Interleukin-21 inhibits dendritic cell activation and maturation

Short title: IL-21 inhibits DC activation

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Abstract

IL-21 is a newly described cytokine with homology to IL-4 and IL-15. They belong to a cytokine family, which use the common \( \gamma \)-chain for signaling but also have their private high-affinity receptors. Since IL-4 is well-known to modulate differentiation and activation of dendritic cells (DC), we analyzed effects of IL-21 in comparison to IL-15 on DC differentiation, maturation and function. Here we show that DCs generated with GMCSF in the presence of IL-21 (IL-21DCs) differentiated into phenotypic and functional altered DCs characterized by reduced MHCII expression, high antigen-uptake and low stimulatory capacity for T-cell activation \textit{in vitro}. Additionally, IL-21DCs completely failed to induce Ag-specific, T-cell mediated contact-hypersensitivity. Furthermore, IL-21 blocked LPS induced activation and maturation of DCs, which was not mediated by release of the anti-inflammatory cytokine IL-10. In contrast, if IL-15 was supplemented to GMCSF, DCs differentiated into mature APCs with low antigen-uptake and high significantly increased capacities to stimulate T-cells \textit{in vitro} and \textit{in vivo}. Taken together these results identify a dichotomous action of these structural related cytokines on DCs, establishing IL-21 as inhibitory cytokine on DC activation and IL-15 as potent stimulator of DC function, making both cytokines interesting targets for therapeutical manipulation of DC-induced immune-reactions.

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Introduction

DCs possess specialized features such as pathogen recognition, Ag capturing and processing machinery, migratory capacity and constitutively expressed costimulatory molecules that allow them to act as professional APCs. Thus, DCs display an extraordinary capacity to initiate T-cell dependent immune responses. In mediating this, DCs pass through different functional activation states. Thereby DCs convert from a high efficient antigen-capture and uptake to an antigen-presenting state. The capacity of mature DCs to prime naïve T-cells and to promote their differentiation is critically attributed to their cytokine-secretion pattern. Vice versa polarizing signals from the microenvironment directly shape the DC maturation. A network of cytokines modulate this sensitive system of cell communication, including IL-15, a pro-inflammatory cytokine which activates APCs like DCs and macrophages.

IL-15 was introduced as a member of the four-helix-bundle cytokine family able to reproduce biological effects of IL-2. This is related to the fact that the IL-15 receptor complex contains beside its private high affinity α-chain the β- and common γ-chain of the IL-2R complex. However, IL-15 was also found to have distinct functions compared to those mediated by IL-2. In addition IL-2 expression is nearly restricted to T-cells whereas IL-15 is hardly detectable in T-cells but produced by a variety of tissues, monocytes and DCs. Therefore IL-15 may be an important candidate for modulating DC-mediated immune responses.

It is established that combining GMCSF and IL-4 promotes DC generation in vitro. Therefore it was of interest to investigate other members of this cytokine family acting on DC development. It has been recently reported that IL-15 can skew monocyte differentiation into DCs with a surface phenotype of Langerhans cells and supports the maturation of monocytes to DCs ex vivo. Interestingly, although IL-4 and IL-15 both mobilize the common γ-chain IL-4 was unable to induce comparable effects. Moreover, IL-15 is produced
by DCs itself after inflammatory stimuli suggesting that IL-15 might be important to link innate response to infection with the initiation of an adaptive immune response by DCs.

IL-21, a novel cytokine has close structural similarities with IL-15, IL-2 and IL-4. Like IL-4 and IL-15, IL-21 has its private high-affinity receptor chain and shares in addition the common γ-chain as functional subunit of its IL-21 receptor. The IL-21R is expressed in lymphoid tissues like thymus, lymph-nodes, leukocytes as well as NK-cells, suggesting that this receptor/ligand pair could play an role in innate and acquired immunity. Interestingly, IL-21R expression was also found in bone marrow (BM) cells matching the fact that IL-21 promotes the differentiation of lymphoid cells. Unlike its receptor IL-21 appeared more restricted; production was found in activated peripheral T-cells. These observations open up the possibility that IL-21 is involved in T-cell dependent immune responses mediated by professional APCs like DCs.

This led us to the question how IL-15 and IL-21 influences differentiation, maturation and function of bone marrow-derived DCs (BMDCs) as well as DC-T-cell interaction. The principle method to generate murine BMDCs in vitro is adapted for many years and yield in high amounts of pure myeloid, CD11c+ DCs. Expansion of in vitro generated BMDCs is promoted by IL-4. Given the structural similarity between IL-21 and the IL-15/IL-4 family of cytokines together with their common use of the γ-chain it was reasonable to speculate that they influence DC development in similar ways.

We report here, that despite structural similarities and shared receptor components, signals from IL-15 during DC generation induced high immunogenic DCs whereas IL-21 kept DCs in an immature state characterized by low T-cell stimulatory capacity.
Methods

**Mice, culture medium.** C57BL/6 mice, 10-12 weeks, were from Charles River (Sulzfeld, Germany). OTII\(^g\) mice, transgenic for a CD4 T-cell restricted T-cell receptor, recognizing OVA\(^{323-339}\) were from IFFA-Credo (France). All *in vivo* experiments were performed in compliance with the national and institutional guidelines.

