Interleukin-18 attracts plasmacytoid dendritic cells (DC2) and promotes Th1 induction by DC2 through IL-18 receptor expression

Short title: Plasmacytoid DC express functional IL-18R

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Abstract

*In vivo* evidence suggests that interleukin-18 (IL-18) shapes the development of adaptive immunity toward T helper cell type 1 (Th1) responses. Monocyte-derived dendritic cells 1 (DC1) preferentially induce a Th1 response, while plasmacytoid DC-derived DC2 have been linked to a Th2 response. We analyzed the role of IL-18 during the initiation phase of a Th response *in vitro* to elucidate the basis of the aforementioned *in vivo* observations. IL-18 was constitutively released from DC1, but not DC2. Neutralization of IL-18 in co-culture experiments of DC1 with allogeneic naïve T lymphocytes did not alter the Th1/Th2 phenotype, while anti-IL-12 efficiently downregulated the Th1 response. Unexpectedly, IL-18R α and β chain were expressed on DC2 lineage. IL-18R expression was functional as IL-18 induced chemotaxis in plasmacytoid DC (pre-DC2), and enhanced the allostimulatory capacity of IL-3-differentiated DC2. Pre-DC2 exposed to IL-18 skewed the development of Th cells toward Th1 in co-culture experiments of DC2 and allogeneic naïve T cells, which was inhibited by IL-12 p70 neutralization. IL-18 might have a profound role during the initiation phase of an immune response by recruiting pre-DC2 and modulating the function of DC2. Herbert.Tilg@uibk.ac.at

[186 words]

**Abbreviations**

DC, dendritic cell; DC1, monocyte-derived DC; DC2, plasmacytoid DC differentiated with IL-3; IL-18-DC2, plasmacytoid DC differentiated with IL-3+IL-18;
Introduction

Interleukin-18 (IL-18) is a polypeptide cytokine identified by its ability to induce interferon-γ (IFN-γ), thus formerly called IFN-γ-inducing factor (IGIF) (1,2). From a structural point of view, IL-18 is related to the IL-1β family of cytokines, and shares caspase-1 (IL-1β converting enzyme, ICE) for processing pro-IL-18 to bioactive IL-18 (3). Furthermore, one of the IL-18 receptor chains (IL-18Rα) is the IL-1 receptor related protein (IL-1Rrp), a member of the IL-1 receptor family (4). Recently, IL-18 binding protein (IL-18BP), a soluble antagonist of IL-18, has been identified (5). IL-18BP shares some homology with the IL-1 type II receptor (5).

From a functional point of view, IL-18 might be more related to IL-12 (6,7). IL-12 and IL-18 synergize in inducing IFN-γ production in CD4+ T lymphocytes (1,8). IL-18 acts as a costimulant for Th1 cells to augment IL-2, GM-CSF, and IL-2Rα production, and it induces cell proliferation, whereas IL-18 has no effect on Th2 clones (7).

Furthermore, IL-18Rα is selectively expressed on the surface of Th1 cells but not of Th2 cells (9). While IL-12 is well established as a major determinant of development of naïve T cells into Th1 cells (10-12), the role of IL-18 in the commitment of naïve Th cells to Th1 lineage is less clear. However, data primarily derived from knock-out experiments indicate that IL-18 might indeed favor a Th1 response in vivo, although the cellular / molecular pathway awaits to be established (13-20).

Induction of primary immune responses and consequently differentiation of naïve Th cells into Th1 and Th2 cells depends on the interaction between dendritic cells (DCs) as antigen-presenting cells and naïve T cells (21). Two distinct types of DC precursors have been identified in humans: myeloid monocytes (pre-DC1) and plasmacytoid DC precursors (pre-DC2) (12,22). Pre-DC1 differentiate into DC1 by IL-
4 and GM-CSF (23), while pre-DC2 require IL-3 for their development into DC2 (24). Activated myeloid DC1 produce large amounts of IL-12 and preferentially induce Th1 development, while activated lymphoid DC2 preferentially induce Th2 development (12,22,25-28). As outlined above, IL-18 might preferentially support the development of a Th1 response \textit{in vivo}, but how this is achieved is currently not understood. Therefore, we designed the current study to elucidate the role of IL-18 during the initiation phase of an immune response.

