Instruction of effector T cell programs by flexible dendritic cells
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CHAPTER 3

IL-4 is a mediator of IL-12p70 induction by human Th2 cells: reversal of polarized Th2 phenotype by dendritic cells

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Abstract
IL-12 is a key inducer of Th1-associated inflammatory responses, protective against intracellular infections and cancer, but also involved in autoimmune tissue destruction. We report that human Th2 cells interacting with monocyte-derived dendritic cells (DC) effectively induce bioactive IL-12p70 and revert to Th0/Th1 phenotype. In contrast, the interaction with B cells preserves polarized Th2 phenotype. The induction of IL-12p70 in Th2 cell - DC cocultures is prevented by IL-4 neutralizing mAb, indicating that IL-4 acts as a Th2 cell-specific cofactor of IL-12p70 induction. Like IFN-γ, IL-4 strongly enhances production of bioactive IL-12p70 heterodimer in CD40L-stimulated DC and macrophages and synergizes with IFN-γ at low concentrations of both cytokines. However, in contrast to IFN-α, IL-4 inhibits the CD40L-induced production of inactive IL-12p40 and the production of either form of IL-12 induced by LPS, which may explain the view of IL-4 as an IL-12 inhibitor. The presently described ability of IL-4 to act as a cofactor of Th cell-mediated IL-12p70 induction may allow Th2 cells to support cell-mediated immunity in chronic inflammatory states, including cancer, autoimmunity, and atopic dermatitis.

**Introduction**

Selection of immune effector mechanisms that are most appropriate for the type of invading pathogen and the type of affected tissue is orchestrated by polarized Th1 and Th2 cells. Th1 cells promote cellular immunity, protecting against intracellular infections and cancer but carrying the risk of autoimmune damage to host's tissues. Th2 cells promote humoral responses, highly effective against extracellular pathogens. While interleukin-4 (IL-4), a Th2- and mast-cell product, is a major factor driving the differentiation of naive Th cells into the Th2 subset, IL-12, produced by several types of APC including dendritic cells (DC), is a key Th1-driving cytokine (1). Induction of the bioactive IL-12p70 heterodimer (composed of p35 and p40 subunits) during DC - Th cell interaction depends on triggering of CD40 by CD40L (CD40L; CD154), present on activated Th cells, but high-level IL-12p70 production requires an additional costimulatory signal that can be provided by IFN-γ (2), a product of Th1 cells, CD8+ T cells and NK cells.

Primary polarization of Th cells occurs already at the moment of their priming in the lymph nodes (3). Although DC are the only APC type involved in priming of naive Th cells, also other APC types, e.g., B cells, are involved in regulating the cytokine profiles of Th cell responses (4,5). This indicates the importance of post-priming events in the modulation of cytokine profiles in committed Th cells, which mechanism is not completely clarified yet.

Compared to their mouse counterparts, human Th2 cells are more flexible and susceptible to the reversal of their polarized cytokine profiles by exogenous IL-12 (6), suggesting the possibility of their therapeutic re-modulation, e.g., in Th2-mediated disorders. However, since committed Th2 cells are deficient in IFN-γ production and produce endogenous Th2-driving factors, e.g. IL-4, that were shown to suppress IL-12 production (7-9), it remains unclear whether and in which conditions such a reversal can also occur in physiologic situations. Here we show that although, in accordance with previous studies, IL-4 is an inhibitor of LPS-induced IL-12 production (7,9) and an inhibitor of CD40L-induced production of inactive IL-12p40 subunit (8), it is a potent enhancer of the production of bioactive IL-12p70 heterodimer in DC and macrophages, allowing human Th2 cells to efficiently induce IL-12p70.