Cells were cultured in RPMI1640 medium plus 10% heat-inactivated FCS (Biochrom, Berlin, Germany), 2mM L-glutamine (Lifetechnologies, Karlsruhe, Germany), 50µM β-mercaptoethanol, 100U/ml penicillin and 100µg/ml streptomycin (PAA, Linz, Austria), at 37°C in 5% CO₂.

**DC preparation.** BMDCs were generated as described\(^{18}\). In brief, BM cells from femurs were isolated and seeded (2x10\(^5\)/ml) in bacterial-grade petri dishes (Falcon, Heidelberg, Germany) and differentiated for 8 days in 10ml medium plus 20ng/ml GMCSF (Tebu, Frankfurt, Germany) alone or plus 20ng/ml IL-15 (RnD, Wiesbaden, Germany) or 20ng/ml IL-21 (ZymoGenetics, Seattle, U.S.A.). At day 3, 10ml medium containing 20ng/ml GMCSF alone or plus cytokines were added. At day 6 half of the cell-free supernatant was exchanged, and fresh medium containing 10ng/ml GMCSF alone or plus cytokines was added. At day 8, cells were harvested using Accutase (PAA).

**Histomorphometry.** Harvested cells (5x10\(^4\)) were transferred in fresh medium onto chamber-slides (Nunc, Wiesbaden, Germany) and incubated for 48h to adhere and stained with Pappenheim.
RT-PCR. RNA was extracted from 5x10^6 cells after 8 days of culture using RNAzol (Lifetechnologies). cDNA was synthesized using random hexanucleotide primers and Superscript preamplification systemII (Lifetechnologies). cDNA was amplified using 1U of AmpliTaq DNA polymerase (Roche, Mannheim, Germany), 250µM of each dNTP and 2µl 10-fold PCR buffer (Roche). The final primer concentration was 0.5µM. Cycling conditions were 5min at 94°C and additional 30s at 94°C. Annealing temperature was 60°C for 30 seconds, followed by 30s at 72°C. 30 cycles were performed and ended with final extension at 72°C for 10min. Primers (Table 1) were from TIB Molbiol (Berlin, Germany) or Metabion (Martinsried, Germany). To exclude contaminations all experiments were run with a mock PCR. β-actin was used to normalize cDNA amount. As positive controls for IL-21 and its receptor cDNA from CD4^+ T-cells from C57BL/6 mice was isolated. For IL-15 and its receptor we used L929 fibroblasts and CTLL-2 cells for all IL-2R chains.

FACS analysis. BMDCs were characterized using anti-CD11c (FITC), MHC class II (I_A/I_E), CD80, CD86, CD11b (PE-conjugated, all from Pharmingen, Heidelberg, Germany). Propidiumiodide (Sigma) was added to exclude dead cells. Anti-CD3, CD8α, CD45/B220, DX5, NK1.1 and F4/80 (Serotec, Eching, Germany) were used as control. Analysis was performed on CD11c^+ gated DCs with a FACSCalibur CELLQuest (Becton Dickinson).

Analysis of endocytosis. To quantify endocytic activity, FITC-dextran uptake (MW: 70.000; MolecularProbes, Göttingen, Germany) was monitored by FACS as described by Stumbles et al.

DC activation. All DC types were cultured for 24h with low-dose LPS (10ng/ml) to induce DC activation and maturation. In addition all DCs were labeled with FITC (12.5µg/ml,
Sigma) as described below. Concentrations were chosen out of several titration experiments. As control, medium was used. After 24h DCs were analyzed by FACS.

**Proliferation assay.** DCs were harvested after 8 days culture and seeded into 96-well flat-bottom plates (Costar Corning, Cambridge, MA) at a density of 1x10^5 /well to adhere for 12h. T-cells were isolated from lymph nodes of OTIItg mice and used at 1x10^5 per well. OVA^{323-339} peptide was supplemented at 0.3µM and 1µM to a final volume of 200µl. Cells were incubated for 72h and labeled for additional 12h with 0.2µCi [³H]thymidine (Amersham, Freiburg, Germany). Proliferation of T-cells was quantified by liquid scintillation counting (Wallac/PerkinElmer, Freiburg, Germany).

**Assay for contact hypersensitivity (CHS) to FITC.** To estimate the capacity of the differently generated DCs in inducing *in vivo* T-cell sensitization and an antigen-specific immune response, 1x10^6 cells/ml were labeled with 12.5µg/ml FITC (Sigma) for 20min at 37°C as described^{22}. 50µl (5x10^5 cells) were injected in one footpad. After five days, mice were challenged by applying 50µl FITC (3.5mg/ml in acetone:dibutylphtalate, 1:1) on the right ear^{22}. As control, the left ear was painted with diluent or unsensitized mice were painted with FITC. The CHS response was determined by measuring ear swelling at 24h, 48h and 72h after challenge using a micrometer (Mitutoyo, Elk Grove Village, USA).