\textbf{Materials and Methods}

\textit{Reagents}

The culture medium used in the present study was RPMI 1640 (Schoeller Pharma, Vienna, Austria) supplemented with 10% heat-inactivated (30min, 56°C) fetal calf serum (FCS, Gibco, Life Technologies, Vienna, Austria), 100 U/mL penicillin G and 100 µg/mL streptomycin (Schoeller Pharma). Recombinant human (rhu) IL-4 was generously supplied by Schering-Plough Research Institute (Kenilworth, NJ), rhuGM-CSF was purchased from Schering-Plough (AESCA, Traiskirchen, Austria), rhuIL-3 and rhuIL-18 were from Peprotech (London, UK), stromal-derived factor-1 (SDF-1) from R&D Systems (Minneapolis, MN). The following antibodies were used: Phycoerythrin (PE)-conjugated anti-IL-18R\alpha mAb (R&D Systems), IL-3R\alpha (CD123) mAb (Santa Cruz Biotechnology, Heidelberg, Germany), anti-CD3 mAb and anti-CD28 mAb (Pharmingen, Hamburg, Germany), fluorescein isothiocyanate (FITC)-conjugated IFN-\gamma mAb, PE-conjugated IL-4 mAb (Pharmingen), FITC-conjugated anti-CD80 mAb, PE-conjugated anti-CD86 mAb, PE conjugated anti-IL-12R\beta1 and PE-conjugated anti-IL-12R\beta2 (Pharmingen), FITC-conjugated HLA-DR mAb (Serotec, Raleigh, NJ), CD40 mAb (Pharmingen) and CD83 mAb (Immunotech,
Instrumentation Laboratories, Vienna, Austria), and FITC-conjugated anti-mouse IgG (Sigma, Vienna, Austria), PE-conjugated mouse IgG1κ and PE-conjugated rat IgG2a (Pharmingen). Lipopolysaccharide (LPS; *Escherichia coli* 055:B5) was obtained from Sigma.

**Dendritic cells**

Peripheral blood mononuclear cells (PBMC) were obtained by density gradient centrifugation (Histopaque 1077, Sigma) of buffy coats obtained from the local blood bank. DC1 precursors (i.e. monocytes) were obtained by immunomagnetic sorting using CD14-coated MACS microbeads (purity > 98%) according to manufacturer’s protocol (Miltenyi Biotec, Bergisch-Gladbach, Germany). DC1 were generated as described (23,29). In brief, monocytes were cultured at a density of $1 \times 10^6$ per mL in culture medium supplemented with $1 \times 10^3$ IU/mL IL-4 and $1 \times 10^3$ IU/mL GM-CSF. Culture medium, IL-4 and GM-CSF were replenished on day+2 and day+5. DC1 were used at day+6 of culture. DC2 precursors (pre-DC2, i.e. plasmacytoid dendritic cells) (12,24) have been recently described to selectively express a novel type II C-type lectin, which is recognized by mAbs termed BDCA2 and BDCA4 (30,31). Pre-DC2 were isolated from PBMC by immunomagnetic sorting of BDCA4$^+$ cells (purity >94%, typical recovery $0.8 \times 10^6$ - $2.5 \times 10^6$ per 500 mL whole blood) as recently described (30). DC2 were cultured for 3 days at a density of $1 \times 10^6$ per mL RPMI / 10% FCS with addition of rhuIL-3 at 10 ng/mL as described (12,24,32,33). RhuIL-18 was added at the onset of DC2 culture as indicated at 100 ng/mL. In selected experiments, DC1 and DC2, respectively, were activated / matured (12) by irradiated (30 Gy) murine myeloma cells transfected with human CD154/CD40 ligand (P3 × TBA7 cells; one P3
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× TBA7 cell per two DC) (34) kindly provided by R.A. Kroczek (Robert Koch Institute, Berlin, Germany) and N. Romani (Dept. of Dermatology, Innsbruck, Austria).

**DC-T cell cocultures**

Allogeneic CD4+CD45RA+ naïve T lymphocytes were isolated from PBMC by immunomagnetic purification. In brief, untouched CD4+ T lymphocytes were obtained through MACS CD4+ T cell isolation kit according to manufacturer's instructions (Miltenyi). In a next step, naïve CD45RA+ T lymphocytes were positively isolated by CD45RA-coated immunomagnetic microbeads (Miltenyi) (12,35). Naïve T lymphocytes were co-cultured with extensively washed DC1 and DC2, respectively, for 6 days at a ratio of 5:1 in culture medium (12,35). Where indicated, DC1, DC2, and IL-18-DC2 were activated through CD40L-expressing irradiated P3 × TBA7 cells as described in the previous paragraph. Anti-IL-18 and anti-IL-12 mAbs (10 µg/mL each) were added at the onset of co-culture of DC1 and T cells as indicated. On day-6, cells were re-stimulated at a density of 1×10^6 per mL with plate-bound anti-CD3 (5 µg/mL in PBS, overnight, 4°C) and anti-CD28 (1 µg/mL) for 5h (for intracellular cytokine staining) and 24h (for quantification of cytokines in the supernatant), respectively (12,35). Figure 1 shows an outline of the performed experiments.

**Flow cytometry analysis**

For intracellular two-color flow cytometry, T cells were re-stimulated as described above. Brefeldin A (1 µg/mL, Sigma) was added into the cultures for 2 hours before the staining to prevent cytokine secretion. Cells were washed and fixed with paraformaldehyde, permeabilized with 1% saponin (Sigma) and incubated with PE-IL-4 and FITC-IFN-γ. After washing, cells were immediately analysed on a FACS Calibur (Becton Dickinson, Vienna, Austria).
For surface flow cytometry of DC, DC were incubated with indicated FITC- or PE-labeled mAbs for 30 min, followed by washing, and immediate analysis, as recently described (29). For CD40 and CD83 staining, unlabeled primary mAbs were used, with FITC-anti-mouse IgG secondary reagent (1:40 dilution) as described previously (29). Data evaluation was performed by Cellquest software (Becton Dickinson).

