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IL-1 Family Members IL-18 and IL-33 Upregulate the Inflammatory Potential of Differentiated Human Th1 and Th2 Cultures

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The IL-1 family members IL-1β, IL-18, and IL-33 are potent cytokines in relationship to amplifying the CD4+ T cell cytokine production. To evaluate their impact on in vitro-differentiated human Th1 and Th2 cultures, such cultures were established from naive T cells, purified from healthy blood donors, and reactivated in the presence of IL-1β, IL-18, or IL-33. Interestingly, we observe modifying responses in Th1 and Th2 cultures induced by IL-18 or IL-33 but not by IL-1β, both contributing to amplifying production of IL-5, IL-13, and IFN-γ. IL-18 or IL-33 stimulation of Th1 cultures resulted in increased IFN-γ and IL-13 production concurrent with reduced IL-10 gene transcription and secretion even though Th1 cultures, in contrast to IL-18Rα, had low ST2L expression. Furthermore, adding IL-18 to Th1 cultures promoted Tbet mRNA expression and production. Th2 cultures stimulated with IL-18 or IL-33 had an increased IL-5 secretion. Interestingly, E4BP4 gene expression and the percentage of E4BP4+ cells of the recently shown IL-10 transcriptional regulator E4BP4 correlated with IL-10 gene expression and protein induction of IL-10 in T cells has been unclear, Motomura et al. (8) recently described the transcription factor E4BP4, also called NFIIL3, as critical for IL-10 production in CD4+ T cells.

Failure to dampen protective CD4+ T cell response after pathogen clearance can result in severe tissue inflammation and release of highly inflammatory cytokines such as IL-18 and IL-33. The IL-1 family members IL-18 and IL-33 are highly inflammatory cytokines constitutively expressed in mucosal or barrier cell types, acting as regulators of innate and acquired immune responses by amplifying both Th1 and Th2 responses with or without TCR activation (9, 10). In a murine bronchial asthma model with Ag-specific Th1 cells, administration of IL-18 induced asthma symptoms (11). Furthermore, IL-18 sera levels are elevated in atopic dermatitis patients as well as in asthmatics (12, 13). IL-33 is an important mediator of allergy by its induction of Th2 cytokines (9, 14, 15) and has been associated with development of severe asthma, with high plasma and bronchial levels of IL-33 located in epithelial and smooth muscle cells (16–18). IL-18 and IL-33 signal their biological activities through the heterodimeric receptors IL-18Rα and IL-33R. IL-33 also acts as a transcriptional regulator independently of IL-33 binding to membrane-localized IL-33R (19). IL-18R and IL-33R contain, respectively, a highly regulated primary binding chain IL-18Rα or IL-1Rα-like 1 (ST2L) and a constitutively expressed IL-18Rβ or IL-1R accessory protein (20). The expression of IL-18Rα and ST2L in the human and murine systems has been described to be expressed on Th1 and Th2 cells, respectively (21–25).

The capacity of IL-18 and IL-33 to amplify the effector Th1 and Th2 cytokine production of IL-5, IL-9, IL-13, IFN-γ, and TNF-α suggests that they are endogenous activators of inflammatory responses, with implications for both autoimmune and allergic disorders (9, 10, 20, 26). Recently, we established an in vitro system to examine tissue factors with regard to modulating the cytokine production by differentiated CD4+ T cell subsets (26). In the present study we used this system to demonstrate that IL-1 family member IL-18 or IL-33 stimulation, in addition to

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Abbreviation used in this article: qRT-PCR, quantitative RT-PCR.

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increasing both Th1 and Th2 proinflammatory cytokine production, represses IL-10 gene transcription and secretion in differentiated Th1 cultures. IL-33 or IL-18 stimulations of Th2 cultures promote additional IL-5 secretion. Furthermore, we find that E4BP4 gene expression and the percentage of E4BP4+ cells correlate with IL-18- and IL-33-mediated downregulation of IL-10 gene transcription and secretion in differentiated Th1 cultures.

