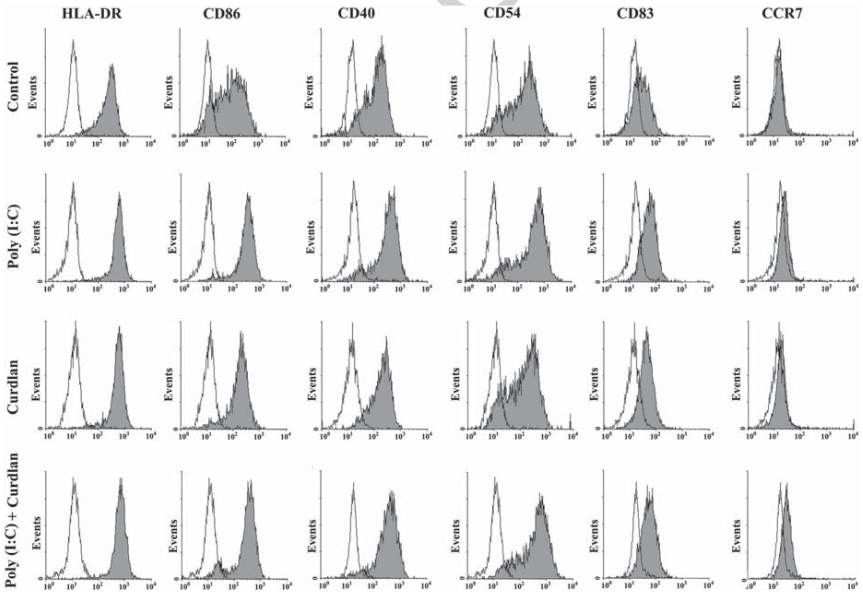

Figure 1. Phenotypic characteristics of MoDC. MoDC were obtained by cultivation of human monocytes for 6 days with GM-CSF (100 ng/mL) and IL-4 (20 ng/mL) and then stimulated with Poly (I:C) (25 μg/mL), curdlan (100 μg/mL) or their combination for an additional 48 h. Non-adherent cells were collected and stained for key DC markers using MAb (anti-HLA-DR–PE, CD86–PE, CD83–FITC, CD40–FITC, CD54–PE and CCR7–FITC) and analyzed by flow cytometry. Results are presented as over-laid histograms within the gated population of one (out of six) experiment.

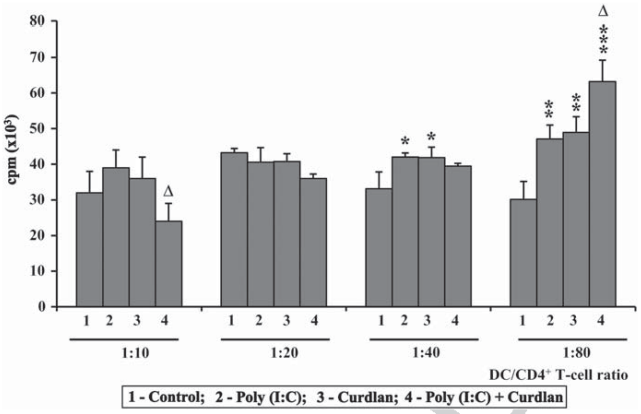

Figure 2. The effect of Poly (I:C), curdlan and their combination on the allostimulatory capability of MoDC. MoDC were generated by the cultivation of monocytes in the presence of GM-CSF (100 ng/mL) and IL-4 (20 ng/mL) for 6 days and Poly (I:C) (25 μg/mL), curdlan (100 μg/mL) or their combination for an additional 48 h. MoDC were co-cultivated in 'U'-bottomed 96-well microtiter plates with allogeneic CD4+ T cells at concentrations of 1×10^5 cells/well at different DC:CD4+ T-cell ratios. Cell proliferation was measured after 5 days using a [^{3}H]thymidine uptake assay. Values are presented as c.p.m. ± SD of triplicates (one representative experiment out of six with similar results). $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ compared with the control. $^{\Delta}P<0.05$ compared with Poly (I:C)-treated MoDC.