**In vivo DC migration.** DCs were labeled with FITC as described above and 5x10^5 labeled DCs were injected s.c. in the hind footpad. To assess that FITC was not taken up by other cells, FITC-labeled DCs were fixed with 0.1% glutaraldehyde and injected s.c in the hind footpad^{23}. After 24h local draining lymph nodes (DLN) were removed and prepared as described^{24}. Cells were stained with anti-CD11c-APC and analyzed by FACS.
**DC activation by LPS.** DCs, generated with GMCSF only were cultured for another 24h in medium or with low dose LPS (10ng/ml) to induce DC activation. To analyze the effects of IL-15 and IL-21 on this *in vitro* process, DCs were incubated with a combination of LPS and IL-15 or IL-21 (100ng/ml). Cytokine and LPS concentrations were chosen out of several titration experiments. As control, the anti-inflammatory cytokine IL-10 (100ng/ml) was used, which inhibits DC maturation25. After 24h, the surface phenotype of the DCs was analyzed by FACS and the supernatant was analyzed for cytokine-production using a Bio-Plex™ kit (BioRad, Munich, Germany). 50µl per sample were analyzed on the Luminex 100™ according to manufacture’s instruction.
Results

**CD11c⁺ DCs differentiate in the presence of IL-15 or IL-21 in vitro**

IL-21R expression was found in BM cells. Therefore we first analyzed whether IL-21 might play a role in generation of myeloid DCs compared to IL-15. Freshly isolated BM cells were cultured following established protocols\(^{18}\) with GMCSF alone (subsequently designated DCs) or GMCSF plus 20ng/ml IL-15 (subsequently designated IL-15DCs) or 20ng/ml IL-21 (subsequently designated IL-21DCs). Cells aggregated within 48h and after 8 days of culture had a characteristic DC morphology (not shown). At day 8, DCs were transferred into microchamber slides. The differentially generated DCs displayed a typical DC phenotype such as cytoplasmic protrusions (**Fig. 1**), indistinguishable between the three types. In FACS analysis after 8d of culture, the IL-15DCs and IL-21DCs showed comparable size and granularity like DCs (**Fig. 1**). Further, the percentage of CD11c⁺ cells (a specific DC marker in mice) was similar high in all conditions (**Fig. 1**). Moreover we assessed by FACS that adding IL-15 or IL-21 to BM cells in combination with GMCSF did not lead to increased differentiation of other cell types like B220⁺B-, NK1.1⁺NK-, CD3⁺T-cells, Gr1⁺granulocytes, Mac3 or CD14⁺macrophages or CD8α⁺ lymphoid DCs (not shown). Thus, IL-15 and IL-21 did neither alter the purity of the generated DCs nor the total yield compared to GMCSF alone (mean of 7.5x10⁶ cells out of 2x10⁶ seeded for all conditions).

**IL-21 and IL-15 receptors are expressed in BM derived DCs**

The IL-15 receptor complex (IL-15R), which is composed of a private α-subunit, the IL-2/IL-15Rβ- and the common γ-chain, is expressed on DCs\(^{13}\). Message-expression for the private IL-21R subunit was previously found in lymphoid tissues\(^{15}\). In order to elucidate whether myeloid DCs express the private receptor subunits and the common γ-chain we performed RT-PCR analysis. We found both private receptor chains, IL-15Rα and IL-21R, expressed...
after 8 days in BMDCs (Fig. 2). Adding IL-15 or IL-21 to the culture did not change expression levels of the IL-21R subunit. Analyzing the expression of IL-15Rα mRNA in the differently generated DC types revealed that all cells showed IL-15Rα transcripts (Fig. 2).

We next determined whether endogenous expression of IL-15 and IL-21 was modulated by the different culture conditions since IL-15 mRNA up-regulation was identified during an increase of DC activation. Transcripts for IL-15 were expressed in all three differentiated DC types at comparable levels, however IL-21 expression was not detected (Fig. 2).

Besides its high affinity receptor, IL-15Rα, IL-15 is also able to signal through the low affinity IL-2Rβ-chain. For both cytokines, the common γ-chain is an indispensable subunit so we also examined expression of the β- and γ-subunit and in addition the IL-2Rα–chain. IL-2Rα mRNA was only low expressed in IL-21DCs compared to DCs or IL-15DCs (Fig. 2). Interestingly, the mRNA expression of the β-subunit was restricted to IL-15DCs (Fig. 2) whereas in DCs or IL-21DCs no expression was detectable, probably since only IL-15 uses - and therefore up-regulates this receptor. All DC types had comparable mRNA levels for the common γ-chain (Fig. 2). Shown are representative data out of 5 experiments. Positive controls exhibit the expected results.