Materials and Methods

Cell cultures

Cells used in this study originate from buffy coat fractions of blood portions from anonymized donors, whose identity has been anonymized for the researcher by the Blood Bank at Copenhagen University Hospital (Copenhagen, Denmark).

PBMCs were purified using Lymphoprep (Axis-Shield, Oslo, Norway) density centrifugation. The cells were washed twice with PBS buffer (PBS without Ca++ and Mg++, Invitrogen, Carlsbad, CA) and supplemented with 0.5% (v/v) EDTA (Bie & Berntsen, Rosdovre, Denmark) and 0.5% (v/v) human serum albumin (ZLB Behring, Marburg, Germany).

Human naive CD4+CD45RA+CD45RO CD25 cells were isolated using the naive CD4+ cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. In short, PBMCs were incubated with a mixture of biotinylated Abs (Miltenyi Biotec), followed by labeling with magnetic anti-biotin-coated microbeads for magnetic depletion. The cells were depleted twice using new columns for each depletion, and subsequently stained and depleted with the same mixture of biotinylated Abs and anti-biotin microbeads to ensure a purity of >99% CD4+CD45RA+CD45RO CD25 cells.

Naive CD4+ T cells (2.5-10^5) were cultured at 37°C in 6- or 48-well flat-bottom plates (Nunc, Roskilde, Denmark) in RPMI 1640 (Sigma-Aldrich, Saint Louis, MO) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin, 1 mM l-glutamine (Invitrogen), 50 μg/ml 2-ME (Sigma-Aldrich), and 5% (v/v) human AB serum (Copenhagen University Hospital). The naive CD4+ T cells were cultured in media and supplemented with mouse fibroblastic L cells (CRL-10680; American Type Culture Collection) stably expressing FcγRII/CDw32 (provided by Eva Steen, Department of Immunology, Lund University, Lund, Sweden).

The fibroblasts were either irradiated by Gammacell 2000 RH (A.E.K. Riso, Roskilde, Denmark) or incubated with 10 μg/ml mitomycin C (Sigma-Aldrich) for 3 h following three wash steps and cultured at a ratio of one fibroblast per five T cells in the presence or absence of the indicated human cytokines added on day 0 and/or day 5: 10 ng/ml IL-4, IL-12, and IL-33 (eBioscience, San Diego, CA); 10 ng/ml IL-2 (Proleukin, Novartis, Basel, Switzerland) and 10 ng/ml IL-1β and IL-18 (Humaxymy, Chicago, IL). Abs used were: 10 μg/ml anti-CD45 (11711), 5 μg/ml anti-IL-12, 10 μg/ml anti-IL-18R (70255), and 10 μg/ml anti-ST2 (97203) (R&D Systems, Minneapolis, MN); 10 μg/ml anti-IL-4 (MP4-25D2) and 5 μg/ml anti–IFN-γ (MD-1; eBioscience); and 0.03 μg/ml anti-CD3 (UCHT1) and 0.03 μg/ml anti-CD28 (CD28.2) (BD Biosciences, Franklin Lakes, NJ). The cultures were restimulated at day 5. At day 3 after restimulation, medium was added 1:1 to the 120 h cultures with the same concentration of Abs and cytokines as at day 5. Prior to flow cytometry and multiplex analysis, the cultures were restimulated for 6 h with 25 ng/ml PMA and 1 μg/ml ionomycin, and for the last 4 h incubation with 10 μg/ml brefeldin A (Sigma-Aldrich). Cell pellets for quantitative RT-PCR (qRT-PCR) analysis of mRNA were snap-frozen in liquid nitrogen and RNA was harvested and used in cDNA synthesis reactions. The cDNA from the three different stimulations was combined and stored at −80°C.