**Materials & Methods**

**Polarized Th1 and Th2 cells**

Generation of polarized Th cell populations was performed in IMDM supplemented with 5% normal human serum (Bio-Whittaker, Walkersville, MD), gentamycin (80 μg/ml; Duchefa, Haarlem, The Netherlands) and rIL-2 (10 U/ml; Chiron, Emeryville, CA). CD45RA+ naive CD4+ Th cells were isolated (purity >98%) from PBMC of healthy donors using negative selection columns (R&D Systems Europe Ltd., Abingdon, U.K.). Th cells were stimulated with immobilized CD3 mAb (1 μg/ml; CLB-T3/3, CLB, Amsterdam, The Netherlands) and soluble CD28 mAb (2 μg/ml; CLB-CD28/1, CLB) and cultured for 10 days in
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the presence of either rIL-4 (1000 U/ml; PBH, Hannover, Germany), for Th2-polarizing conditions, or rIL-12 (100 U/ml; a gift from Dr. M. K. Gately, Roche, Nutley, NJ) and neutralizing anti-IL-4 (1 µg/ml; CLB-IL-4/6 (5B5), CLB), for Th1 polarizing conditions. House dust mite-specific Th2 clone (RDC41), isolated from peripheral blood of an atopic patient and selected on the basis of its strongly pronounced deficit in IL-12 responsiveness and a total inability to produce IFN-γ, was described previously (10).

DC and macrophages

To obtain immature DC, monocytes (5 x10^5 cells/ml) were cultured for 6 days in 24-well plates (Costar) in IMDM with 10% FCS (Hyclone) and gentamycin (80 µg/ml; Duchefa) supplemented with rhuGM-CSF (500 U/ml; a gift of Schering-Plough, Uden, The Netherlands) and rhuIL-4 (250 U/ml; PBH), as described (2,11). At day 6, the cultures consisted of uniformly HLA-DR^+, CD83^+, CD40^{high} immature DC, without detectable CD3^+ cells. Over 90% of the cells expressed high levels of CD1a. Macrophages were obtained in parallel GM-CSF-supplemented cultures that did not contain IL-4, as described (2). They showed CD14 expression, lack of CD1a, lower levels of CD80, CD86, and CD40 expression, in accordance with previous report (2). Cell-surface phenotype was analyzed by flow cytometry, after labelling of cells with appropriate mAb, as described (2,11).

B cells

Peripheral blood B cells were isolated using the StemSep™ antibody enrichment cocktail (StemCell Technologies Inc., Vancouver, Canada) for human B cells in combination with StemSep™ magnetic colloid, accordingly to the manufacturer's instructions. Isolated B cells (>95% positive for CD20, CD3 < 1%, CD14 <1%, CD16 < 1%) were cultured overnight before irradiation (2500 Gy) and co-culture with Th cells.

APC-Th cell cocultures

At day 12 after priming in either Th1- or Th2-driving conditions, Th cells were harvested, washed, and cocultured (10^5 cells per well) with DC (2x10^4) or B cells (6 x 10^4) in the presence of SEB (1ng/ml; Serva, Heidelberg, Germany) in a final volume of 0.2 ml, as described (2,11). When indicated, rhuIL-12 (100 U/ml), was added at the beginning of the cocultures. To test the impact of the interaction of Th2 cells with different APC populations upon their subsequent cytokine profile, such differentially restimulated Th2 cells were expanded for additional 8 days before their tertiary stimulation with PMA/ionomycin. To compare the IL-12p70-inducing capacity of Th1 and Th2 cells, they were cocultured (10^5 cells per well in 0.2 ml) for 48 hours with DC (2x10^4) or B cells (6 x 10^4) in the presence of SEB. To study the induction of IL-12p70 by a totally IFN-γ-deficient Th2 clone (RDC41, ref.10), a model was used where DC presented a stimulatory mouse anti-human CD3 mAb (IgG2a) via FcγR (12). Some cultures contained 1 µg/ml of IL-4-neutralizing mAb (CLB-IL-
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4/6) or control irrelevant mouse IgG1 (DL-5, a gift from Dr. P. H. van der Meide, BPRC, Rijswijk, The Netherlands), as indicated.