Taken together, BM derived myeloid DCs expressed the private receptor chains IL-15Rα and IL-21R plus the common γ-chain and therefore may respond to IL-21 and IL-15, where the latter was expressed by DCs itself. IL-21 was not expressed in any condition, supporting the finding, that IL-21 is mainly produced by activated T-cells. Since no antibodies against the IL-21R are currently available, the protein expression remains to be determined.
IL-21 reduced MHCII and CCR7 expression

Antigen presentation to and co-stimulation of T-cells by DCs in innate and acquired immunity are mediated by MHC- and a variety of co-stimulatory molecules on DCs. To assess the repertoire of surface molecules on DCs we did phenotyping by FACS after 8 days of culture. Interestingly, generation in the presence of IL-21 resulted in a significantly decreased expression of MHCII (Fig. 3A) compared to DCs or IL-15DCs.

In contrast, all three differently generated DCs showed comparable levels of CD80 and slightly reduced CD86 expression (Fig. 3A). Moreover, all DCs did not differ in the expression of OX40L, CD95, CD95L, CD11b, and CD54 (not shown). Although adding IL-21 or IL-15 during generation did not alter the quantity and morphology of DCs, the phenotype of the DCs shown by expression of the functional relevant MHCII antigen-presenting molecule was modulated.

Chemokine receptors are regulated during DC maturation and modulate trafficking of DCs. Immature DCs express high CCR1 and CCR5 whereas mature DCs up-regulate CCR7, which mediates migration to the DLN. Incubating normal DCs with IL-21 for 24h increased CCR1 and CCR5 but decreased CCR7 expression (Fig. 3B). IL-21DCs presented the same phenotype, supporting their immature state compared to IL-15DCs (Fig. 3B).

High endocytosis in IL-21DCs versus low activity in IL-15DCs

The phenotypic changes of DCs generated in the presence of IL-21, particularly the significantly reduced expression of the MHCII molecule, point to different maturational stages and suggest that IL-21 might modulate APC functions. In vitro, immature DCs are characterized by an increased antigen-capture activity. Therefore, we studied the endocytic activity using a fluorescent model-antigen. FITC-dextran uptake (at 37°C) was monitored
after 8 days of DC-culture as described\textsuperscript{21}. IL-21DCs showed a significantly increased FITC-dextran uptake (MFI: 495) compared to DCs (Fig. 4). Prolonged incubation did not increase the uptake (not shown). In contrast to IL-21DCs, FITC-dextran uptake was strongly reduced in IL-15DCs (MFI: 59) compared to normal DCs (MFI: 156). To control passive FITC diffusion, all experiments were additionally performed on ice, showing very low FITC-dextran uptake. Internalization was further confirmed by fluorescence microscopy (not shown). In addition, using the hapten FITC, IL-21DCs also showed highest uptake \textit{in vitro} (not shown). Thus, the presence of IL-21 \textit{in vitro} leads to the differentiation of “functional immature” DCs, characterized by high unspecific antigen uptake whereas IL-15 induces DCs with a mature phenotype, accompanied by lower unspecific antigen uptake.

**IL-21DCs keep their immature phenotype after antigen uptake and LPS stimulation**

The high FITC-dextran uptake, showed above, indicates that IL-21DCs have functional alterations. To study this more in detail we investigated the activation of DCs by different stimuli. It is known that contact sensitizer trigger DC activation \textit{in vitro}\textsuperscript{26} as well as bacterial products like LPS\textsuperscript{27}. DC activation and maturation is accompanied by up-regulation of MHC and co-stimulatory molecules.

To examine whether IL-21DCs are blocked in maturation by keeping their immature phenotype even after strong stimulation by LPS or after antigen-uptake we incubated all DC types with the contact sensitizer FITC (Fig. 5A) and with low dose LPS (Fig. 5B) for 24h. FACS analysis of CD80, CD86 and of MHCII revealed that IL-21DCs showed a significantly inhibited expression of these representative molecules in all conditions (Fig. 5A and B as compared to unstimulated cells, see Fig. 3). This inability of IL-21DCs to up-regulate these molecules was not due to altered TLR2 and TLR4 LPS receptor expression (which was comparable to DCs and IL-15DC; not shown) or due to insufficient Ag-uptake, which we have shown above to be rather enhanced. In contrast, DCs and IL-15DCs showed clearly an
up-regulation of CD80 and MHCII suggesting that IL-21DCs were blocked in their expression, suggesting that they are unable to “mature” after these stimuli.

**IL-21DCs inhibit antigen specific T-cell proliferation**

The capacity to induce specific T-cell activation and proliferation is a functional hallmark of mature DCs whereas immature DCs fail to prime T-cell responses. To assess whether IL-21DCs are inhibited to induce T-cell response we set up an antigen-specific T-cell proliferation assay. The different DCs after 8 days of culture were pulsed with OVA\(^{323-339}\) peptide and co-cultured for 72h with T-cells from lymph nodes of syngenic OTII\(^{tg}\) mice. We compared the T-cell stimulatory capacity of DCs that had been cultured with GMCSF alone or in combination with IL-15 or IL-21. As shown in Fig. 6, IL-15DCs showed a high significantly increased ability to prime T-cell proliferation, compared to DCs, with a maximum at OVA\(^{323-339}\) peptide concentration of 0.3µM. In contrast IL-21DCs induced significantly lower proliferation. This reduced ability of IL-21DCs to prime specific T-cells could refer to the above described reduced MHCII expression supporting the deduction that DCs generated in the presence of IL-21 are less mature and - most important – do not acquire an immunogenic, T-cell activating phenotype after Ag uptake.