The effects of simultaneous treatment of MoDC with Poly (I:C) and curdlan on cytokine production

Control immature MoDC produced low levels of IL-10 and IL-27, whereas the production of IL-12, IL-23, TNF-α and IL-6 was almost undetectable. Ligation of TLR3 by Poly (I:C) triggered a high production of IL-12, IL-27, TNF-α and IL-6. The level of IL-23 was moderately increased compared with the control, whereas the production of IL-10 was not significantly changed. Curdlan up-regulated the production of IL-23 and IL-27 and down-regulated the production of IL-10, compared with untreated MoDC, whereas the production of other cytokines was not significantly modulated. Stimulation of immature MoDC with the combination of Poly (I:C) and curdlan was followed by synergistic up-regulation of IL-12, IL-23 and IL-10 production. In contrast, the levels of IL-6 and IL-27 were lower compared with their levels in MoDC cultures treated with Poly (I:C), alone (Figure 3).

The combination of Poly (I:C) and curdlan stimulates MoDC to promote Th1 and Th17 immune responses

The capability of MoDC to polarize Th immune responses was measured by the production of cytokines in DC:CD4+ T-cell co-cultures. Total CD4+ T cells in co-culture with control MoDC produced a significant quantity of all the Th cytokines examined. Poly (I:C)-treated MoDC enhanced the production of IFN-γ and IL-17 but decreased the production of IL-10 and IL-5 by allogeneic CD4+ T cells, compared with control cultures. Allogeneic CD4+ T cells in co-culture with MoDC treated with curdlan produced significantly higher levels of IFN-γ, IL-17 and IL-2 and lower levels of IL-5, compared with control cultures. The stimulatory effect of curdlan-treated MoDC on IL-17 production was higher compared with Poly (I:C)-treated MoDC. The opposite results were observed with IFN-γ. The treatment of MoDC with both compounds resulted in up-regulation of IFN-γ and IL-10 production in co-cultures, compared with the levels of these cytokines in co-cultures of MoDC treated with single agonists. A similar tendency towards an additional increase in the production of IL-17 was also observed. In contrast, the production of IL-2 was down-regulated (Figure 4A).

The intracellular cytokine staining showed that MoDC treated with a combination of Poly (I:C) and curdlan stimulated the expansion of both single IFN-γ- and IL-17-producing allogeneic CD4+ T cells, but also double-positive IFN-γ+ IL-17+ cells. In contrast, MoDC pre-incubated with single agonists mostly expanded effector CD4+ T cells of separate (IFN-γ+ or IL-17+) phenotype (Figure 4B).

When the production of cytokines in co-culture of MoDC with allogeneic CD4+ CD45RA (naive) and CD4+ CD45RO (memory) T cells was investigated, the results varied depending on the CD4+ T-cell subsets. Poly (I:C)-treated MoDC stimulated the production of IFN-γ by both naive and memory CD4+ T cells, but the response of naive CD4+ T cells was stronger. In contrast, curdlan-treated MoDC stimulated the

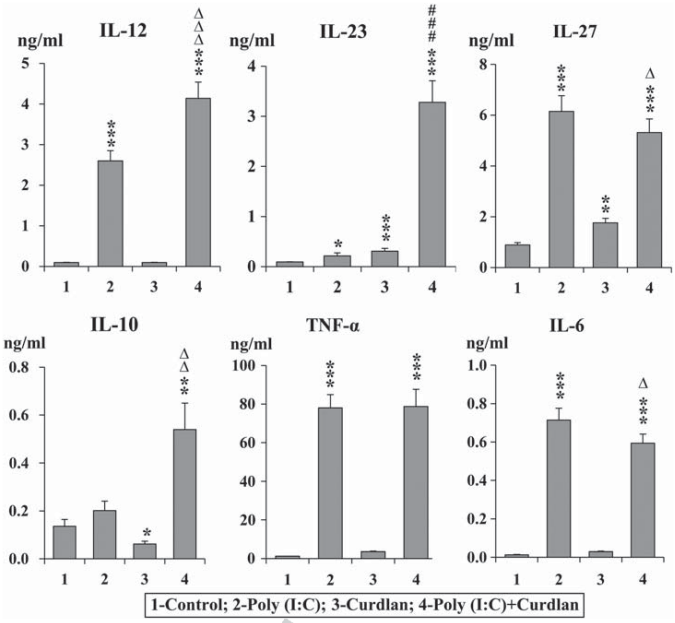

Figure 3. Cytokine production by MoDC. MoDC were obtained by cultivation of human monocytes for 6 days with GM-CSF (100 ng/mL) and IL-4 (20 ng/mL) and then stimulated with Poly (I:C) (25 μg/mL), curdlan (100 μg/mL) or their combination for an additional 48 h. Levels of secreted IL-12, IL-23, IL-10, TNF-α, IL-6 and IL-27 were determined in cell-free supernatants by ELISA. Values are given as mean ± SD ($n = 6$ donors). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared with the control. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.005$ compared with Poly (I:C)-treated MoDC. $^{\#}P < 0.05$ compared with curdlan-treated MoDC.