Statistical analysis

Bar graphs are represented as means ± SEM. One- or two-way matched Bonferroni corrected ANOVAs were used for statistical analysis; data from qRT-PCR and supernatant were log transformed prior to analysis. Statistical significance is designated as follows: *p < 0.05, **p < 0.01, ***p < 0.001. Statistics were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

Results

IL-18Ra and ST2L expression on differentiated CD4+ T cells

IL-18Ra and ST2L are described to be tightly regulated, whereas their coreceptors are constitutively expressed; furthermore, IL-18Ra and ST2L receptor expression has been shown to be Th1 a and Th2 restricted, respectively (28). To confirm these findings in differentiated Th1 and Th2 cultures, we studied the expression of these genes in cultures under Th0, Th1, and Th2 culture conditions for a period of 5 d. The Th1 and Th2 cultures were established at day 5 (Fig. 1A) with high expression of the master transcription factors T-bet and GATA3, respectively. As described by others (21, 25), IL-18Ra gene transcription and the percentage of IL-18Ra+ cells correlated with the expression of a Th1 subtype (Fig. 1B, 1C). Of interest, all differentiated cultures expressed the gene encoding IL-18Ra (Fig. 1C). Unlike IL-12–mediated expression of IL-18Ra under Th1 culture conditions, ST2L was weakly induced by IL-4 (Fig. 1B). In contrast to flow cytometry data, ST2L mRNA expression was upregulated under Th1 and Th2 culture conditions (Fig. 1C).

IL-1 family members IL-18 or IL-33 induce an inflammatory phenotype in differentiated Th1 and Th2 cultures

To test whether Th1 and Th2 cultures respond to stimulation by the IL-1 family member IL-1β, IL-18, or IL-33, differentiated Th1 and Th2 cultures were further stimulated with none or one of the cytokines for an additional 5 d.
Stimulation of Th1 cultures with IL-18 increased the secretion of the Th1 signature cytokine IFN-γ (Fig. 2A). Surprisingly, IFN-γ secretion was also increased in Th1 cultures stimulated with the Th2-associated IL-1 family member IL-33 (Fig. 2A). Furthermore, adding IL-18 or IL-33 to the differentiated Th1 cultures attenuated IL-10 secretion compared with the classical Th1 cultures (Fig. 2A). Overall, 5 d stimulation of the Th1 cultures with IL-18 or IL-33 but not IL-1β induced and repressed the same cytokines, indicating activation of the same signal pathway. Incubating Th2 cultured cells with IL-18 or IL-33 resulted in additional secretion of the Th2 subtype cytokine IL-5 (Fig. 2B). In contrast to the Th1 results, IL-10 secretion was not reduced in the Th2 cultures stimulated with IL-18 or IL-33, although stimulation with IL-33 showed a tendency of reduced IL-10 secretion. Collectively, these data indicate that IL-18 or IL-33 stimulation results in amplification of both Th1 and Th2 subtype cytokines concurrent with repression of IL-10 production in the Th1 cultures. In contrast, stimulation of Th1 and Th2 cultures with IL-1β did not increase production of Th1 and Th2 subtype-associated cytokines, and IL-1β was omitted in the following experiments.

To address the kinetics of the IL-18– and IL-33–mediated cytokine regulation, 5 d differentiated Th1 and Th2 cultures were incubated with none, IL-18, or IL-33 during a period of up to 5 additional days (Fig. 3).

Th1 cultures stimulated with IL-18 resulted in markedly divergent responses regarding IFN-γ and IL-10 secretion (Fig. 3A, 3C). The same pattern, even though delayed, was observed by stimulation of Th1 cultures with IL-33 (Fig. 3A, 3C). To address whether the observed delay (Fig. 3C) was a result of different biological activity of IL-18 and IL-33, Th1 cultures were stimulated with increasing concentrations of IL-18 or IL-33 (Supplemental Fig. 1). The inhibition capacity of IL-10 secretion of Th1 cultures of IL-18 or IL-33 was positively correlated with increasing protein concentrations, and IL-18 was shown to be 2- to 3-fold more biologically active than IL-33 (Supplemental Fig. 1). As for IL-18– or IL-33–mediated induction of IFN-γ in Th1 cultures, IL-5 secretion was promoted by both IL-1 family members in Th2 cultures after 24 h (Fig. 3E). In Th2 cultures IL-10 secretion was weakly repressed and seen after 120 h incubation with IL-33 or IL-18 (Fig. 3G). Analyzing IL10 mRNA expression showed IL-18– and IL-33–mediated inhibition in Th1 cultures and no change in the Th2 cultures (Fig. 3D, 3H). IL-13 secretion followed the same kinetics as IL-5 and was induced early by IL-18 stimulation in both Th1 and Th2 cultures (Fig. 3B, 3F). Representative flow cytometry plots after 24 h stimulation (Supplemental Fig. 2) showed IL-18– or IL-33–mediated induction of IL-13 mainly within the T-bet+ or GATA3+ population in the Th1 or Th2 cultures, respectively. Additionally, IL-18– or IL-33-mediated