**Apoptosis assessment**

Apoptosis was determined by FACS analysis via propidium iodine (Sigma) exclusion test or TUNEL staining (Roche Applied Science, Mannheim, Germany) according to manufacturers’ protocols.

**Cytokine determination in the supernatant**

Commercially available matched antibody pairs and recombinant protein standards were used according to manufacturers’ instructions for enzyme-linked immunosorbent assays (ELISA) for the following cytokines: IFN-γ, IL-4, IL-12 p70, IL-10 (Pharmingen), IL-18 and IFN-α (both Bender MedSystems, Vienna, Austria).

**Polymerase chain reaction**

IL-18R α and β chain, IL-12 p35 and p40, IL-23 p19, and IL-27 p28 and EBI3 chain cDNAs were obtained by reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was extracted from indicated cell types (Th1-polarized cells, obtained by co-culture of allogeneic naïve Th cells with DC1 for 6 days, which have been shown by FACS analysis to express IL-18Rα abundantly (data not shown), served as a positive control for IL-18R α and β chain amplification). Reverse transcription was performed with Omniscript reverse transcriptase (RT) (Qiagen, Hilden, Germany) using a random hexanucleotide mix (Roche, Basel, Switzerland).
Complementary DNA PCR was performed with Hot Star Taq Polymerase (Qiagen) using 10 mM dNTPs (Amresco, Solon, OH, USA), 15 pmol of each sense and antisense primer in 30 cycles (IL-18Rα, IL-18Rβ, β-actin) or 33 cycles (all others) of 95°C for 1 min, respective annealing temperatures (IL-18Rα 57°C; IL-18Rβ 59°C; IL-12 p35 58,4°C; IL-12 p40 59,4°C; IL-23 p19 59,4°C; IL-27 p28 63,4°C; EBI3 63,4°C; β-actin 59°C) for 1 min and 72°C for 1 min, respectively, in a total volume of 50 µl. Primers were as follows: IL-18R α chain 5'-TGA CTC CAG AAG GCA AAT GGC-3' (position 833) and 5'-AAA GAG ATT TAT CGG CCT TCC-3' (position 1523); IL-18R β chain 5'-GCA TCC TGT GAG TAT TCC GCA TC-3' (position 940) and 5'-CAG CAC GGC CAC CAG GGT CC-3' (position 1589); IL-12 p35 5'-ACC TGC CGC GGC CAC AGG TC-3' (position 704); IL-12 p40 5'-GGT ATC ACC TGG ACC TTG GAC C-3' (position 211) and 5'-TGC TGG CAT TTT TGC GGC AG-3' (position 939); IL-23 p19 5'-CAG CAC CCC TGC CTG GAC TC-3' (position 225) and 5'-AAT TTT CAA CAT ATG CAG GTC CC-3' (position 833); IL-27 p28 5'-CGC CAG GAA GCT GCT CTC CG-3' (position 159) and 5'-CCC TGT AAG GCG CTG CCC AG-3' (position 553); EBI3 5'-TCG GTA CCC GAT CGC CGT GG-3' (position 127) and 5'-AGG TTG CCC GGC AGC TCA GC-3' (position 823); β-actin 5'-GTG ACG AGG CCC AGA AGA G-3' and 5'-AGG GGC CGG ACT CAT CGT ACT C-3'.

**Proliferation assays**

Proliferation was assessed in triplicate by [3H]methylthymidine (NEN, Perkin Elmer, Freiburg, Germany) incorporation after 3 days of culture. Cells were pulsed with 1 µCi/well for 19h in 96 well plates.
Chemotaxis assays

Migration of cells into nitrocellulose to gradients of soluble attractants was measured using a 48-well Boyden microchemotaxis chamber (Neuroprobe, Bethesda, MD) in which a 5 µm pore-sized filter (Sartorius, Göttingen, Germany) separated the upper from the lower chamber (36).

As indicated, cells were incubated with antibodies to IL-18 receptor for 20 min prior to migration experiments. To exclude non-specific migration, IL-18 was co-incubated with antibodies to IL-18 in the lower chamber as indicated. Migration time into the filters was 180 min, subsequently filters were dehydrated, fixed, and stained with hematoxylin-eosin (29). Migration depth of cells into the filter was quantified by microscopy, measuring the distance (µm) from the surface of the filter to the leading front of cells, before any cells had reached the lower surface (leading-front assay). Data are expressed as 'chemotaxis index', which is the ratio of the distance of stimulated and random migration of leukocytes into the nitrocellulose filters.

Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation was performed by Student’s t test and for chemotaxis experiments with Kruskal-Wallis ANOVA and post-hoc Mann Whitney U test using SPSS 9.0 software package (SPSS, Chicago, IL), and P < 0.05 considered significant.