**Th cell-independent DC stimulation**

DC were washed and stimulated (2 x 10^4 cells in 200 ml) with one of the following stimuli: CD40L-transfected J558 plasmacytoma cells (J558-CD40L, 5 x 10^4 cells per well; a gift of Dr. P. Lane, Birmingham, UK), which were previously shown to induce IL-12p70 in an IFN-γ-independent way (13), soluble recombinant CD40 ligand trimer (sCD40L, 1 mg/ml; a gift of Dr. E. K. Thomas, Immunex Corp., Seattle, WA), known to require IFN-γ to induce high-level IL-12p70 production, in analogy to CD40L expressed on Th cells (2,11), or with LPS (250 ng/ml; Difco). RhuIFN-γ (a gift of Dr. P. H. van der Meide, BPRC,) or rhuIL-4 were added to the cultures, as indicated.

**Cytokine measurements**

Analysis of Th cell cytokine production at a single cell basis was performed after 6 hour stimulation with PMA (10 ng/ml, Sigma) and ionomycin (1 mg/ml; Sigma,) in the presence of Brefeldin A (10 μg/ml; Sigma). The cells were fixed with 2% paraformaldehyde, permeabilized with 0.5% saponine (Sigma), and labeled with FITC-coupled IFN-γ mAb (Becton Dickinson) and PE-coupled IL-4 mAb (Becton Dickinson). The cells were evaluated by FACSscan (Becton Dickinson). IL-12 p70 ELISA (sensitivity, 3 pg/ml (9)) was performed with use of p70-specific mAb 20C2 (a gift from Dr. M. K. Gately, Hoffmann-La Roche, Nutley, NJ) and p40-specific C8.6 mAb (a gift from Dr G. Trinchieri, The Wistar Institute, Philadelphia, PA). IL-12p40-, IFN-γ-, and IL-4-specific ELISAs were described before (2,9,11).

**Results and Discussion**

**Reversal of polarized phenotype in DC-restimulated Th2 cells does not require exogenous IL-12.**

Priming of human naive Th cells in the presence of IL-4 yielded strongly polarized human Th2 cells, that produced IL-4 but not the Th1-type cytokine IFN-γ (Fig. 1a, left). Although B cells and DC are both efficient APC for memory Th cells, the restimulation by these distinct APC populations yielded different outcomes for the cytokine profiles of such committed Th2 cells. Th2 cells restimulated by B cells preserved their polarized Th2 cytokine profiles, as evidenced by their selective production of IL-4 but not IFN-γ after a subsequent, tertiary stimulation (Fig. 1a, top, Fig. 1b). The presence of exogenous IL-12p70 during the B-cell-mediated restimulation of Th2 cells shifted their cytokine profiles, resulting in Th0/Th1-type cells, producing IFN-γ upon subsequent stimulation. These findings are in line with the previous observations that human Th2-polarized cells are more flexible than their mouse counterparts and can revert to Th0/Th1-like cells in response to exogenous IL-12 (6), becoming resistant to IL-12 only in a fraction of terminally polarized clones (10). The mechanism of reversal of Th2 phenotype within polarized Th cell population and the
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definition of the point-of-no-return for individual Th2-biased human cells need further analysis at clonal level.

Unexpectedly, the restimulation of Th2 cells by DC led to the similar reversal of polarized Th2 phenotype, already in the absence of any exogenous IL-12p70 (Fig. 1a, bottom, Fig. 1b), indicating the intrinsic tendency of Th2 cells to revert to Th0/Th1-type cells following the interaction with DC. The addition of exogenous IL-12p70 to DC-restimulated Th2 cell cultures did not have any additional effect (Fig. 1a, bottom-right).

**Figure 1: Loss of polarized Th2 phenotype as a result of the interaction of newly committed Th2 cells with DC but not with B cells.** (A) Th2 cells (left panel), obtained by priming of naive Th cells in Th2-driving conditions (see M&M) were restimulated by SEB-coupled B cells or DC. RhuIL-12 was added to some cultures, as indicated. After additional 8 days, such differentially restimulated Th2 cells were all stimulated with the combination of PMA and ionomycin to analyze their ability to produce IL-4 and IFN-g at a single cell level. Data from one experiment of three that all gave similar results. (B) Acquisition of the ability to secrete high levels of IFN-g by DC-restimulated Th2 cells. Polarized Th2 cells were restimulated by SEB-coupled B cells or DC. After additional 8 days, the production of IFN-g was induced in such differentially restimulated Th2 cells by the combination of CD3 and CD28 mAb. The supernatants were harvested after 24 hours.