**IL-21DCs are unable to prime in vivo contact hypersensitivity**

Small molecules, which act as antigens after protein binding are designated as haptens, and induce a contact hypersensitivity (CHS) in the skin. Following application to skin, epidermal DCs take up hapten-protein complexes, process them and migrate towards the regional DLN, to prime antigen-specific T-cells. During this process, DCs convert from an immature into an activated functional state\(^{28}\). In addition to the *in vitro* data, we examined whether IL-15- and IL-21DCs showed also a modulated capability for T-cell priming *in vivo*. Therefore we labeled the different DCs *in vitro* with the fluorescent hapten FITC and injected them...
subcutaneous in the footpad of syngenic C57BL/6 mice. After 5 days mice were challenged at one ear with FITC and the ability to initiate an antigen-specific, T-cell mediated immune response was examined by measuring the FITC-specific ear swelling 24, 48 and 72h after challenge. Unsensitized mice served as negative controls for unspecific ear swelling.

Mice that had been actively sensitized with IL-15DCs showed a high significant increase in the CHS ear swelling response 24h after challenge compared to mice, which had been injected with DCs or IL-21DCs (Fig. 7A). In contrast injection of IL-21DCs resulted in high significantly reduced CHS response at all three time points i.e. no swelling above unsensitized controls was observed.

To confirm that sensitization was based on active migration of the injected, viable DCs from the footpad to DLN, additional control mice were injected with FITC labeled, glutaraldehyde fixed (dead) DCs which failed to induce any CHS response (not shown).

Two weeks after the first challenge, a second challenge was performed by painting FITC on the other ear and analyzing ear swelling as before (Fig. 7B). Mice, sensitized by DCs and IL-15DCs showed in repetition a strong ear swelling, even more pronounced as after the first challenge. However, mice sensitized with IL-21DCs again did not mount a significant response compared to negative controls.

These findings are completely in line with our previous in vitro data stressing the fact that combining GMCSF and IL-15 generates “high immunogenic” DCs while IL-21 induces “functional immature” DCs, unable to mature to fully effective, T-cell-priming APCs after antigen uptake neither in vitro nor in vivo.
**Comparable migration of all DC types to DLN**

To exclude, that the differences in CHS responses are due to altered migratory DC migration, we investigated their abilities to enter DLN *in vivo*\(^{29}\). For this purpose FITC-labeled DCs were injected in the hind footpad. After 24h cell suspensions from the DLNs were prepared as described\(^{24}\), stained with anti-CD11c antibody and analyzed by FACS. Double positive cells (FITC\(^+\)/CD11c\(^+\)), which migrated in the DLN, are given in percent of total CD11c\(^+\) DCs (the total number of DCs in the DLN did not differ). As control, unlabelled DCs were injected. All DCs showed similar migratory capacities; 25-30\% of the total lymph node CD11c\(^+\) DCs migrated within the 24h from the periphery into DLNs (Fig. 7C). This exhibits that the reduction of CHS responses by IL-21DCs *in vivo* is not attributed to limited migration.

In contrast, footpad-injected glutaraldehyde-fixed (i.e. “dead”) FITC labeled DCs did not reach the DLN and no FITC uptake by surrounding DCs of recipient mice was observed (not shown), indicating that the observed migration into DLN is an active and specific process.

**IL-21 inhibits LPS induced activation and release of pro-inflammatory cytokines by normal DCs**

Since IL-21, when present during the entire differentiation prevents DC maturation, we subsequently elucidated whether IL-21 could act also in short time course on DC activation. To this end, we studied the effects of IL-21 and IL-15 when given in parallel to LPS. Therefore DCs, differentiated 8 days with GMCSF only were activated for additional 24h with low dose LPS (10ng/ml) alone or in combination with 100ng/ml of IL-15 or IL-21. As control we used the anti-inflammatory cytokine IL-10 that inhibits DC activation\(^{25}\). To proof, that IL-15 and IL-21 were taken up by DCs, we did confocal microscopy and found both cytokines internalized after 30 min (not shown).
It was evident that adding IL-21 showed similar properties like IL-10 in blocking the LPS induced up-regulation of CD80, CD86 and MHCII molecules, which were expressed 40%, 70% respectively 90% higher when LPS was used alone (Fig. 8A). This inhibitory effect was already seen at IL-21 concentrations of 1ng/ml (not shown). These observations strongly support the evidence that IL-21 prevents also in vitro induced maturation by LPS. Supplementing IL-15 to LPS did not further enhance the effects mediated by LPS alone, which is likely due to maximal activation reached by LPS. Additionally we analyzed the effects of the cytokines alone and stimulated DCs with the IL-15, IL-21 and IL-10 (100ng/ml) for 24h and did subsequently FACS analysis. We found no pronounced up-regulation of the indicated markers due to the stimulation with solely cytokines and no induction of apoptosis by IL-21 at concentrations up to 200ng/ml (not shown).