Results and Discussion

IL-18 secreted by DC1 cells does not contribute to Th1 development

As expected from previously published data (37,38), experiments studying the effect of maturation of DC1 revealed that the activation process results in substantial
upregulation of IL-18 mRNA (data not shown). In contrast, IL-18 protein secretion is not upregulated by maturational stimuli but rather constitutive in DC1 cells (data not shown), which might be related to downregulation of the enzyme processing IL-18, namely caspase-1 (data not shown). This is in apparent contrast to IL-12 p70, which is markedly upregulated during maturation (data not shown). Furthermore, it should be noted that far less IL-18 (sometimes below the detection limit of our assay) than IL-12 p70 was released from DC1 (data not shown). In contrast to DC1, DC2 did not release detectable amounts of IL-18 and IL-12 p70 (data not shown).

To identify the role of IL-18 during the induction phase of an immune response driven by DC1, anti-IL-18 mAb was added to co-culture experiments with allogeneic naïve Th cells. After 6 days of DC1-Th co-culture, Th cells were harvested and restimulated with anti-CD3 and anti-CD28 to determine the Th phenotype. The outline of these experiments is shown in Fig. 1 and is virtually identical to previously reported experimental approaches (12,28,35). As expected, DC1 preferentially induced a Th1 phenotype, releasing high amounts of IFN-γ and low amounts of IL-4 (Fig. 2ab) (12). Intracellular FACS staining revealed a predominance of IFN-γ-secreting Th1 cells, and few IL-4-secreting Th2 cells (Fig. 2c). As shown in Figure 2, neutralization of IL-18 with anti-IL-18 mAb during the co-culture did not significantly change the Th1 phenotype, while neutralization of IL-12 significantly reduced the amount of IFN-γ released and the number of IFN-γ+ Th1 cells (Fig. 2). Interestingly, the percentage of IFN-γ-producing cells could not be reduced below 20-40%, suggesting that Th1-driving factors other than IL-12 might be present (39). These data are essentially in accordance with those of Stoll et al, demonstrating that IL-18 released from DC1 and acting on naïve Th cells might not have a role in their commitment to Th1 lineage (37,39).
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However, there is increasing in vivo evidence that IL-18 indeed influences the induction phase of an immune response and seems to tip the balance toward Th1 (13-17,19,20). Since we (Fig. 2abc) and others (8,37,39) have ruled out the possibility that IL-18 might act directly on naïve Th cells in this context, we asked whether IL-18 might act one step earlier, namely by instructing DC. IL-18 signals through IL-18 receptor, constituting of the ligand-binding α chain (formerly called IL-1 receptor related protein [IL-1Rrp]) (4,40) and the signal-transducing β chain (formerly called IL-1 receptor accessory protein-like protein [IL-1RAcPL]) (41). Cytofluorometric analysis revealed abundant IL-18Rα expression on pre-DC2 (Fig. 3a), which were isolated by immunomagnetic sorting of BDCA4+ cells. Identity and purity of pre-DC2 was determined by CD123 (IL-3Rα) (Fig. 3b) and HLA-DR (Fig. 3c) expression and the absence of lineage markers (data not shown) (12,24). Pre-DC2 faintly expressed IL-12Rβ1, while IL-12Rβ2 was absent (Fig. 3de). DC2 did also express IL1 8Rα as shown in Fig. 3f. In contrast to DC2 lineage, DC1 lineage cells do not express IL-18Rα chain, as demonstrated on pre-DC1 (i.e. monocytes) (Fig. 3g) and DC1 (Fig. 3h). In accordance, DC1 do not express IL-18Rα mRNA at any time during their differentiation (Fig. 3i), while IL-18Rα mRNA was readily detected in pre-DC2, DC2, and CD40L-matured DC2 (Fig. 3i). A similar mRNA expression pattern was detected for the signal-transducing IL-18Rβ chain, with the exception that IL-18Rβ mRNA was also expressed in pre-DC1 (Fig. 3i). Altogether, this suggested that IL-18R could be functional in DC2 lineage.
IL-18 down-regulates proliferation of pre-DC2

The expression of the receptor of the “Th1-cytokine” IL-18 on DC2 but not DC1 is somewhat unexpected and raises the question whether IL-18 might play a role in the regulation of DC2 differentiation. Recently Rissoan et al. demonstrated that the Th2-cytokine IL-4 enhances DC1 differentiation but kills pre-DC2, an effect that is blocked by the Th1 cytokine IFN-γ (12). It was concluded that a negative feedback loop from mature Th cells may selectively inhibit prolonged Th1 or Th2 responses by regulating survival of the appropriate DC subset (12). Since IL-18 is not a product of the “final pathway” of Th immunity (i.e. not released from Th1 cells), but in contrast primarily secreted from activated macrophages (and DC1), one would expect IL-18 to down-regulate the development of DC2 lineage cells. As reported previously, IL-3 induces the proliferation of pre-DC2 (12,24). Indeed, IL-18 down-regulated the proliferative response of pre-DC2 cultured with IL-3 as demonstrated in Fig. 4a. Interestingly, this did not result in a decrease in the number of DC2 recovered on day 3, as shown in Fig. 4b. Since the number of DC decreases similarly over time in the IL-3 and IL-3+IL-18 group, a lower proliferation index in the IL-3+IL-18 group might be explained by increased survival in this group compared to the IL-3 group, which might make up for lower proliferation in the IL-3+IL-18 group. However, there is no difference in the apoptosis rate 4h and 24h after culture of pre-DC2 with IL-3 vs IL-3+IL-18 as determined by propidium iodine exclusion and TUNEL staining (data not shown). In conclusion, a consistent regulatory “feedback loop” might not be functional, although IL-18 obviously interacts with the differentiation pathway of DC2.

