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IL-12p35 Subunit Contributes to Autoimmunity by Limiting IL-27–Driven Regulatory Responses

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Contrasting results have emerged from studies performed using IL-12p35−/− mice. Animals lacking the IL-12p35 subunit can either be protected from or develop exacerbated autoimmune diseases, intracellular infections, and delayed-type hypersensitivity responses. In this study, we report that mice lacking the IL-12p35 subunit develop a significantly milder Ag-induced arthritis compared with wild-type (WT) mice. Lack of severe inflammation is accompanied by an increase in the mRNA levels of the Ebi-3 and p28 subunits and increased secretion of IL-27 and IL-10. This anti-inflammatory environment contributed to increased differentiation of regulatory T and B cells with intact suppressive function. Furthermore, IL-12p35−/− mice display reduced numbers of Th17 cells compared with WT arthritic mice. Neutralization of IL-27, but not the systemic administration of IL-12, restored inflammation and Th17 to levels seen in WT mice. The restoration of disease phenotype after anti–IL-27 administration indicates that the IL-12p35 subunit acts as negative regulator of the developing IL-27 response in this model of arthritis. The Journal of Immunology, 2011, 187: 3402–3412.
IL-23), anti–IFN-γ-alkaline phosphatase (clone XM13.G1.2), and anti–IL-17-PE (clone TC11-18H10) were purchased from BD Pharmingen. Anti–IL-27-carboxyfluorescein (clone 234.205) was purchased from R&D Systems.

**Animals**

IL-12p35−/− and C57BL/6 WT mice were purchased from The Jackson Laboratory. These animals were bred and maintained under specific pathogen-free conditions at the animal facility at University College London (London, U.K.). These studies have been reviewed and approved by the UK Home Office.

**Induction and assessment of AIA**

Female C57BL/6 mice (8–12 wk old) were used in all experiments. Animals were immunized with 100 μg methylated BSA (mBSA; Sigma-Aldrich) dissolved in 50 μl PBS and emulsified in an equal volume of CFA (Sigma-Aldrich), containing 3 mg/ml Mycobacterium tuberculosis (Difco). This was followed, 7 d later, by intra-articular injection of 10 μl 20 mg/ml methylated BSA in the right knee. Additionally, the second knee was injected with PBS alone to observe the swelling due to trauma. Knee swelling was measured using calipers and "Ag-specific" swelling was calculated by deducting the size of the PBS-injected knee from the mBSA-injected knee. Knee joints were removed postmortem and fixed with 10% (w/v) buffered formalin and decalcified in 5% EDTA. After decalcification, the paraffin sections were stained with H&E. Two independent observers evaluated the slides histologically. The gradation of arthritis was scored from 0 to 4 according to the intensity of lining layer hyperplasia, mononuclear cell infiltration, and pannus formation as reported (16): 0, normal; 1, normal synovium with occasional mononuclear infiltrates; 2, a few layers of flat to round synovial lining cells and scattered mononuclear infiltrates; 3, clear hyperplasia of the synovium with three or more layers of loosely arranged lining cells and dense infiltration with mononuclear cells; 4, severe synovitis with pannus and erosion of articular cartilage and subchondral bone.

**Induction and assessment of CIA**

Female C57BL/6 mice (8–12 wk old) were used in all experiments. CIA was induced as described previously (17). Briefly, animals were immunized with 200 μg chicken collagen emulsified in an equal volume of CFA (Sigma-Aldrich), containing 3 mg/ml M. tuberculosis (Difco). This was followed, 14 d later, by 200 μg chicken collagen emulsified in an equal volume of IFA (Sigma-Aldrich).

**Treatment with rIL-12 and neutralizing IL-27**

C57BL/6 WT and IL-12p35−/− mice received 100 ng recombinant mouse IL-12 (R&D Systems) i.p. at days −8, −7, −6, −1, 0 and 1 after AIA onset. Injection of PBS was used as a control. For neutralization of IL-27, 20 μg polyclonal goat anti–IL-27 Ab (R&D Systems) was administered i.p 4 h before AIA induction. Polyclonal goat IgG was used as an isotype control.