**Th2 cells effectively induce IL-12p70 production in an IL-4-dependent mechanism.**

The above findings suggested a possibility that endogenous IL-12p70 production was induced during Th2 cell - DC interaction. Indeed, cocultures of Th2 cells with DC (but not with B cells) resulted in the production of surprisingly similar amounts of IL-12p70 as the DC - Th1 cocultures (Fig. 2a). This was accompanied by the onset of IFN-γ production in Th2 - DC cocultures visible already within 48 hours (not shown). To test whether the IL-12-inducing capacity is also present in Th2 cells devoid of any residual ability to produce IFN-γ, we used an IL-12-unresponsive Th2 clone generated from an atopic patient (RDC41), that was totally deficient in IFN-γ production (10). In contrast to naive Th cells that induce only trace amounts of IL-12p70, due to their low IFN-γ production (2), RDC41 clone proved itself as an efficient IL-12p70 inducer (Fig. 2b), despite the fact that it selectively produced high IL-4 levels during the stimulation with CD3 and CD28 mAb (1.2 ± 0.09 ng/ml) and during
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the interaction with DC (2.81 ± 0.04 ng/ml) but not any detectable IFN-γ (<70 pg/ml) both after the CD3/CD28 mAb stimulation and during the 48 hour interaction with DC. This suggested that a) the ability of human Th2 cells to induce IL-12 is not restricted to the situations when their IL-4 production is low, as proposed before (15), but is a standard phenomenon, and b) that human Th2 cells utilize a separate, different from IFN-γ, costimulatory mechanism that provides them with the ability to induce IL-12p70 production. In support of this possibility, neutralization of IL-4 in Th2 cell - DC cocultures, resulted not in an enhancement but in a profound suppression of IL-12p70 production (Fig. 2b), indicating that IL-4 produced during the Th2-cell-DC interaction acts as Th2-cell-specific costimulatory factor for the induction of IL-12p70, instead of being an IL-12 inhibitor. The key role of IL-4 in the induction of IL-12p70 production by Th2 cells signifies that this ability is intrinsic to polarized Th2 phenotype.

Figure 2:
IL-4 mediates the induction of high-level IL-12p70 production in DC interacting with Th2 cells. (A) Both Th1 and Th2 cells are potent inducers of IL-12p70. Th1 and Th2 cells were prepared by priming of naive Th cells in Th1- or Th2-driving conditions. Th1 or Th2 cells from two different donors (see the inset for the levels of IL-4 and IFN-γ production after the CD3/CD28-mediated stimulation) were cocultured with SEB-coupled DC or B cells. Culture supernatants were harvested after 48 hours. Similar data were obtained in two additional experiments, each utilizing Th cells from separate single donors. (B) Induction of IL-12p70 by Th2 cells depends on IL-4. Polarized Th2 cells were co-cultured with SEB-coupled DC. RDC41, an IL-12-unresponsive Th2 clone of atopic origin (see ref.10) was cocultured with DC presenting stimulatory CD3 mAb (24). Some cultures contained IL-4-neutralizing mAb or control irrelevant IgG1 (both 1 µg/ml), as indicated. Supernatants were harvested after 48 hours. Data is shown as mean (±SD) of triplicate cultures. Similar results were obtained with Th2 cell lines from two other donors.
IL-4 enhances of IL-12p70 production in CD40L-stimulated DC and macrophages: synergism with IFN-γ.