![FIGURE 2](http://www.jimmunol.org/)
ated induction of IL-13 in the Th1 cultures was mainly reproduced with IFN-γ (data not shown).

IL-18 increases frequency of CD4^+ T-bet^+ and reduces the level of CD4^+GATA3^+ in Th1 cultures

We then asked whether induction of the master transcription factor for Th1 and Th2 differentiation of GATA3 and T-bet, respectively, was associated with the IL-18– and IL-33–dependent cytokine induction and repression. Five-day-primed Th1 and Th2 cultures were incubated with none, IL-18, or IL-33 for the indicated periods (Fig. 4). The frequency of CD4^+T-bet^+ cells was additionally increased in the period from 12 to 48 h simultaneously with early reduced percentage of CD4^+GATA3^+ cells in response to the addition of IL-18 to Th1 cultures (Fig. 4). Although the percentage of CD4^+GATA3^+ cells increased after restimulation of the Th2 cultures, IL-18 or IL-33 stimulation did not increase CD4^+GATA3^+ cells (Fig. 4B).

The positive feedback effects of IL-18 or IL-33, in relationship to inducing IL-18Rα or ST2L expression, were investigated by flow cytometry and real-time quantitative PCR. Th1 cultures exhibited higher expression of IL-18Rα compared with Th2 cultures during the investigated period (Supplemental Fig. 3A). ST2L expression was unexpectedly low in both Th1 and Th2 cultures with and without IL-1 family stimulation, but it was surprisingly moderately higher in Th1 cultures after 72 h (Supplemental Fig. 3B). In contrast to the flow cytometry data, IL-18 stimulation of Th2 cultures resulted in upregulated IL18Rα and ST2L (Supplemental Fig. 3C, 3D). As previously reported (25), IL-33 stimulation of Th2 cultures leads to upregulated ST2L mRNA expression (Supplemental Fig. 3D). Notably, IL-18 or IL-33 stimulation of Th1 did not affect the IL18Rα and ST2L mRNA expression (data not shown).

To determine whether we could block the action of IL-18 or IL-33, we added commercially available blocking Abs against IL-18Rα or ST2L to the culture conditions (Supplemental Fig. 4). The stimulating capacity of IL-18 was partially blocked by Abs against IL-18Rα (Supplemental Fig. 4). However, adding ST2L Abs did not reduce the IL-33–mediated inhibition of IL-10 secretion (Supplemental Fig. 4).

IL-10 suppression correlates with E4BP4 expression in Th1

We finally wanted to examine whether transcription factor E4BP4 expression correlated with IL-10 production. Five-day-primed Th1 and Th2 cultures were incubated with none, IL-18, or IL-33 for the indicated periods (Fig. 5).

Culturing naive CD4^+ T cells under Th2 culture conditions for 5 d resulted in higher E4BP4 gene expression than that seen for the Th1-cultured cells (Fig. 5A). This is in agreement with earlier studies describing IL-10 as a Th2 subtype cytokine (4). We found that IL-18– or IL-33–mediated inhibited IL-10 secretion was closely associated with E4BP4 gene transcription and the percentage of CD4^+T-bet^+E4BP4^+ cells in Th1 cultures (Fig. 5B, 5C). In addition to being involved in IL-10 gene regulation, E4BP4 has been associated with both increased and decreased IL-13 production (8, 29). However, we observed no relationship between IL-13 production and E4BP4 expression in Th1 cultures stimulated with IL-18 or IL-33 (Figs. 2B, 5C).