IL-18 induces chemotaxis in pre-DC2

While myeloid DC are recruited by a variety of inflammatory chemokines, the same chemokine receptors expressed on plasmacytoid DC are largely non-functional (42).
Among a wide variety of chemokines tested for their chemotactic properties on pre-DC2, only the lymph-node homing chemokine stromal-derived factor-1 (SDF-1, CXCL12) consistently induced migration (28,42-44). The mode of recruitment of pre-DC2 toward sites of foreign antigen-uptake, i.e. inflammatory sites, is currently unclear. As depicted in Fig. 5a, IL-18 dose-dependently induced a migratory response in Boyden chamber microchemotaxis experiments, which exceeded the response induced by SDF-1. Receptor blockade by anti-IL-18Rα mAb (Fig. 5b) as well as blocking of IL-18 by anti-IL-18 mAb (Fig. 5c) dose-dependently abrogated migration, demonstrating the specificity of IL-18-induced chemotaxis of pre-DC2. These data unambiguously demonstrate the functionality of IL-18 receptor on pre-DC2, and suggest an unexpected role of IL-18 in the recruitment of pre-DC2 to sites of inflammation. A chemotactic function of IL-18 on T lymphocytes has recently been described by Komai-Koma et al (45). Of note is the fact that activated plasmacytoid DC2 themselves secrete chemokines preferentially attracting Th1 cells (CCR1/CCR5 ligands) (42).

**IL-18 increases the allostimulatory capacity of DC2**

To further characterize the functional consequences of IL-18 exposure of pre-DC2, we compared DC2 (differentiated from pre-DC2 for 3 days with IL-3) and IL-18-DC2 (differentiated with IL-3 + IL-18) for their allostimulatory capacity. DC2 and IL-18-DC2 were washed, subsequently activated/matured through CD40L (transfected in P3 × TBA7 cells) as indicated in Fig. 6, and co-cultured with allogeneic naïve Th cells for another 3 days. As shown in Fig. 6, IL-18-stimulation of pre-DC2 increased the proliferative capacity of mature DC2 as assessed by ³H-thymidine incorporation into Th cells. FACS analysis ruled out the possibility that increased HLA-DR expression
on CD40L-activated IL-18-DC2 vs. CD40L-activated DC2 might account for the difference in allostimulatory capacity (data not shown).

**IL-18 skews DC2 toward induction of a Th1 phenotype**

Selective induction of Th2 cells from naïve Th cells has been ascribed to DC2 cells (12,22,28), although the extent of Th2 over Th1 induction might vary (46). Furthermore, it should be noted that other factors than DC lineage might also contribute to Th1/Th2 balance (47-50). Based on the results described in the previous paragraphs, we asked whether IL-18 might influence Th1/Th2 differentiation via modulation of the functional properties of DC2. As described above for DC1, we co-cultured DC2 (either differentiated from pre-DC2 with IL-3 ["DC2"] or IL-3+IL-18 ["IL-18-DC2"] and allogeneic naïve Th cells for six days, recovered Th cells and restimulated them with anti-CD3 and anti-CD28, revealing their Th phenotype (see Fig. 1 and (12,28,35)). In our hands, DC2 induced a Th phenotype releasing substantially more IL-4 than Th cells differentiated through DC1 cells (Fig. 2b), although DC2 still induced both, IFN-γ-secreting Th1 cells and IL-4-secreting Th2 cells at varying ratios (Fig. 2ab and Fig. 7a). Interestingly, CD40 ligation of DC2 with CD40L-transfected P3×TBA7 cells did not significantly alter the induced Th1/Th2 ratio as compared to DC2 applied without CD40 ligation (Fig. 7c). Notably, IL-18-DC2 induced a higher proportion of IFN-γ secreting Th1 cells than DC2 (13.74% ± 2.25% vs 20.65% ± 3.68%, 56% relative increase, \( P < 0.05 \)), while the number of IL-4 secreting Th2 cells showed a tendency to decrease (Fig. 7a), although this did not reach statistical significance (6.3% ± 1.82% vs 5.09% ± 1.18%, 12% relative decrease, \( P = 0.09 \)). Accordingly, restimulated Th cells released significantly more IFN-γ, while IL-4 release was unaffected (Fig. 7b). Comparable results were obtained with CD40L-matured DC2: IL-18-DC2 induced 24% more IFN-γ-secreting Th1 cells...
\(P < 0.05, n = 3\), while a statistically non-significant 10% decrease in IL-4-producing Th2 cells was noted (Fig. 7c). Altogether, these results might be interpreted that IL-18 shifts the Th1/Th2 differentiation pathway toward Th1 through modulation of DC2 function. This pathway of IL-18 action might undeceive the in vivo promotion of a Th1 response by IL-18 in the absence of IL-18R on naïve Th cells.