To confirm this unexpected activity of IL-4 in a more simple model, DC were stimulated with CD40L-transfected J558 cells (J558-CD40L), that are known to induce substantial IL-12p70 production already in the absence of any additional factors (13). Indeed, also in this model IL-4 dose-dependently increased the production of bioactive IL-12p70, with a similar efficacy as IFN-γ (Fig. 3a). However, while the IL-12 p70-enhancing activity of IFN-γ was accompanied by the enhancement of an overall IL-12p40 production (mostly composing an inactive p40-p40 homodimer (1)), the elevation of IL-12p70 production by IL-4 was accompanied by a dose-dependent suppression of p40 production. This reciprocal regulation of the two IL-12 subunits by IL-4 indicates that IL-12p40 is a poor marker of bioactive IL-12 production. In contrast to IL-12p70 heterodimer, the p40 homodimer, produced in huge excess over IL-12p70, lacks the biological activity of IL-12 and can act as a competitive IL-12 inhibitor (1).

The IL-12-enhancing activity of IL-4 is not a phenomenon restricted to monocyte-derived DC. It was also observed in monocyte-derived macrophages (Fig. 3b), that show lower CD40 expression than DC ((2), current data not shown) and lower overall levels of IL-12p70 product. However, IL-4 showed a synergism with IFN-γ in costimulating IL-12p70 production in J558-CD40L-stimulated macrophages at low, perhaps more physiological, concentrations of these cytokines (Fig.3c-top). A similar synergism between low concentrations of IL-4 and IFN-γ could be observed in DC, especially after their stimulation with sCD40L (Fig.3c-bottom), that provides a weaker IL-12-inducing signal, compared to J558-CD40L (2,11). A requirement for both IL-4 and IFN-γ in the optimal induction of IL-12p70 production may explain the surprising observation that IL-4 knock-out mice show a deficit of some Th1 functions (see below), reversible upon exogenous IL-4 treatment (16).
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Figure 3: IL-4 is a potent enhancer of IL-12p70 production in CD40L-stimulated DC and macrophages. (A) IL-4 enhances the production of IL-12 p70 in CD40L-stimulated DC, but inhibits the production of p40 subunit of IL-12. DC were stimulated by J558-CD40L in the presence of increasing concentrations of either IL-4 or IFN-γ. The supernatants were harvested after 24 hours, and analyzed for the contents of IL-12p70 and p40. The data are shown as means ±SD of triplicate cultures from a representative experiment of four. (B) IL-4 enhances the IL-12p70 production in monocyte-derived macrophages. Macrophages (see M&M) or DC were stimulated by J558-CD40L, in the absence or the presence of IL-4 or IFN-γ (both at 1000 U/ml), as indicated. The supernatants were harvested after 24 hours. Results from a representative experiment of four. (C) Synergism between low concentrations of IL-4 and IFN-γ in inducing high-level IL-12p70 production in CD40L-stimulated DC and macrophages. Monocyte-derived macrophages or DC were stimulated with J558-CD40L or sCD40L, as indicated, in the presence of either low or high concentrations of either IL-4 or IFN-γ or their combination. The supernatants were harvested after 24 hours. The data are shown as means ±SD of triplicate cultures from a representative experiment of three.
Despite strongly upregulating the CD40L-induced IL-12p70 production, IL-4 significantly inhibited the LPS-induced production of IL-12p70 (Fig. 4, p<0.01), in accordance with previous reports (7,9). This differential regulatory effect upon the IL-12p70 production induced either by CD40 ligation or by bacterial products, together with the ability of IL-4 to inhibit the CD40L-induced production of the inactive p40 subunit of IL-12 (see Fig.3a, and ref. 8), may explain the current view of IL-4 as an inhibitor of IL-12 production.

**Figure 4: IL-4 inhibits the LPS-induced IL-12p70 production.** DC were stimulated with LPS for 24 hours in the presence of either IL-4 or IFN-γ (both at 1000U/ml). The data, shown as means ±SD of triplicate cultures, represents one experiment of three that all gave similar results.

