Interleukin-23 Promotes a Distinct CD4 T Cell Activation State Characterized by the Production of Interleukin-17\textsuperscript{*}

Received for publication, July 26, 2002, and in revised form, October 31, 2002
Published, JBC Papers in Press, November 3, 2002, DOI 10.1074/jbc.M207577200

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Interleukin (IL)-17 is a pro-inflammatory cytokine that is produced by activated T cells. Despite increasing evidence that high levels of IL-17 are associated with several chronic inflammatory diseases including rheumatoid arthritis, psoriasis, and multiple sclerosis, the regulation of its expression is not well characterized. We observe that IL-17 production is increased in response to the recently described cytokine IL-23. We present evidence that murine IL-23, which is produced by activated dendritic cells, acts on memory T cells, resulting in elevated IL-17 secretion. IL-23 also induced expression of the related cytokine IL-17F. IL-23 is a heterodimeric cytokine and shares a subunit, p40, with IL-12. In contrast to IL-23, IL-12 had only marginal effects on IL-17 production. These data suggest that during a secondary immune response, IL-23 can promote an activation state with features distinct from the well characterized Th1 and Th2 profiles.

Interleukin (IL)-17 is a T cell-derived pro-inflammatory molecule that stimulates epithelial, endothelial, and fibroblastic cells to produce other inflammatory cytokines and chemokines including IL-6, IL-8, G-CSF, and MCP-1 (1–8). IL-17 also synergizes with other cytokines including tumor necrosis factor-\(\alpha\) and IL-1\(\beta\) to further induce chemokine expression (7, 9). Interleukin-17 levels are found to be significantly increased in rheumatoid arthritis synovium (10, 11), during allograft rejection (12–15), and in other chronic inflammatory diseases including multiple sclerosis (16) and psoriasis (17–19). Although clearly produced by activated T cells, previous reports have not provided clear classification of IL-17 within the paradigm of Th1 and Th2 polarized cytokine profiles.

We have examined the possibility that IL-17 is expressed in response to signals distinct from those associated with the Th1 or Th2 response. We observe a previously unrecognized activity of the recently identified cytokine IL-23 (20). IL-23 is a heterodimeric cytokine that shares one subunit, p40, with IL-12. The initial characterization of this cytokine has suggested it can promote proliferation within the memory T cell population. Subsequent work demonstrated that transgenic over-expression of the second component of IL-23, p19, was sufficient to induce systemic inflammation and premature death (21). In addition, the mice had markedly elevated levels of circulating neutrophils. Interestingly they did not exhibit consistent elevation of IFN-\(\gamma\), a hallmark effect of IL-12. These data suggest that IL-23 may have a biological role substantially distinct from that of IL-12. In this report we present evidence that IL-23 acts to induce a distinct T cell activation state that produces IL-17 as a principle effector cytokine.

EXPERIMENTAL PROCEDURES

Cell Culture—Single cell suspensions of spleen were prepared from C57/BL-6 mice, and mononuclear cells were isolated from suspended splenocytes by density gradient centrifugation. 2 \(\times\) 10\(^6\) cells/ml were cultured with IL-2 (100 units/ml) in the presence or absence of various stimuli (for times indicated in figure legends), following which the cells were collected and analyzed for IL-17 using ELISA (R&D Systems, Minneapolis, MN). Dendritic cells were derived from macrophages (obtained as adherent population from splenocyte suspension) by treating macrophages with rGM-CSF (2 mg/ml) and rIL-4 (1000 units/ml) for 4 days, washing and re-activating using LPS (0.5 \(\mu\)g/ml). Memory and naive T cells were isolated by staining mononuclear cells isolated from single cell suspension of murine splenocytes with CyC-CD4 + PE-CD44 or CyC-CD4 + PE-CD62L and sorting for CD4+ cells that were either CD44\(^{hi}\)/CD62L\(^{lo}\) for memory phenotype or CD44\(^{lo}\)/CD62L\(^{hi}\) for naive phenotype.