production of this cytokine only by memory CD4$^+$ T cells. Poly (I:C)-treated MoDC stimulated the production of IL-17 by memory CD4$^+$ T cells. Curdlan-treated MoDC stimulated the production of this cytokine by both CD4$^+$ T subsets, but its effect on memory CD4$^+$ T cells was stronger. Simultaneous treatment of MoDC with curdlan and Poly (I:C) potentiated IFN-γ production by naive and memory CD4$^+$ T cells and IL-17 production by memory CD4$^+$ T cells (Figure 5A). The combined effect of these agonists was not dependent on the changes in the proportion of CD4$^+$ CD45RA$^+$ and CD4$^+$ CD45RO$^+$ cells in cultures (Figure 5B) nor was it a consequence of the different proliferation rate of these cell subsets (Figure 5C).

Discussion

Simultaneous targeting of two or more PRR is a promising tool for generating immunogenic DC (2). In this context we examined, for the first time, the combined effect of Poly (I:C), a TLR3 agonist, and curdlan, a Dectin-1 agonist, on the maturation and functions of human MoDC. This is an interesting immunotherapeutic approach, because no microbe or fungal pathogen that expresses or generates ligands for both TLR3 and Dectin-1 has been found so far.

Numerous studies have shown that Poly (I:C), which mimics the effects of viral dsRNA, induces very potent maturation of DC, which produce high quantities of IL-12 and polarize a strong Th1 immune response (9,13–15). We confirmed these results and additionally showed that Poly (I:C)-treated MoDC produced high levels of two other members of the IL-12 family (IL-23 and IL-27). Consistently with this cytokine profile, we showed that Poly (I:C)-treated MoDC up-regulated Th1 and Th17 and down-regulated Th2 immune responses. These findings are in agreement with the current concept of reciprocal regulation of the Th1:Th2 balance (16). However, the functional significance of the Th17 immune response, triggered by TLR3 agonists, or natural viral ligands for TLR3 has been investigated less.

The transmembrane receptor Dectin-1 is able to recognize soluble and particulate β-glucan (17,18), which makes up to 50% of the fungal cell wall. The role of Dectin-1 in modulating the biologic functions of human DC is reported in only a few studies