**Isolation of synovial cells**

Cells infiltrating the AIA-affected joint were isolated from the synovial membrane 3 d after disease onset as described (18). Briefly, knee joints were removed and synovial membrane was excised under a dissecting microscope and digested with collagenase A (1 mg/ml; Sigma-Aldrich) and DNAse type IV (150 μg/ml; Roche) for 45 min at 37°C. The cells were then washed and passed through a cell strainer before using for flow cytometry and cell culture.

**Flow cytometric analysis**

Intracellular cytokine analysis was performed as previously described (19). Briefly, splenocytes, inguinal lymph node (LN), or synovial cells (3 d after disease onset) were suspended at 5 × 10^6±10^6 cells/well and cultured in complete medium with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and GolgiPlug (BD Biosciences) for 6 h. Cells were then stained for extracellular markers followed by fixation (PBS/2% PFA), permeabilization (permeabilization buffer from eBioscience), and incubation with anti-mouse IL-10-allophycocyanin, IFN-γ-allophycocyanin, or IL-17-PE mAbs. The cells were acquired with a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software.

**Anti-mBSA IgG ELISA**

Blood was obtained postmortem. After blood coagulation, serum was separated and analyzed for levels of anti-mBSA IgG by a sandwich ELISA assay. Microplates were coated with mBSA (Sigma-Aldrich), diluted in PBS, and incubated overnight. Non-specific binding sites were saturated with 1% gelatin (Sigma-Aldrich) for 1 h. Serial dilutions of tested sera were incubated at 37°C for 2 h. Following washes, peroxidase-conjugated streptavidin detected bound complexes by using 3,3’,5,5’-tetramethylbenzidine (Sigma-Aldrich) and the reaction was stopped by adding H2SO4. Absorbance was read at 450 nm. Washing steps were conducted in 0.05% Tween 20 in PBS.

**Cytokine ELISA and cytokine bead assay**

Draining LN and synovial cells were cultured at 5 × 10^5±10^5 cells for 48 h without any stimulation or with mBSA (50 μg/ml). Supernatants from cell suspensions were harvested analyzed for levels of secreted cytokines using standard sandwich ELISA kit (R&D Systems) or cytokine bead assay (CBA; Bender MedSystems) performed according to the manufacturer’s instructions.

**Real-time PCR**

Cells were cultured at 5 × 10^5±10^5 cells for 48 h without any stimulation or with mBSA (50 μg/ml). RNA was extracted using RNeasy columns (Qiagen) and treated with DNase 1 (Promega). cDNA was obtained using a reverse transcriptase kit (Qiagen). RNA (100 ng) was analyzed for the expression of IL-10, IL-12p35, IL-27p28, and Ebi-3 using TaqMan expression assays and performed according to the manufacturer’s instructions.

**Treg suppression assay**

LN s were excised postmortem from IL-12p35−/− and WT mice 3 d after disease onset. Tregs and effector CD4+ cells were isolated using a mouse CD4+CD25+ Treg isolation kit (Miltenyi Biotec). Purity and expression of Foxp3 in CD4+CD25+ regulatory and CD4+CD25− effector cells were determined by FACs and are shown in Fig. 5c. Cells were plated with a total cell number of 2 × 10^5±10^5 cells/well. Effector T cells were only wells contained 2 × 10^5±10^5 Tregs/well; Treg-only wells contained 2 × 10^5±10^5 Tregs/well; and mixed wells contained 1 × 10^5±10^5 Tregs/well (ratio 1:1). Purified cells were cultured for 60 h without any stimulation or with mBSA (50 μg/ml) or anti-CD3 (1 μg/ml). Supernatants were harvested and analyzed for TGF-β secretion by ELISA (R&D Systems) Additional cultures were left for 96 h and then pulsed overnight with 1 μCi [3H]thymidine, harvested, and counted in a scintillation counter (LKB Instruments).

**Adaptive transfer of B cell subsets and transitional 2-marginal zone precursor Bregs functional assay**

B cells isolated from the spleens of WT or IL-12p35−/− mice in the re-inoculation phase of AIA (day 12 after disease onset) were stained with CD19, CD23, CD21, and CD24. B cell subsets were FACS sorted using gates drawn according to previous reports (20). Seven days prior to cell transfer/intra-articular injection, all recipients were immunized with mBSA in CFA. Transitional 2-marginal zone precursor (T2-MZP) Bregs and MZB cells (2 × 10^5±10^5) were transferred i.v. to syngeneic WT C57BL/6 mice on the day of intra-articular injection. The control group (no transfer) received a PBS injection.