In vitro Induction of T Cell Differentiation—CD\(^{+}\) T cells were purified from spleen of wild type C57/BL6 mice using anti-CD4 magnetic beads (Miltenyi Biotec). Purified T cells (2 \(\times\) 10\(^6\) cells/ml) were activated for 3 days by plating on plates coated with 5 \(\mu\)g/ml anti-CD3 and 1 \(\mu\)g/ml anti-CD28 antibodies. The cultures were supplemented with IL-2 and treated with IL-12 (20 nm) + anti-IL-4 (0.5 \(\mu\)g/ml) (for Th1 differentiation), IL-4 (1000 units/ml) + anti-IFN-\(\gamma\) (0.5 \(\mu\)g/ml) (for Th2 differentiation), or IL-23 (10 nm) (for IL-17 production). Following initial activation, the cell cultures were washed extensively and re-stimulated with anti-CD3 (1 \(\mu\)g/ml) for another 24 h, following which the cell supernatants were analyzed for various secreted cytokines using ELISA.

IL-12p40 Antibody Inhibition of IL-17 Induction—Anti-IL-12 antibody (R&D Systems, cat no. AF-419-NA) or an unrelated control antibody (anti-FGF-8b (R&D Systems, cat no. AF-423-NA)) were pre-incubated with IL-23 (100 ng/ml) or conditioned media of LPS-stimulated dendritic cells (10\% v/v) for 1 h at 37 \(^\circ\)C and then incubated for another 5–6 days with mononuclear cells isolated from mouse spleen (2 \(\times\) 10\(^6\) cells/ml). Supernatants were collected and levels of IL-17 measured using ELISA.

Purification of IL-23—Murine IL-23 component was produced by co-expression of carboxyl-terminal His-tagged p19 and FLAG-tagged p40 in human embryonic kidney cells (293 cells), and secreted protein was purified by nickel affinity resin. Endotoxin levels were undetectable at less than 0.2 endotoxin units per \(\mu\)g.

RESULTS

We first examined the ability of various microbial products to stimulate the production of IL-17. Increased IL-17 has recently been observed by Infante-Duarte et al. (22) in response to microbial lipopeptides from a Lyme disease causing spirochete,
**Borrelia burgdorferi.** We observed that spleen cell cultures in the presence of various microbial products including LPS (Gram-negative bacteria), lipoteichoic acid (Gram-positive bacteria) or lipopeptide (bacterial lipopeptide) resulted in the production of IL-17 (Fig. 1). Neither purified T cells alone nor purified macrophages themselves produced IL-17. Purified T cells were obtained from murine splenocytes following positive selection of fluorescence-activated cell-sorted, CD4-labeled cells. These cells were cultured (1 x 10⁶ cells/ml) in presence or absence of plate-bound anti-CD3 (5 µg/ml) or supernatant from activated dendritic cells (LPS-treated) for 3 days and culture supernatants collected and analyzed for IL-17 levels using an ELISA kit. Representative results from three independent experiments are shown.

**Fig. 1. IL-17 production in different cell types.** A, mononuclear splenocytes were cultured in the presence or absence of microbial lipopeptide (100 ng/ml), LPS (100 ng/ml), or lipoteichoic acid (100 ng/ml) for 3 days, following which the cells were collected and analyzed for IL-17 using ELISA. B, purified T cells were obtained from murine splenocytes following positive selection of fluorescence-activated cell-sorted, CD4-labeled cells. These cells were cultured (1 x 10⁶ cells/ml) in presence or absence of microbial lipopeptide (100 ng/ml), LPS (100 ng/ml), or lipoteichoic acid (100 ng/ml) for 3 days, following which the cells were collected and analyzed for IL-17 using ELISA. Representative results from three independent experiments are shown.