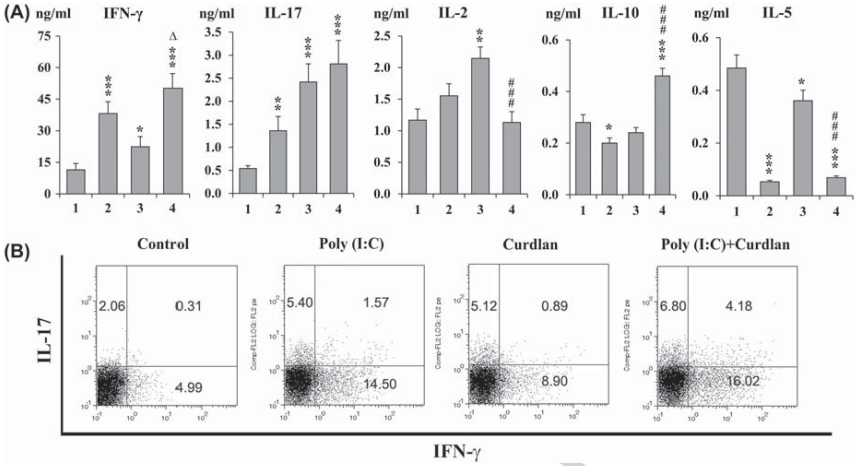

Figure 4. The combined effect of Poly (I:C) and curdlan on the Th polarization capability of MoDC. Allogeneic CD4$^+$ T cells were co-cultured with MoDC (1×10^4 cells/well) at a concentration of 1×10^5 cells/well. Co-cultivation lasted for 5 days, after which PMA (20 ng/mL) and ionomycin (500 ng/mL) were added for an additional 8 h. (A) Cell-free supernatants were collected and used for detection of IL-17 by ELISA, and IFN-γ, IL-2, IL-5 and IL-10 by FlowCytomix Human Th1/Th2 11plex. Cytokine levels are given as mean ± SD ($n=6$ donors). (B) In order to evaluate intracellular expression of cytokines, cells were harvested from parallel CD4$^+$/MoDC co-cultures and incubated with monensin sodium for 6 h. The cells were then stained with anti-IFN-γ–FITC and anti-IL-17–PE. Cell fluorescence was analyzed using an EPICS XL-MCL flow cytometer. The results are presented as dot-plots. The percentage of single- and double-positive cells are indicated (one representative experiment out of three with similar results). 1, control; 2, Poly (I:C); 3, curdlan; 4, Poly (I:C) + curdlan. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.005$ compared with the control. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.01$, $^{\Delta\Delta\Delta}P<0.005$ compared with Poly (I:C)-treated MoDC. $^{\#\#\#}P<0.005$ compared with curdlan-treated MoDC.

(12,19,20). It has been shown that mouse or human DC, stimulated with curdlan, increases the expression of co-stimulatory molecules and produces a number of pro-inflammatory (IL-1β, IL-6, IL-12 and IL-23) and anti-inflammatory (IL-10) cytokines (11,12,19–21).

We found that activation of the Dectin-1 pathway by curdlan was followed with maturation of MoDC, increased production of IL-23 and IL-27 and decreased production of IL-10 by MoDC. The reason for these differences between ours and previously published results (12,21) might be explained by the difference in applied concentrations of curdlan and the mode of its preparation, both at the manufacturer level and in the laboratory (22). It is interesting that, although the level of IL-12 was undetectable, curdlan-treated MoDC promoted a moderate Th1 response. This finding might be explained by stimulation of IL-12 production during direct contact of MoDC with CD4$^+$ T cells in co-culture, predominantly through CD40–CD40L interactions (23). On the other hand, a relatively stable phenomenon, which has already been published (12), was the ability of curdlan to stimulate the production of IL-23 by MoDC and to promote the Th17 immune response.

The crucial finding of our study was that simultaneous engagement of TLR3 and Dectin-1 was followed by amplification of IL-12, IL-23 and IL-10 production by MoDC, whereas the production of IL-27 and IL-6 was down-regulated. As expected, such activated MoDC increased the production of both IFN-γ and IL-17, compared with the effect of single agonists. These results were more obvious when naive (higher producers of IFN-γ) and memory (higher producers of IL-17) CD4$^+$ T cells were used in the co-culture experiments. The effect was not associated with the change in the ratio of CD4$^+$ CD45RA$^+$/CD4$^+$ CD45RO$^+$ subsets, nor their proliferative capability in culture.

The enhancement of cytokine production was a consequence of expansion of both single-positive IFN-γ$^+$ and IL-17$^+$ CD4$^+$ T cells, and also double-positive IFN-γ$^+$ IL-17$^+$ CD4$^+$ T cells. IFN-γ$^+$ IL-17$^+$ double-positive cells are found in significantly elevated numbers in inflamed tissue and blood from patients with chronic inflammatory disease. They are phenotypically unstable in culture and could become IL-17 or IFN-γ single-producing cells (24). Based on these findings we hypothesize that cytokines of the IL-12 family, produced by MoDC following the simultaneous engagement of TLR3 and Dectin-1, stimulated polarization of the immune response, at least partly, by inducing the differentiation of IFN-γ$^+$ IL-17$^+$ double-positive CD4$^+$ T cells. Although the