**Concluding remarks**

In contrast to the view of IL-4 as an inhibitor of IL-12 production, the present findings show that IL-4 acts as a Th2-specific coinducer of the production of bioactive IL-12p70, resulting in an intrinsic tendency of human Th2 cells to revert to IFN-γ-producing Th0/Th1 phenotype upon the interaction with DC. They suggest that an initial commitment of lymph node-based naive Th cells to the Th2 subset results in a flexible population of effector cells, the functions of which remain under control of distinct APC populations encountered in peripheral tissues. This feature may allow Th2 cells to induce different effector mechanisms of immunity depending on the requirements met in distinct locations. The interaction with IL-12-deficient B cells can result in helper signals for Ig production but also in a preservation, or an enhancement, of the polarized Th2 phenotype. The maintenance of polarized Th2 cytokine profiles can also occur when Th2 cells meet other APC populations in the tissues rich in IL-12-suppressing factors, e.g. IL-10, TGF-β, or prostaglandin E₂, like the mucosa of the airways and the alimentary tract, or tumor tissues. DC isolated from the airways and from the gut-associated lymphoid tissues have been recently shown to be IL-12 deficient (17,18). Repetitive (re)stimulations in such conditions may allow Th2 cells to proceed to the stage of IL-12 resistant Th2 cells, a phenotype observed in a small fraction of human Th cells in atopic disease (10).

In contrast to the above situation, migration of Th2-polarized cells to the tissues which can accommodate the potentially dangerous Th1 responses and which allow for local IL-12 production, such as the skin, can result in encountering APC that are able to produce IL-12 in response to CD40L- and IL-4-mediated signals. This can allow Th2 cells to effectively contribute to the local inflammatory-type responses, by abandoning their Th2-
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polarized phenotype and supporting the IL-12- and IFN-g-dependent mechanisms of cellular immunity.

Such a differential control of Th2 cell function by distinct tissues and APC types may possibly be observed in pathological conditions in atopic dermatitis. Allergen-specific Th2 cells efficiently support IgE production in atopic individuals, consistent with the absence of IFN-g (that suppresses IgE production (19)) during their interaction with B cells. In contrast, activation of allergen-specific Th2 cells in the skin results in a not completely understood phenomenon of an early wave of Th2-type cytokine production, followed by the late-phase of IL-12 and IFN-g production (20). The presently described IL-4-mediated IL-12 induction by infiltrating Th2 cells and their resulting repolarization may explain the mechanism of this process.

Although mouse Th2 cells, that (in contrast to their human counterparts (6)) typically produce high levels of IL-10, can suppress IL-12p70 production (21), the classical view of IL-4 and IL-10 as two equivalent Th2 cell-derived IL-12 inhibitors may need reevaluation also in the mouse system. Although earlier studies (22) showed a partial inhibitory effect of IL-4 upon the IL-12 production induced in DC by CD40 mAb, recent data obtained in a more physiological model of OVA-specific TCR-transgenic animals (21) indicated that mouse IL-4, in contrast to IL-10, lacks the IL-12p70 suppressing activity, while another mouse study showed the ability of IL-4 to enhance IL-12 production (23).

In accordance with a possible role of murine IL-4 as an IL-12p70 inducer, Schuler et al. (16) have recently shown that IL-4-knock-out mice have impaired tumor immunity, associated with an impaired development of tumor-specific CTL responses, reduced production of IFN-g and reduced levels of tumor-reactive IgG2a, the hallmarks of impaired Th1-cell function. This deficit was reversible upon the administration of exogenous IL-4, but the link between IL-4 deficiency and the observed phenotype remains to be established. IL-4-deficient mouse also show an impaired resistance to *Candida albicans* (24) associated with impaired production of IFN-γ and reduced levels of IL-12. Although the administration of rIL-4 corrected these deficits and primed neutrophils for increased IL-12 production, it needs to be established if IL-4 can also contribute to the induction of IL-12 during the interaction of APC with *C. albicans*-specific T cells, since IL-4 was shown ineffective upon peritoneal macrophages (24). Similarly, it remains to be tested whether the IL-4-mediated induction of IL-12 plays a role in the paradoxical ability of some Th2 cells to promote autoimmune destruction of own tissues (25,26), and whether it contributes to the recently described ability of IL-4 to enhance the production of endogenous IFN-γ in CD40L-stimulated DC (27).

The presently-described flexibility of Th2 cells and their functional dependence on distinct APC types underline the importance of the post-priming check-points controlling the
quality of the immune response and suggest additional possibilities of therapeutic induction of Th1 responses in Th2-associated diseases.

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