To check, how IL-21 influences release of pro-inflammatory cytokines and whether the observed effects of IL-21 were mediated by the release of the inhibitory cytokine IL-10, we analyzed the supernatant of the stimulated DCs after 24h. As shown in figure 8B, addition of IL-21 could high-significantly suppress the pro-inflammatory cytokines IL-1β, IL-12, IL-6 and TNF-α but did not significantly enhances DC-production of IL-10. Incubation of DCs with IL-21 alone (using increasing concentrations from 1-100ng/ml) for 24h resulted in no significant increase of the analyzed cytokines compared to medium alone and had no toxic effect as cell survival was checked by AnnexinV/PI staining (not shown).

In conclusion, IL-21 not only inhibits DC maturation when present during the generation but also the activation and maturation of DCs in the presence of high-potent stimuli such as LPS. These findings could also be verified for human DCs, suggesting that these effects of IL-21 are not species-specific (SBP, manuscript submitted).
Discussion

Our results show that the newly described cytokine IL-21 and its structural relative IL-15 modulate DC differentiation, maturation and function in vitro and in vivo. Given IL-21 to BM cultures in addition to GMCSF provided phenotypic and functional “immature” DCs with reduced MHCII expression, elevated endocytic activity and limited T-cell stimulatory ability. This is the first report, showing that IL-21 in combination with GMCSF is able to modulate differentiation of myeloid BMDCs and exhibits a regulatory impact on APC function, therefore expanding results from literature demonstrating that IL-21 influences also lymphoid cell development and function. By contrast IL-15, which is structural related and shares the common γ-chain with IL-21, mediated opposite effects as IL-21 by promoting maturation of DCs in vitro and significantly enhanced DC mediated antigen-specific T-cell response in vitro and in vivo.

DCs are able to respond directly to pathogens like microbial cell-wall components and indirectly sensing infection through inflammatory cytokines. In response to these “danger” signals, DCs are activated to enter maturation. Our data imply that IL-21 is able to modulate maturation of DCs. Thus we showed that LPS induced activation of DCs, characterized by an elevated expression of functional relevant molecules like MHCII, CD80, CD86, and production of pro-inflammatory cytokines (IL-6, IL-12, IL-1β and TNF-α) was significantly reduced when IL-21 was given in parallel to LPS. Other cytokines in the extra cellular environment, notably from T-cell released IL-10, have been implicated in impeding DC maturation which was associated with the retention of an “immature” phenotype. Concomitant incubation of DCs with LPS and IL-10 blocked maturation likewise and impaired expression of antigen- and costimulatory molecules, suggesting that IL-21 has comparable inhibitory effects like IL-10. We further could show that the inhibition by IL-21
is not mediated by IL-10 release from DCs, but rather seems to be a direct effect of IL-21. This is in line with recently published data showing that IL-21 did not induce IL-10 or TGF-β release from T-cells. However, further attempts have to be undertaken to elucidate the exact actions of IL-21 in blocking DC activation.

Nevertheless, our data provide first evidences that IL-21 is an important negative regulator of DC activation in response to microbial stimuli. In contrast IL-15 did not enhanced the LPS action, most probably due to the fact, that IL-15 is already produced by LPS-stimulated DCs, thus exogenous added IL-15 could not further enhance the activation.

Because IL-21 was shown to support differentiation of myeloid NK cells from BM progenitor cells and act in this respect synergistically with IL-15 we investigated whether IL-21 and its structural relative IL-15 have modulatory impact on DC differentiation. Moreover IL-15 and IL-21 belong to a receptor family, which shares a common γ-chain in its receptor complex. With the knowledge that IL-4, another member of this receptor-sharing family, modulates DC differentiation, we seek after comparable influence of IL-15 and IL-21. Our studies revealed completely contrary outcomes of DCs generated in the presence of either IL-15 or IL-21. We observed on the one hand highly immunogenic IL-15DCs, which represent mature DCs after several, by an exhaustive body of literature, defined criteria. On the other hand IL-21DCs were blocked in their activation and were unable to enter maturation after various stimuli.