IL-18 might skew DC2 toward Th1 induction via IL-12 and other Th1-promoting cytokines

To gain some clues in the understanding of this Th1-skewing property of IL-18-DC2, we evaluated the surface phenotype and cytokine secretion pattern of DC2 and IL-18-DC2 (Fig. 8). While FACS analysis revealed no differences between DC2 and IL-18-DC2 in surface staining of HLA-DR, CD83, CD80 (B7.1) and CD86 (B7.2), IL-18-DC2 showed increased surface expression of CD40 compared to DC2 (Fig. 8a-e).

While IFN-\(\gamma\) secretion was absent in 5 of 6 DC2 supernatants tested, IL18-DC2 commonly secreted faint amounts of IFN-\(\gamma\) (data not shown). Little IL-4 detected in some culture supernatants of DC2 was non-significantly reduced in IL-18-DC2 (data not shown). While IL-12 p70 was not detected in DC2 or IL-18-DC2 supernatant (data not shown), both populations equally released small amounts of IL-10 and IFN-\(\alpha\) (data not shown). In contrast to IL-12 p70 protein, CD40L-stimulated DC2 express IL-12 p35 and p40 mRNA, which are both upregulated by IL-18 in CD40L-stimulated IL-18-DC2 (Fig. 8f). In co-culture experiments of DC2 and allogeneic Th cells, minute IL-12 p70 levels just at the detection limit of our ELISA were observed (data not shown). Notably, addition of a neutralizing anti IL-12 p70 mAb to the co-cultures described in the previous paragraph reduced both, baseline Th1-induction in CD40L-stimulated DC2 as well as the Th1-promoting capacity of CD40L-stimulated IL-18-DC2 (Fig. 7c), suggesting that trace amounts of IL-12 p70 released during the co
culture might be sufficient to induce a Th1 phenotype. Recently, two novel IL-12-like Th1-promoting cytokines have been described: IL-23 (consisting of IL-12 p40 and p19 chain), primarily acting on memory Th cells (51); and IL-27, composed of EBI3 (Ebstein-Barr virus-induced gene 3, an IL-12 p40 homologue) (52) and p28, a newly discovered IL-12 p35-related polypeptide (53). IL-27 has been proposed to drive rapid clonal expansion and Th1 induction of naïve but not memory Th cells (53), although data derived from EBI3−/− mice called this into question (54). Notably, CD40L-stimulated DC2 express mRNAs for all of the aforementioned polypeptides, and their expression is upregulated in IL-18-DC2 stimulated with CD40L. It might be speculated that IL-27 and IL-23 might have an adjunctive role to IL-12 in Th1 induction by DC2 as well as IL-18-DC2, although the formal proof awaits the availability of neutralizing antibodies or respective knock-out mice (55).

In the context of the initiation phase of an immune response, what is the cellular source that releases IL-18, which might recruit plasmacytoid pre-DC2 and alter the properties of DC2? Many cell types have been reported to produce IL-18, including macrophages and DC (37), Kupffer cells (56), astrocytes and microglia (57), intestinal and airway epithelial cells (58), keratinocytes (59) and osteoblasts (60). The factors leading to release of IL-18 have not been extensively studied, but IL-18 is found after bacterial and viral infection and in many other infectious diseases (61). Therefore it might be anticipated that foreign antigen binding to respective pattern recognition receptors on cells other than DC might recruit pre-DC2 and shape the properties of DC2 via release of IL-18. Our data are another indication of the importance of the cytokine microenvironment for the steering of DC and subsequent Th1/2 induction: for example Soumelis et al recently showed that epithelial-derived TSLP (thymic
stromal lymphopoietin) potently activates CD11c⁺ DC and induces Th2 development, which might have a prominent role in allergic inflammation (35).

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**Figure legends**

Figure 1. Experimental design.

Figure 2. IL-18 secreted from DC1 does not drive a Th1 response. ab. Th1 (IFN-γ) and Th2 (IL-4) cytokine release from polarized Th cells. As outlined in Fig. 1, allogeneic naïve Th lymphocytes were co-cultured with DC1 (either immature ["DC1 w/o CD40L"] or matured by a further 24h culture with CD40L-expressing P3 × TBA7 cells ["DC1 + CD40L"] or DC2 as indicated for 6 days. During this period, neutralizing antibodies (anti-IL-12, anti-IL-18, and isotype-matched control-Ig) were present as indicated. On day-6, Th cells were harvested, counted and restimulated for 24h with anti-CD3 and anti-CD28. IFN-γ and IL-4 released into the supernatant were detected by ELISA. Neutralization of IL-12 resulted in a substantial decrease of IFN-γ release, while anti-IL-18 mAb had no significant effect. Th cells obtained through co-culture with DC2 secreted substantially more IL-4 than those from DC1 co-cultures. One of two experiments is shown. c. Th phenotype determined by intracellular FACS. Th cells generated through co-culture of naïve allogeneic Th cells with DC1 as described above were restimulated for 5h and intracellular accumulation of IFN-γ and IL-4 analyzed. The percentage of positive cells is given in the respective quadrants. Anti-IL-12 mAb during co-culture significantly decreased the number of IFN-γ+IL-4- cells. One experiment of two is shown.