**Fig. 2.** IL-23 stimulates production of IL-17. A, mononuclear cells isolated from splenocytes were cultured with IL-2 in presence or absence of various concentrations of IL-23 (0.1–1000 ng/ml) for 6 days. Levels of IL-17 accumulated in culture supernatants were measured using ELISA. Experiments were performed at least six times with similar results. Inclusion of ConA in the culture conditions substantially increased the level of IL-17 produced in response to IL-23 but also increased basal production of IL-17 and other cytokines in the absence of IL-23 (not shown). B, changes in mRNA levels for IL-17 in response to IL-23 treatment were measured by quantitative RT-PCR. Plotted is the relative change in Ct (cycle threshold) of the PCR reaction. Data for each sample is normalized to the glyceraldehyde-3-phosphate dehydrogenase mRNA level present in each sample and then normalized again between samples to the level of IL-17 mRNA present in the time zero unstimulated condition. As each Ct corresponds to a PCR cycle, one Ct is approximately equal to a 2-fold change in mRNA abundance. The approximate mRNA fold difference for 5 Ct and 10 Ct changes are indicated in parenthesis. The experiment was performed with splenocytes from 4 mice, and the individual data points are represented with x and the average Ct change is indicated by bar columns. C, changes in mRNA levels of the IL-17 family member IL-17F in response to IL-23 treatment were measured by quantitative RT-PCR as in the legend to Fig. 2B.
cells, upon receptor cross-linking using plate-bound anti-CD3 and treatment with supernatants from activated macrophages/dendritic cells, produced increased IL-17, indicating the presence of an unidentified factor(s) released by these cells that acts on T cells to promote IL-17 production.

In profiling the expression of candidate molecules that might be responsible for this IL-17 promoting activity, we observed 100–1000-fold increased mRNA expression of the IL-23 (20) components, p19 and p40, in activated dendritic cells using real-time RT-PCR (not shown), hence, the effect of IL-23 was examined. Murine spleen cell cultures, in the presence of IL-23, resulted in high levels of IL-17 production in a dose-dependent manner (Fig. 2A). However, when these cells were cultured under IL-12-stimulated Th1-inducing conditions, they resulted in marginal IL-17 production, whereas under Th2-inducing conditions there was no increased production of IL-17 over controls (Table I). IL-23 also resulted in higher levels of GM-CSF than observed under Th1-inducing conditions. In contrast, IFN-γ levels were significantly lower than those obtained under Th1-inducing conditions. Tumor necrosis factor-α levels were similar to Th1 conditions. IL-12p40 alone did not result in any IL-17 production (data not shown). IL-23 promoted ele-

| Table I Cytokine levels in Th cells in response to IL-23 |
|---------------------------------------------------------|
| Purified CD4+ T cells were incubated in presence of IL-2 (100 units/ml) and activated as described under “Experimental Procedures” under Th1-inducing conditions (IL-12+ anti-IL-4), Th1-inducing conditions (IL-4+ anti-IFN-γ), or purified IL-23 (10 nM) for 3 days, following which the cultures were washed and re-stimulated with anti-CD3 for another 24 hours. Levels of various cytokines were measured using ELISA. The levels less than the lowest dilution of the standard curve range of ELISA kit were recorded as not detectable (N.D.). The results below are representative of three experiments performed independently. |
| Control | IL-12 | IL-4 | IL-23 |
|---------|-------|------|-------|
| IL-17   | N.D.  | 58 ± 82 | 64 ± 91 | 1191 ± 569 |
| IL-4    | 50 ± 26 | 396 ± 17 | 3259 ± 118 | 101 ± 100 |
| IFN-γ   | 341 ± 0 | 2757 ± 1016 | 489 ± 502 | 580 ± 813 |
| GM-CSF  | N.D.  | 46 ± 13 | 365 ± 516 | 882 ± 169 |
| TNF-α   | N.D.  | 174 ± 40 | 214 ± 314 | 205 ± 85 |

**Fig. 3.** IL-23 acts on memory T cells to induce IL-17 production. CD4-positive memory (CD44high/CD62Llow) or naive (CD44low/CD62Lhigh) T cells were cultured with IL-2 in the presence or absence of IL-23 (or its boiled prep as a control) and plate-bound anti-CD3 (5 μg/ml) and anti-CD28 (1 μg/ml) for 3 days (6 days for naive cell population), washed, and re-stimulated with anti-CD3 antibody for another 24 h. Supernatants were collected, and IL-17 levels were measured using ELISA.