It is well known that DC maturation is directly linked to T-cell stimulatory capacities. Indeed our experiments revealed that antigen-specific T-cell response was significantly reduced with IL-21DCs. By contrast, IL-15DCs mediated high significantly increased T-cell proliferation. While IL-15 led to more mature DCs characterized by enhanced capability to prime T-cell response, IL-21 silenced DC functions in this respect. The immature phenotype
of IL-21DCs was also stable after in vitro incubation of IL-21DCs with the contact sensitizer FITC, where normal DCs and IL-15DCs changed their phenotype by up-regulating B7 and MHC molecules, which has been expected from recent reports.26, 35

The CHS28 model provides a unique approach to assess multiple functions of DCs in vivo. DCs transport antigens from the periphery to the DLN to prime T-cells, which then become effectors of cell-mediated immunity. Indeed IL-15DCs induced a significantly increased CHS response whereas the same number of IL-21DCs failed to induce CHS. To exclude that the inability of IL-21DCs to induce a CHS response was due to reduced migration capacity we examined the local draining lymph nodes and found IL-21DCs traffic to same extends to the local DLN compared to DCs and IL-15DCs. Therefore the lack of T-cell stimulation by IL-21DCs is most likely attributed to their “immature” phenotype with reduced MHCII expression and not due to a defect in homing to DLN. This is supported by the observation that the lack of IL-21 signaling in IL-21R-/- mice results in an increased delayed type hypersensitivity (DTH) response.36 This correlated with a marked increase in IFN-γ production by T-cells. Wurster and coworkers showed also that IL-21 treatment dampened the responsiveness of T-cells to IL-12 through a reduction of STAT-4 which could extended by our data, showing that IL-21 also directly inhibits IL-12 release by DCs. We reported that DCs developing under the aegis of IL-21 displayed immunosuppressive functions by abrogating Ag specific CHS. In addition, mice that had been injected with IL-21DCs even after a second application of FITC two weeks after DC injection were unable to mount an ear swelling. This implicates that IL-21DCs not only failed to undergo maturation after antigen uptake and migration to DLNs but also miss to establish a T-cell response and hence might not be able to induce memory T-cells. It has to be clarified in future experiments, if or to which extent IL-21DCs may induce anergy and therefore may be used for tolerance induction. Interestingly, we further observed that normal DCs also act immunosuppressive in vivo when
pre-incubated with IL-21 only for a short time (unpublished data). Thus we could state that IL-21 plays not only a role in a discrete developmental window but also is able to act in short time courses making it an attractive therapeutical target.

Indeed, preliminary experiments showed that injection of IL-21 in mice significantly down-regulates expression of costimulatory molecules like CD80, CD86 and MHCII up to 50% in spleenic DCs (unpublished data). Subsequently antigen presentation by these immature DCs might induce differentiation of naïve T-cells toward suppressor/ regulatory phenotype\textsuperscript{37, 38}.

Moreover we also observed a suppressive effect of IL-21 on macrophage function, which further support our data that IL-21 has silencing properties on APCs (unpublished data).

Because IL-15 was found to induce DC activation after short time incubation\textsuperscript{3, 13} it has been considered to be important in maturation processes. We here present evidences that during generation of DCs, permanently given IL-15 induced high potent DCs with regard to T-cell stimulation. Since IL-15 is produced \textit{in vivo} by BM- and lymph node stromal cells\textsuperscript{4}, the presence of this cytokine in the extracellular matrix may shape DC phenotype during hematopoiesis and DC function during a variety of immune responses. That DC-derived IL-15 indeed is essential for induction of Th1 immune responses could be shown recently by us, using IL-15 and IL-15R\textsubscript{α} deficient DCs (RR, unpublished data).

Despite the fact, that IL-15 and IL-21 share structural motives and the common γ-chain in they mediate completely contrasting effects on DCs which are most likely due to their unique private receptor α-chains that complete the IL-15Rαβγ and IL-21R complexes and thereby allow differential responsiveness depending on the ligand and high affinity receptor expressed. How the γ-chain in the multipart-receptors of these cytokines is involved in the DC
differentiation has to be determined, however, it is known, that the effects of IL-4 on DCs are independent from the γ-chain and its associated kinase Jak3\textsuperscript{39}. In this respect it will be necessary to unravel the modulation of GMCSF signaling pathway by IL-15 and IL-21.

Alternatively, blocking of IL-21 and its private receptor could give more insight into the mechanisms, which keep DCs controlled in an immature state. Moreover it is reasonable to speculate that T-cells release IL-21 during or after contact with DCs, providing a negative feedback signal keeping DCs in vivo in an immature state. In addition to suppressing DC maturation, IL-21 was also shown to prevent IL-15-mediated proliferation of murine CD44\textsuperscript{+}CD8\textsuperscript{+} memory T-cells and the up-regulation of receptors for IL-2, IL-15 and IFN-γ\textsuperscript{40}.

In vivo, the tolerogenity of immature DCs could lead to therapeutical applications for instance in prolonging allograft survival\textsuperscript{41} or treating autoimmune diseases. Vice versa IL-15DCs might serve as potent immune stimulating DCs and possibly provide new or expanded properties for immunotherapy based on the injection of antigen-pulsed DCs\textsuperscript{42,43}.