**Statistical analysis**

For the statistical analysis of the data, unpaired t tests and the Fisher test were applied. Results in which p < 0.05 were considered significant.

**Results**

**IL-12p35−/− mice are protected from developing AIA**

To study the direct effect of IL-12p35 on acute inflammatory arthritis, IL-12p35−/− mice and C57BL/6 WT mice were immunized with mBSA with CFA, followed by an intra-articular inoculation of mBSA 1 wk later (16). IL-12p35−/− mice displayed a significantly reduced joint swelling and total arthritis index score as compared with age and gender matched WT mice (Fig. 1A–C). Histopathological evaluation of the WT mice revealed a substantial accumulation of inflammatory cells in the affected joints, causing severe cartilage damage and bone erosion. In contrast, IL-12p35−/− mice showed only a minor cell infiltrate and an overall preservation of joint structure (Fig. 1B, 1C). Significantly lower numbers of inflammatory cells were recovered from the affected synovia of IL-12p35−/− compared with WT mice (Fig. 1D). IL-12p35−/− mice had reduced levels of mBSA-specific Abs as
compared with WT mice (Fig. 1E), despite mBSA-induced proliferation of spleen and draining LN lymphocytes having been comparable in both mouse strains (Fig. 1F).

**IL-12p35 deficiency impairs Th1 and Th17 responses while favoring IL-10–dependent anti-inflammatory responses**

To gain more insight into the potential mechanisms modulating the development of AIA in IL-12p35−/− mice, we assessed the release of cytokines associated with inflammation in the draining LNs. In response to in vitro mBSA restimulation, lymphocytes from the draining LN of IL-12p35−/− mice displayed significantly reduced IFN-γ production compared with lymphocytes from WT mice (Fig. 2A). However, the expression of IL-23p19 mRNA (Fig. 2B) was not significantly affected. Additionally, the production of IL-23 measured by ELISA (data not shown) or assessed by intracellular staining (Supplemental Fig. 1A) was also not changed. Further cytokine analysis revealed that significantly lower amounts of IL-17, IL-6, and IL-1β were released in IL-12p35−/− compared with the levels measured in WT mice (Fig. 2C). In contrast, increased levels of the anti-inflammatory cytokines IL-10 and IL-5 were observed in IL-12p35−/− mice as compared with WT mice (Fig. 2C).

To assess the cellular source of cytokine production, we next analyzed T cell responses by intracellular FACS staining and demonstrated that CD4+ T cells from IL-12p35−/−-immunized mice display reduced differentiation into Th1 and Th17 cells (Fig. 2D, 2E).

Next, we studied how the lack of IL-12p35 influenced the cytokine profile at the site of inflammation, namely the synovial membrane. Th1 or Th17 cells were virtually absent in the synovial infiltrate of IL-12p35−/− mice, whereas they were abundantly present in the inflamed joints of WT mice (Fig. 2F–I). No detectable levels of IL-23 could be measured in the synovia of WT or IL-12p35−/− mice (data not shown).

**Increased numbers of functional Tregs in IL-12p35−/− mice**

CD4+CD25+Foxp3+ Tregs were found with increased frequency in both the LN and at the site of inflammation in IL-12p35−/− mice compared with WT mice (Fig. 3A–C). In addition to the increased frequency, CD4+CD25+Foxp3+ Tregs infiltrating the synovia of IL-12p35−/− mice expressed increased levels of Foxp3 (Fig. 3D), which has been correlated to increased suppressive activity of regulatory cells (21).