Discussion

Recently, it has been shown that innate tissue cells can release alarmins with the ability to modulate homed effector CD4^+ T cells to produce a diverse range of cytokines, all being important to eradicate encountered pathogens (30). In this study, using in vitro-differentiated human Th1 and Th2 cell cultures, we demonstrate that stimulation with the IL-1 family alarmins IL-18 or IL-33 results in amplification of powerful inflammatory cytokines as well as in Th1 cultures blocking production of the regulatory cytokine IL-10. It has been shown in vitro and in vivo that IL-10...
can be coproduced by effector Th subsets after multiple restimulations, indicating induction of tolerance, which is exemplified by coproduction of IL-10 in specific Th1 and Th2 cells after continuous exposure of high-dose bee venom (31). If our results can be reproduced in vivo, inhibition of IL-10 production concurrently with amplification of CD4+ T cell-produced cytokines would indicate an important mechanism of the innate cells to switch a response from tolerance toward eradicating encountered pathogens. In the case of IL-33, it has been shown to be important in promoting Th2-oriented immunity, and together with it is the release by necrotic tissue cells, fitting with a mechanism to combat chronic parasite infections by modulating the local CD4+ T cells to increase cytokine production (20, 32, 33). Therefore, dysfunctional regulation of IL-18 or IL-33 could contribute to development of chronic inflammations.

We observe heterogeneity in responses of Th1 and Th2 cultures induced by IL-18 or IL-33, both contributing to amplify production of Th1- and Th2-type cytokines, in contrast to IL-1β. IL-1β has been associated with development of Th17 cells, explaining why stimulations of Th1 and Th2 cultures did not induce secretion of the investigated Th1 and Th2 cytokines (10). Interestingly, Zielinski et al. (34) recently identified IL-1β as essential in induction of human proinflammatory Th17 cells characterized by inhibited IL-10 production.

In relationship to IL-13, we found a moderate increase in its secretion in Th1 cultures stimulated with IL-18. Nakanishi et al. (35) described that stimulation of Th1 cells with IL-18 induces so-called “super” Th1 cells coproducing IFN-γ and IL-13, and these cells are involved in the development of an allergic inflammation. Moreover, other studies have shown that chronically activated Th1 cells start to coproduce IL-10 and IL-13 under the control of E4BP4, indicating that IL-10 is produced by Th1 cells as a result of negative feedback (8). Several publications support our observations of heterogenic IL-18-/IL-33-induced cytokine production of CD4+ T cell subsets; for example, in the absence of IL-12, IL-18 administration contributed to the development of a spontaneous atopic dermatitis-like phenotype, as well as increasing circulating IL-4, IL-13, and IgE, in a mouse model (36, 37). Additionally, neutralizing IL-18 signaling in an IL-18–deficient mouse model diminished allergen-induced chronic inflammation (38). Furthermore, it has been shown that Th2-skewed human CD4+ T cells produce IFN-γ in response to activation of IL-33 and Ag (9). Our Th2 cultures have added anti–IFN-γ, making us unable to detect any potentially upregulated IFN-γ production.

It has been reported that IL-18Rα and ST2L are selectively upregulated and expressed by Th1 and Th2 cells, respectively (21–25). We confirm the association of Th1 cells and high IL-18Rα expression, but we were unable to reproduce that Th2 cells have

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**FIGURE 4.** IL-18 increases frequency of CD4+T-bet+ and reduces the level of CD4+GATA3+ in Th1 cultures. Naive CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th1 (IL-12 and anti–IL-4) and Th2 (IL-4 and anti–IFN-γ) conditions for 5 d and restimulated for indicated periods with none, IL-18, or IL-33. (A and B) Flow cytometry analysis of frequencies of T-bet (A) and GATA3 (B) cells in Th1 or Th2 cultures for indicated periods with none, IL-18, or IL-33. Data are from three independent experiments using cells from two donors (n = 6). *p < 0.05, **p < 0.01.