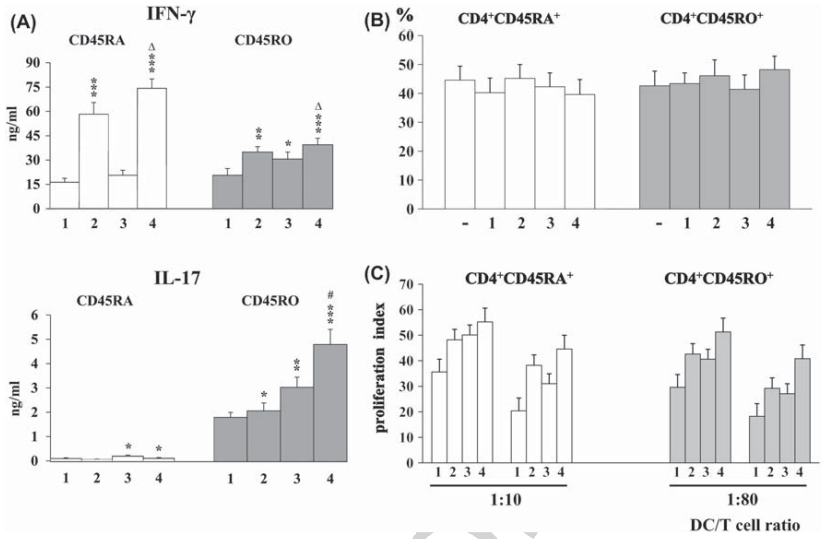

Figure 5. The combined effect of Poly (I:C) and curdlan on the capability of MoDC to influence cytokine production (A), frequency (B) and proliferation (C) of $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells in co-cultures. Purified $CD4^+$ $CD45RA^+$ (naive) and $CD4^+$ $CD45RO^+$ (memory) T cells (1×10^5 cells/well) were co-cultivated with MoDC (1×10^4 cells/well) for 5 days, when PMA (20 ng/mL) and ionomycin (500 ng/mL) were added for an additional 8 h. (A) The levels of IFN-γ and IL-17 were determined in cell-free supernatants of $CD4^+$ $CD45RA^+$/MoDC and $CD4^+$ $CD45RO^+$/MoDC co-cultures, as described for Figure 4A. Cytokine levels are given as mean ± SD ($n = 6$ donors). (B) Proportions of $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ cells in cultures of total $CD4^+$ T cells were determined before and after co-cultivation with MoDC pre-treated with the agonists, by using anti-CD45RA–FITC and anti-CD45RO–PE MAb. The emitted fluorescence was analyzed using an EPICS XL-MCL flow cytometer. The results are presented as the mean value of the percentage of positive cells ± SD ($n = 3$). (C) To estimate the proliferation rate of naive and memory $CD4^+$ T-cell subsets, purified $CD4^+$ $CD45RA^+$ and $CD4^+$ $CD45RO^+$ T cells (1×10^5 cells/well) were co-cultivated with MoDC at different DC:$CD4^+$ T-cell ratios (1:10 and 1:80). Cell proliferation was measured after 5 days using a [^{3}H]thymidine uptake assay. Values are presented as c.p.m. ± SD of triplicates (one representative experiment out of three with similar results). –, before culture; 1, control; 2, Poly (I:C); 3, curdlan; 4, Poly (I:C) + curdlan. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$ compared with the control. $^{\Delta}P < 0.05$ compared with Poly (I:C)-treated MoDC. $^{\#}P < 0.05$ compared with curdlan-treated MoDC.

combined effect of Poly (I:C) and curdlan on DC has not been investigated yet, a number of studies have shown that the combination of Dectin-1 ligands with other TLR ligands modulates DC functions, but the final outcome of the immune response differs significantly depending on investigated species, type of targeted PRR and culture conditions (21,22,25,26). The potentiated effect of TLR3 and Dectin-1 agonists on cytokine production by MoDC could be associated with the molecules involved in signaling through PRR. One of them could be the caspase recruitment domain-containing protein 9 (CARD9). This is an adaptor protein that is activated by purified microbial compounds, including Poly (I:C) (27) and Dectin-1 agonists (28). Activation of either mitogen-activated protein kinase (MAPK) or nuclear factor-kappaB (NF-κB) by CARD9 modifies the gene expression and fine-tune the cytokine responses, including additional secretion of p40 (29), a subunit common to both IL-12 and IL-23. However, it remains to be studied whether other mechanisms are involved and how signaling through an individual receptor modifies the expression of the other receptor (30).