It has been shown that IL-35 is released by CD25+Foxp3+CD4+ Tregs and contributes to their development and suppressive function (22). Despite their inability to release IL-35, CD4+CD25+Foxp3+ Tregs isolated from IL-12p35−/− mice efficiently suppressed in vitro mBSA-mediated proliferation of CD4+CD25− Teffs isolated from the draining LN of WT mBSA-immunized arthritic mice (Fig. 3E). These results are in agreement with those previously published showing that Tregs in Ebi-3−/− mice, also lacking IL-35, are normal during *Listeria monocytogenes* infection (23). However, in agreement with the data of Vignali and colleagues (24), the suppressive capacity of IL-12p35−/− Tregs was significantly reduced when effector cells were polyclonally stimulated with anti-CD3 (Supplemental Fig. 1B, 1C). Therefore, these data suggest that IL-35−/− Tregs retain their capacity to suppress if activated in the presence of triggering autoantigen. Of interest, the
FIGURE 2. IL-12p35 deficiency impairs Th1 and Th17 responses while favoring IL-10–dependent anti-inflammatory responses. A, Draining LN from WT or IL-12p35−/− mice on day 3 after disease onset were obtained, cultured for 60 h with mBSA (50 μg/ml), and the production of IFN-γ was measured in the supernatant by CBA. One of three independent experiments is shown and data are expressed as mean values ± SEM (n = 5). The p values were determined by an unpaired t test. B, Draining LN cells were isolated from IL-12p35−/− and WT mice at day 3 after disease onset and cultured for 60 h with mBSA. mRNA expression of IL-23p19 was assessed by real-time PCR and normalized to GAPDH. Results are expressed as relative expression calculated using the value obtained from unstimulated WT LN cell expression. Data are expressed as mean values ± SEM (n = 5). These data are representative of three independent experiments, with similar results. C, Draining LNs from WT or IL-12p35−/− mice on day 3 after disease onset were obtained, cultured for 60 h with mBSA (50 μg/ml), and supernatants were harvested and cytokine production was measured by CBA. Data are expressed as mean values ± SEM. One experiment representative of three independent experiments is shown (n = 5). The p values were determined by an unpaired t test. D, Draining LN cells were isolated from IL-12p35−/− and WT mice at day 3 after disease onset. Cells were cultured for 5 h with PMA and ionomycin in the presence of brefeldin A. Representative dot plots show the percentage of CD4+IFN-γ+ and CD4+IL-17+ cells. E, Bar charts show means ± SEM of percentage and absolute values of CD4+IFN-γ+ and CD4+IL-17+ cells. Data are representative of two independent experiments (n = 5). The p values were determined by an unpaired t test. F, Synovial membrane obtained from WT or IL-12p35−/− mice at day 3 after disease onset was dissected and digested. Cells were cultured for 5 h with PMA and ionomycin in the presence of brefeldin A. Representative dot plots show the percentage of CD4+IFN-γ+ and CD4+IL-17+ cells.
lack of IL-35 secretion appeared to be balanced by an increased production of TGF-β during Ag-specific responses (Fig. 3E).

**Lack of IL-12p35 alters the balance between regulatory T2-MZP Bregs and pathogenic MZB cells**

IL-12–producing Bregs have been shown to protect mice from colitis (25). Next, we assessed whether, in addition to Tregs, the lack of IL-12p35 also affected the number and function of T2-MZP Bregs previously identified as the predominant Breg subset in IL-12p35−/− mice as compared with WT mice (Fig. 4A, 4B). The latter B cell subset has been previously attributed with pathogenic properties (29). The number of follicular B cells remained unchanged in both groups (Fig. 4C). Transfer of T2-MZP Bregs isolated from WT or IL-12p35−/− mice, obtained from the spleen at the remission stage of AIA, to WT syngeneic mice with arthritis show equivalent suppressive capacity, thus excluding a role of IL-12p35, IL-12, or IL-35 in the suppressive function of T2-MZP Bregs (Fig. 4D).

**Administration of IL-12 fails to restore inflammation and DTH response in IL-12p35−/− mice**

Other groups have demonstrated that IL-23 is pivotal in the differentiation of Th17 cells and in the pathogenesis of autoimmune diseases (30). However, our data so far support a previously unappreciated role for IL-12p35 in governing DTH in this model of acute inflammation and joint damage. The IL-12 family of heterodimeric cytokines includes IL-12 (comprising p40/p35), IL-23 (p40/p19), IL-27 (p28/Ebi-3), and IL-35 (Ebi-3/p35). The lack of IL-12p35 ablates IL-12 and IL-35 production, but it may indirectly impinge in the release of IL-23 and IL-27. Two conceivable explanations could be put forward to explain the reduced inflammatory response, in favor of the regulatory environment, observed in IL-12p35−/− mice. First, the IL-12p35 subunit, via the release of bioactive IL-12, contributes to the differentiation of an inflammatory environment. Second, owing to the promiscuous sharing of the same chain, we suggest that in the absence of the IL-12p35 subunit, Ebi-3 may be more “available” for coupling to p28, resulting in increased production of IL-27. This may overshadow bioactive IL-23 in the IL-12p35−/− mice and ultimately block Th17 differentiation in this acute model of inflammatory arthritis.
To determine whether exogenous rIL-12 could restore the proinflammatory cascade in IL-12p35−/− mice, murine rIL-12 was given i.p. at days −1, 0, and 1 of mBSA/CFA and intra-articular immunizations as previously described (31). Administration of IL-12 increased IFN-γ secretion in IL-12p35−/− mice (Fig. 5A). However, reconstitution with rIL-12 failed to restore the production of IL-23 or IL-17 in IL-12p35−/− mice (data not shown) and arthritis, as shown by a very mild inflammation and infiltration in the affected joint (Fig. 5B–D). These results suggest that the proinflammatory effect of the IL-12p35 subunit is not due to coupling with the p40 subunit resulting in the release of bioactive IL-12.