In conclusion, we found, that despite structural similarities, IL-15 and IL-21 have completely opposite functional consequences for DC biology including maturation and antigen presentation in vitro and in vivo. Our results display that IL-15 induces high immunogenic DCs and IL-21 release may lead to inhibition of DC activation and maturation.
Acknowledgements

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Figure 1. CD11c+ DCs differentiate in the presence of IL-15 or IL-21. Murine BM cells were induced to differentiate to DCs by GMCSF alone (DCs), or by addition of IL-15 (IL-15DCs), or IL-21 (IL-21DCs) during the entire culture period of 8 days. Cells were analyzed by FACS at day 8. Shown is one representative out of 10 experiments. Morphology was assessed by culturing the DCs for 48h on micro chamber slides following Pappenheim staining. The scale bar is equal to 100µm.
Figure 2. Expression of IL-15/IL-21 receptor components in DCs. Eight-days cultured DCs (lane 1), IL-15DCs (lane 2) and IL-21DCs (lane 3) were analyzed by RT-PCR for mRNA expression of IL-21R, IL-15Rα, IL-15, IL-21, and IL-2Rα- and β-chain as well as for the common γ-chain. β-actin message expression was used to normalize the cDNA amount. To exclude contaminations all experiments were run with a mock PCR and found negative (not shown). The correct size of PCR product was assessed with a marker (M). As positive controls (C) for IL-21 and its receptor cDNA from CD4+ T-cells from C57BL/6 mice was isolated. For IL-15 and its receptor we used cDNA from L929 fibroblasts and CTLL-2 cells were positive reference for all IL-2R chains. Shown is one representative experiment out of 5.
Figure 3. IL-21DCs express low MHC II and CCR7 mRNA. A): DCs, IL-15DCs, and IL-21DCs were analyzed by multicolor flow cytometry (gated on CD11c positive cells). Shown are representative data out of 10 experiments. Given in each histogram is the percentage of positive cells. Unstained cells or isotype control antibodies were used as negative controls. B): IL-21 enhances CCR1 and CCR5 but suppresses CCR7 expression. Chemokine receptor expression was analyzed in DCs, IL-21-stimulated DCs (100ng/ml for 24h), IL-15DCs or IL-21DCs by PCR.
Figure 4. High versus low antigen uptake (endocytosis) by IL-21DCs or IL-15DCs. Cells were incubated 30 min at 37°C with FITC labeled dextran, washed and analyzed for FITC-dextran uptake by FACS. Shown is one representative out of 3 experiments.
Figure 5. IL-21DCs are blocked in their maturation. All three generated DC types were stimulated with A) FITC and B) LPS for additional 24h after 8 days of culture. Given is the percentage of positive (CD86) or high positive (CD80, MHCII) cells (fluorescence channel >10²) with high surface density/number defined by the marker M1. Shown is one representative out of two experiments.
Figure 6. High versus low antigen-specific T-cell stimulation in vitro by IL-15DCs or IL-21DCs. DCs, IL-15DCs and IL-21DCs were labeled with different OVA$_{323-339}$ peptide concentrations and incubated with lymph-node cells from OTII mice. After 72h incubation, cells were labeled with 0.2µCi [3H]thymidine. Shown is one representative out of 3 experiments. Significance compared to DCs was calculated using student’s t-test (** p ≤ 0.01).
Figure 7. Failed versus enhanced induction of T-cell mediated contact hypersensitivity by IL-21DCs or IL-15DCs in vivo. DCs, IL-15DCs and IL-21DCs were labeled in vitro with FITC and injected into the hind footpad.
Fig. 7A. 1\textsuperscript{st} challenge: 5 days later, mice were challenged with FITC-painting to the ear and after 24, 48, and 72h the ear swelling reaction was analyzed. Painting of unsensitized mice served as control.

Fig. 7B. 2\textsuperscript{nd} challenge: 14 days after the first challenge, the mice were painted again on the other ear, and swelling was analyzed as before.

Fig. 7C. Migration of DCs was analyzed by FACS of the draining lymph nodes, 24h after injection of 5x10\textsuperscript{5} FITC labeled DCs. Cells were double stained for CD11c. Shown are the CD11c/FITC\textsuperscript{+} DCs as percent of total CD11c\textsuperscript{+} cells in the DLN. Controls were injected with unlabelled DCs.

The experiments were repeated at least twice, shown is the mean±SD of 12 mice. Significance between IL-15DCs and IL-21DCs compared to DCs was calculated using student’s t-test (*p≤0.05; **p≤0.01).
Figure 8. IL-21 inhibits short-time, LPS-induced DC activation and pro-inflammatory cytokine production in vitro. Immature DCs (after 8 days of culture with GMCSF only) were activated with low-dose LPS (10ng/ml) or with LPS plus IL-15, IL-21 or IL-10 for 24h.
A): Surface expression of costimulatory and MHC II molecules were analyzed by FACS. Given is the percentage of high positive cells, defined by the marker M1. B): Cytokines were analyzed in the DC-culture supernatant. Significance was calculated using Student-t-test and shown is the mean±SD (note different scale for IL-6 concentration). Shown is one representative out of four experiments.
Table

Table 1. Primer sequences.

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References


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