Figure 3. DC2 lineage express IL-18R. a-h. FACS surface analysis. a. IL-18Rα surface expression on pre-DC2 (i.e. plasmacytoid DC). Identity and purity of pre-DC2 as determined by IL-3Rα (CD123, b) and HLA-DR (c) expression. Pre-DC2 were
negative for lineage markers (not shown). IL-12Rβ1 (d) and IL-12Rβ2 (e) expression on pre-DC2. IL-18Rα surface expression on DC2 (f), pre-DC1 (g), and DC1 (h). 

PCR analysis of IL-18Rα and IL-18Rβ chain on DC1 and DC2 lineage, and Th1-polarized cells [positive control]. An IL-18Rα fragment spanning nucleotides 833 – 1523 was amplified in DC2 lineage as a 690bp band. Please note that in pre-DC2 in addition to the expected band another smaller band was reproducibly co-amplified. Nucleotides 940-1589 of IL-18Rβ chain were amplified in DC2 lineage and in pre-DC1, resulting in a band at 649bp. As a positive control for IL-18Rα and β, mRNA was obtained on day 6 of co-culture of naïve Th cells with DC1, at which time Th cells abundantly express surface IL-18Rα as determined by FACS analysis (data not shown). Control amplification was performed for β-actin.

Figure 4. Effect of IL-18 on DC2 differentiation. 

a. Proliferation. Pre-DC2 were cultured with indicated agents for 3 days, and subsequently pulsed with 3H thymidine for 19h. IL-18 significantly inhibited proliferation of pre-DC2 expanding with IL-3. n = 3. 

b. Cell count. Pre-DC2 (initially 250,000 cells) were cultured with indicated cytokines and counted at the given time-points. n = 2.

Figure 5. IL-18 induces chemotaxis in pre-DC2. 

a. Direct chemotaxis of cells toward IL-18. Pre-DC2 were allowed to migrate into nitrocellulose toward various concentrations of IL-18 present in the lower wells of a Boyden microchemotaxis chamber. SDF (1µg/mL) served as positive control. 

b. IL-18 receptor antibodies abrogate pre-DC2 migration toward IL-18. Pre-DC2 were pre-incubated with anti-IL-18Rα mAb at indicated concentrations, and allowed to migrate toward a concentration gradient of IL-18 (10ng/ml). 

c. Anti-IL-18 antibodies abrogate pre-DC2
migration toward IL-18. Anti-IL-18 mAb was added to the lower wells of a
microchemotaxis chamber containing IL-18 (10ng/ml), and pre-DC2 allowed to
migrate into nitrocellulose. Migration time periods for all experiments were 180 min.
Migration depth was quantified microscopically by the leading front assay. Data are
expressed as 'chemotaxis index', which is the ratio of the distance of stimulated and
random migration of leukocytes into nitrocellulose filters. $n = 6$. * indicates $P < 0.05$ in
the Mann Whitney U test after Kruskal Wallis ANOVA.

Figure 6. Allogeneic proliferation of naïve Th cells. DC2 were obtained by culture of
pre-DC2 with IL-3 ("DC2") or IL-3+IL-18 ("IL-18-DC2"). After washing, DC2 and IL-18-
DC2 were either immediately co-cultured with allogeneic naïve Th cells for 3 days
("w/o CD40L"), or alternatively matured by addition of CD40L-expressing irradiated
P3 × TBA7 cells ("+ CD40L"), followed after 24h by addition of Th cells. Proliferation
of Th cells was determined on day 3 of Th / DC co-culture by addition of $^{3}$H thymidine
for 19h. $n = 4$.

Figure 7. Th phenotype induced by IL-18-DC2. a. Th phenotype determined by
intracellular FACS. DC2 were obtained by culture of pre-DC2 with IL-3 ("DC2") or IL-
3+IL-18 ("IL-18-DC2"). After washing, DC2 / IL-18-DC2 were co-cultured with
allogeneic naïve Th cells for 6 days. Subsequently, Th cells were harvested and
restimulated with anti-CD3 and anti-CD28 for 5h. Intracellular FACS staining was
performed as described in Materials and Methods. The percentage of IFN-$\gamma^+$ and IL-
4$^+$ cells is given for each quadrant. The experiment shown is representative of 5
performed. b. IFN-$\gamma$ and IL-4 secretion by restimulated Th cells. Experiments were
performed as described above, with restimulation of Th cells for 24h instead of 5h,
and cytokine secretion determined by ELISA. IFN-$\gamma$ and IL-4 secretion induced in Th
Plasmacytoid DC express functional IL-18R