Another important finding of this study is related to the increased secretion of IL-10 and decreased production of IL-27 by MoDC treated with the combination of Poly (I:C) and curdlan. IL-27 was originally shown to initiate the differentiation of Th1-type $CD4^+$ T cells (31). Certain results indicate the ability of IL-27 to counteract IL-23 and suppress the production of IL-17 by activated $CD4^+$ T cells, and that the balance between IL-23 and IL-27 fine tunes the Th17 response. It was assumed that IL-23 versus IL-27 is to the Th17 differentiation just as IL-12 versus IL-4 is to the Th1:Th2 differentiation (32). Therefore, in our culture model, decreased IL-27 production may be relevant for further enhancement of the Th17 response.

On the other hand, IL-10, as a potent anti-inflammatory and immunosuppressive cytokine, plays

a significant role in self-limitation of Th1 and Th17 cell-mediated immunopathology in conditions of strong inflammatory stimuli (33,34). Based on this concept, an increased level of IL-10, observed in our study, using a combination of TLR3 and Dectin-1 ligands might be considered a significant mechanism for balancing the immune response. IL-10 might also be responsible for inhibition of the alloreactive capability of MoDC. Namely, in spite of the high expression of adhesion and co-stimulatory molecules and the production of high amounts of stimulatory cytokines (IL-12 and IL-23) by MoDC, the proliferation of allogeneic $CD4^+$ T cells in MLR was low at higher DC:CD^+ T-cell ratios. At the lowest DC:CD^+ T-cell ratio (1:80), with decreased DC numbers in cultures, the enhanced proliferation could be explained by the prevalence of stimulatory soluble factors, because the expression of co-stimulatory molecules by DC was not additionally up-regulated. The only significant phenotypic characteristic of MoDC co-ligated with TLR3 and Dectin-1 agonists, was an up-regulation of CCR7 expression. This result suggests that cross-talk between these two PRR induces the phenotypic maturation of MoDC with improved migratory properties.

Our results have possible clinical significance for conditions where both Th1 and Th17 immune responses are desired. The current protocols using DC as cancer vaccines are based on the preparation of immunogenic DC with the ability to polarize the Th1 immune response strongly with the development of potent and specific anti-tumor cytotoxicity (35). Poly (I:C) is such a candidate that has already been applied in clinical trials (36). The precise role of Th17 cells in tumor immunity is still highly controversial, as a series of reports has suggested pro-tumor (37,38) and anti-tumor functions for IL-17 (39). Anti-tumor effects of Th17 cells, which are documented in many studies, are attributed to their capability to enhance inflammatory responses, increase antigen presentation by DC, promote leukocyte homing to tumors and facilitate $CD8^+$ T-cell priming and effector differentiation (40,41).

In conclusion, our results show that signaling through TLR3 and Dectin-1 receptor potentiates the capability of human MoDC to promote the development or expansion of Th1- and Th17-producing cells *in vitro*. It remains to be studied whether such an effect will be operative *in vivo* and what could be the consequence of these interactions on the final outcome of the immune response.

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9. BIOGRAFIJA AUTORA

Ana Ž. Dragičević je rođena 08.11.1982. u Nišu. Nakon završetka gimnazije „Bora Stanković“ u Nišu, upisala je studije na Biološkom fakultetu, Univerzitet u Beogradu, smer Molekularna biologija i fiziologija školske 2001/2002. školske godine. Po završetku studija upisala je doktorske studije na Biološkom fakultet, Univerzitet u Beogradu, modul: Neurobiologija sa neuroimunologijom školske 2007/2008 i specijalističke studije na Biološkom fakultetu, Univerzitet u Beogradu, smer: Imunobiologija sa mikrobiologijom.

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Ana Ž. Dragičević je do sada bila autor i koautor u sedam naučnih publikacija u vrhunskim časopisima međunarodnog značaja, kao i u 10 saopštenja na skupovima međunarodnog značaja i šest saopštenja na skupovima nacionalnog značaja iz uže naučne oblasti.

Прилог 1.

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Име и презиме аутора Ана Драгичевић

Број уписа КА 07/02

Студијски програм Неуронауке, модул: Неуробиологија са неуроимунологијом

Наслов рада Модулација функције хуманих дендритских ћелија комбинованом применом агониста ендозомних Toll-сличних рецептора, дектин-1 рецептора и проинфламаторних цитокина

Ментор академик Миодраг Чолић, др Биљана Божић

Потписани Ана Драгичевић

изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла за објављивање на порталу **Дигиталног репозиторијума Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског звања доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

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Ana Dragičević

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

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Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигитални репозиторијум Универзитета у Београду могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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