**Fig. 4.** IL-12p40 antibody blocks IL-23-dependent IL-17 production. A, IL-12 antibody interacts with p40 subunit present in IL-12 and IL-23. Shown is the Western blot analysis using anti-IL-12 antibody demonstrating interaction with purified recombinant p40 and the p40 subunit present in IL-12 and IL-23. The relative positions of molecular mass markers, in kDa, are indicated. B, increasing concentrations of anti-IL-12p40 antibody or an unrelated control antibody were pre-incubated with IL-23 and then incubated for another 5–6 days with mononuclear splenocytes. Supernatant were collected and levels of IL-17 measured using ELISA (left panel). Optimum concentrations of anti-IL-12p40 antibody or control antibody were pre-incubated with conditioned media of LPS-stimulated dendritic cells and then incubated for another 5 days with mononuclear splenocytes. Supernatant were collected and levels of IL-17 measured using ELISA (right panel). C, mononuclear cells isolated from splenocytes of wild type mice (C57/BL6) or mice lacking one of the components of IL-12, i.e. IL12a−/− (p35 knockout) or IL12b−/− (p40 knockout) were cultured in the continuous presence of ConA for 3 days and IL-17 levels measured in supernatants using ELISA.
IL-12 Promotes IL-17 Production

IL-23 Promotes IL-17 Production

This article discusses the role of IL-23 in promoting IL-17 production. It explains that IL-23, a cytokine produced by antigen-presenting cells, plays a crucial role in the induction of IL-17 production in T cells. The article highlights the interaction between IL-23 and IL-12, another cytokine, in promoting IL-17 production.

The article also mentions the importance of IL-17 as an effector cytokine and its role in the promotion of a distinct T cell activation state. IL-23 is shown to stimulate IL-17 production, which is important for the immune response against intracellular and extracellular pathogens. The article further discusses the role of IL-12 in IL-17 production and how IL-23 and IL-12 cooperate to promote IL-17 production.

Discussion

Taken together, these data suggest a role for IL-23 in the promotion of a distinct T cell activation state that expresses IL-17 as an effector cytokine. The Th1 and Th2 paradigms have been described as promoting cell-mediated versus humoral immune responses. These responses provide important defense for intracellular and extracellular pathogens, respectively, and defects in either of these responses are associated with increased susceptibility to specific pathogens. In contrast, IL-23 may serve to promote an adaptive immune response to pathogens that is characterized by a heavy reliance on cells thought to function primarily as mediators of the innate immune response. IL-17, as a principle effector cytokine of this response, is able to promote the more rapid recruitment of monocytes and neutrophils through induced chemokine production. In addition, the GM-CSF production observed in response to IL-23 supports the production of additional myeloid cells. The character of this adaptive response is, however, not an exclusive reliance on phagocytic cells of the myeloid lineage as IL-17 is known to promote the induction of ICAM thereby providing important co-stimulation of further T cell responses. The actions of IL-23 appear to be restricted to memory T cells. However, it remains to be determined whether there exist co-stimulatory signals that enable action of IL-23 on naive T cell populations. Further analysis with TCR transgenic animals will help to clarify these issues.

Recently several studies have pointed out significant differences between mice deficient in p35 and mice deficient in p40. These studies share the observation that loss of p40 is generally more deleterious than loss of p35 in the immune-mediated clearance of a variety of model organisms. For example, Elkins et al. (32) found that although p35 KO mice were readily able to clear the intracellular bacterium Francisella tularensis LVS, p40 KO mice were unable to completely clear the bacteria and exhibited chronic infection. In light of the results presented here, these observations may reflect the capacity of IL-12 and IL-23 to promote unique and complementary immune responses that facilitate the complete clearance of bacteria and perhaps other pathogens. Interestingly, recently reported p19 transgenic mice display a profound systemic inflammation and neutrophilia (21). This phenotype is consistent.
with the results reported here and further indicates the potential of this signal to mediate profound changes in the balance of immune function. IL-17 production is not completely abrogated in p40 KO mice, indicating that there exist other IL-23-independent pathways to its expression. It will be very important to investigate whether IL-23 has similar functions in human and mice and to establish its relevant role in human immune function. A great deal of attention has been given to the Th1 paradigm and its relationship to major unmet human diseases. Given the clear association of IL-17 expression with most of these same inflammatory diseases it may be that the relationship between IL-12 and these diseases should be re-evaluated and additional work undertaken to understand the role of this new cytokine axis in human disease.

Acknowledgments—We thank Andy Chan, Iqbal Grewal, Sherman Fong, Wenjun Ouyang, and Paul Godowski for their comments and suggestions.

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