cells by “DC2” was set at 1 in each case for further analysis due to the large inter-individual variation (medium baseline IFN-γ: 23 ± 9 ng/mL, baseline IL-4: 661 ± 324 pg/mL). The y axis shows the ratio of Th cytokine secretion induced by “IL-18-DC2” per “DC2”. Differentiation of allogeneic naïve Th cells with “IL-18-DC2” significantly increased IFN-γ release upon restimulation as compared with “DC2”. n = 6. c. Th phenotype and effect of IL-12 neutralization after differentiation with CD40L-matured DC2. Experiments were performed as described in (a), with the exception that CD40L-activated DC2 and IL-18-DC2 were added for 24h before the onset of co-culture with allogeneic naïve Th cells. Anti-IL12 mAb or isotype-matched control Ig was present during the co-culture as indicated. The percentage of IFN-γ⁺ and IL-4⁺ cells is given for each quadrant. The experiment shown is representative of 3 performed with respect to CD40L-matured DC2 / IL-18-DC2, and representative of additional 2 with respect to the effect of anti-IL-12.

Figure 8. Effect of IL-18 on DC2 phenotype. a-e. FACS surface analysis of DC2 and IL-18-DC2. DC2 were obtained by culture of pre-DC2 with IL-3 (“DC2”) or IL-3+IL-18 (“IL-18-DC2”) for 3 days, and stained with indicated antibodies. Surface expression on DC2 is compared with IL-18-DC2. f. IL-27, IL-23 and IL-12 mRNA expression in DC1 and DC2. DC1 were obtained by culture of monocytes for 6 days in the presence of IL-4 and GM-CSF (“DC1”), and further stimulated with LPS (1 µg/mL) and CD40L-expressing irradiated P3 × TBA7 cells (“+ CD40L”) as indicated. DC2 were derived from plasmacytoid DC by culture with IL-3 (“DC2”), or IL-3+IL-18 (“IL-18-DC2”) for 3 days, and further matured as indicated by addition of CD40L-expressing irradiated P3 × TBA7 cells (“+ CD40L”). Messenger RNA expression of IL-27 (consisting of p28 and EBI3 chains), IL-23 (p19 and IL-12 p40) and IL-12 (p35 and p40) was analyzed by PCR as outlined in Materials and Methods. Control
amplification was performed for β-actin. The figure is representative of four independent experiments.
Figure 1

pre-DC1 (i.e. monocytes) \( \rightarrow \) pre-DC2 (i.e. plasmacytoid DCs)

\[ \text{6d} \rightarrow \text{IL-4 + GM-CSF} \quad \text{IL-3} \quad \text{IL-3 + IL-18} \quad \text{3d} \]

DC1 \quad DC2 \quad IL-18-DC2

\pm \text{maturation through CD40L-expressing P3xTBA cells} \quad \text{24h}

+ \quad + \quad +

allogeneic naïve CD4\(^+\) T cells

\pm \text{anti-IL-12} \quad \downarrow \quad \downarrow \quad \downarrow \quad \text{6d}

\pm \text{anti-IL-18}

committed Th cells

\downarrow \quad \downarrow \quad \downarrow \quad \downarrow \quad \text{Restimulation}

5h (intracellular IFN\(\gamma\), IL-4)

24h (Supernatant: IFN\(\gamma\), IL-4)
Figure 3
Figure 4

a

\[
P < 0.001 \quad \text{and} \quad P < 0.001
\]

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b

- Null
- IL-3
- IL-18
- IL-3 + IL-18

Cell count vs. time (d0, d1, d3)
Figure 5

(a) Graph showing the relationship between IL-18 (mg/mL) and Chemotaxis index. The graph indicates a significant increase in Chemotaxis index with increasing concentrations of IL-18, marked by asterisks.

(b) Graph showing the effect of anti-IL-18R mAb (μg/mL) on Chemotaxis index. The Chemotaxis index decreases as the concentration of anti-IL-18R mAb increases, with significant decreases indicated by asterisks.

(c) Graph showing the effect of anti-IL-18 mAb (μg/mL) on Chemotaxis index. The Chemotaxis index also decreases with increasing concentrations of anti-IL-18 mAb, marked by asterisks.
Figure 6

![Graph showing cpm values for DC2, IL-18-DC2, DC2, and IL-18-DC2 with and without CD40L.]

- DC2: 10,000 cpm
- IL-18-DC2: 15,000 cpm
- DC2: 10,000 cpm
- IL-18-DC2: 20,000 cpm

Statistical significance:
- P = 0.27 (DC2 vs. IL-18-DC2)
- P < 0.05 (IL-18-DC2 vs. DC2)

Legend:
- w/o CD40L
- + CD40L
Figure 8

(a) HLA-DR
(b) CD83
(c) CD40
(d) CD80 (B7.1)
(e) CD86 (B7.2)

For specific Ig:
- DC2
- IL-18-DC2

For control Ig:
- DC2
- IL-18-DC2

(f) Gene expression analysis:
- IL-27 p28
- IL-27 EBI3
- IL-23 p19
- IL-12 p35
- IL-12 p40
- β-actin
Interleukin-18 attracts plasmacytoid dendritic cells (DC2) and promotes Th1 induction by DC2 through IL-18 receptor expression

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