**FIGURE 5.** IL-10 suppression correlates with E4BP4 expression. Naive CD4+ T cells were stimulated with anti-CD3/CD28 under classical Th0 (anti–IFN-γ), Th1 (IL-12 and anti–IL-4), and Th2 (IL-4 and anti–IFN-γ) conditions for 5 d and restimulated for indicated periods with none, IL-18, or IL-33. (A) E4BP4 mRNA expression at day 5 relative to Th0. (B) E4BP4 mRNA expression in Th1 cultures with IL-18 or IL-33 relative to the Th1 cultures at the indicated time point. (C) Flow cytometry analysis of frequencies E4BP4 cells in Th1 cultures with IL-18 or IL-33 relative to the Th1 cultures at the indicated time point. Data are from one (C), two (B), or five (A) independent experiments using cells from two donors (n = 2, 4, or 10). *p < 0.05, ***p < 0.001.
increased ST2L expression. This can be explained, however, by the PMA restimulation used prior to harvest, as PMA restimulation is associated with reduced cell surface expression of ST2L and IL-18Rα (21). Interestingly, Pecaric-Petkovic et al. (39) found that even though they could not detect ST2L expression, the investigated basophils responded to IL-33 stimulation. In contrast to the flow cytometry data, we do find mRNA expression of IL18Rα and ST2L in both Th1 and Th2 cultures, indicating that these cultures have the potential to express the receptors on the cell membrane. Moreover, consistent with our mRNA findings, Th1 cultures from a mouse model expressed ST2L, albeit at lower levels than in the Th2 culture (10). We tried to block the interaction of IL-18 or IL-33 to IL-18Rα or ST2L, respectively, with blocking Abs added to the culture medium. However, even though the IL-18–mediated decreased IL-10 secretion was inhibited by adding Abs added to the culture medium, we do find mRNA expression of IL-10. We tried to block the interaction of ST2L with Abs against human ST2, ST2-Flag-His, or ST2-Fc fusion proteins.

The observed heterogeneity in the cytokine pattern induced by IL-18 or IL-33 stimulation could be explained by IL-18R and IL-33R signaling activating NF-κB, JNK, and p38 MAPK cascades through MyD88 and TRAF6 pathways (20). Furthermore, IL-18– or IL-33–mediated regulation of cytokine production could be a result of higher T-bet and GATA3 expression. Stimulation of Th1 cultures with IL-18 induced additional expression of T-bet in the Th1 culture, contrary to no effect on GATA3 expression in Th2 cultures after IL-33 stimulation, indicating that T-bet can potentially be involved in the regulation of E4BP4 gene expression; however, further studies are needed to clarify this. E4BP4 has recently been linked with IL-10 and IL-13 production in CD4+ T cells. Similar to Motomura et al. (8), we observed a correlation between E4BP4 expression and IL-10 production in Th1 cultures. However, the early IL-18–induced secretion of IL-13 in both Th1 and Th2 cultures did not result in additional E4BP4 expression (data not shown for the Th2 cultures). Additionally, Kashiwada et al. (29) has reported that in vitro-differentiated Th2 E4BP4+/- cells have increased IL-5 and IL-13 production. In light of these studies and our findings, E4BP4 appears to represent an important transcription factor in the regulation of IL-10, in contrast to IL-13, where the results are unclear and need further investigation.

An important question is whether the effector CD4+ T cell plasticity shown in our in vitro system has relevance in vivo. In a recently study with stimulation of PBMCs from immune thrombocytopenia patients, neutralizing the biologic activity of IL-18 signaling by inhibiting the IL-18R coreceptor with IL-18BP/Fe resulted in downregulation of IFN-γ while permitting the production of IL-10 (40), basically finding the same pattern of IL-18–mediated downregulation of IL-10 as we found in our Th1 cultures.

In summary, we hypothesize that IL-18 or IL-33 stimulation of CD4+ T cell subsets, in addition to amplifying proinflammatory cytokine production, also switch off the default induction of IL-10 in at least Th1 cultures, thus indicating an important role of innate immune cells in producing alarmins that modulate the local immune response.

Disclosures

The authors have no financial conflicts of interest.

References

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