IL-12p35−/− mice have increased IL-27 production, and in vivo neutralization of IL-27 restores Th17 development and inflammatory response in IL-12p35−/− mice

IL-27 is a member of the IL-12 family of heterodimeric cytokines that comprises the Ebi-3 and p28 chains. IL-27 plays an ambiguous role, as it can promote as well as inhibit autoimmune disease and DTH responses (32, 33). We next investigated the effect that the lack of IL-12p35 subunit has on the expression levels of Ebi-3 and p28, unique to IL-27. mRNA was isolated from the draining LN and analyzed for the expression of Ebi-3 and IL-27p28. Expression of mRNA encoding for both Ebi-3 and p28 was upregulated in the IL-12p35−/− mice compared with WT mice (Fig. 6A). We also noted a significant increase in the frequencies of IL-27–producing cells in IL-12p35−/− compared with WT mice as well as an increase in secreted IL-27 as measured by ELISA (Fig. 6B, 6C). Previous reports have identified the major producers of IL-27 as dendritic cells and B cells (34, 35). In our experiments we also identified the CD19+ B cell subset as producers of IL-27 (Fig. 6D).

Next, we tested whether we could restore AIA in IL-12p35−/− mice by blocking the release of IL-27 in vivo. IL-12p35−/− and WT mice were treated with anti–IL-27 or with an irrelevant isotype control at the time of mBSA/CFA immunization. In vivo neutralization of IL-27 resulted in a restoration of arthritis and cellular infiltration, causing joint damage to levels observed in WT mice (Fig. 7A–C).
IL-27 drives the differentiation of CD4+ T cells producing IL-10, leading to a decrease of Th17 differentiation (36). In vivo neutralization of IL-27 restored Th17 responses in the draining LN of IL-12p35−/− mice to WT levels (Fig. 7D). Release of IFN-γ and IL-23 was not affected by the anti–IL-27 treatment (data not shown). Interestingly, neutralization of IL-27 production led to a significant decrease in IL-10 production in IL-12p35−/− but not in WT mice (Fig. 7E, 7F). Moreover, anti–IL-27 administration impaired the accumulation of CD4+Foxp3+ cells in the draining LN of IL-12p35−/− mice (Fig. 7G). The findings showing that neutralization of IL-27 in WT mice did not affect the frequencies of Th17, Tregs, or the levels of IL-10 produced in the draining LNs, suggesting that in the presence of an intact IL-12p35 pathway, the protective function of IL-27 is overshadowed by the proinflammatory effect of other members of this family, including IL-23.

One of the many outstanding questions is why during CIA, IL-12p35−/− mice develop an exacerbated disease, whereas the same mice undergoing a different immunization regimen, for example, AIA, produce to opposite results. It has been previously shown that the CIA can be ameliorated upon IL-27 administration (37, 38). Because we have demonstrated that IL-27 is crucial in restraining inflammation in this model, we hypothesized that IL-27 could be a pivotal factor determining disease versus resistance in the two different models of arthritis. Comparison of IL-27 production in CIA, as well as in AIA, highlighted that during AIA, IL-12p35−/− mice produce higher levels of IL-27 than do WT animals (Fig. 6B–D), whereas the scenario is reversed in CIA, with IL-12p35−/− animals showing a reduced level of IL-27 compared with WT animals (Fig. 7H, 7I). The differential IL-27 response may help to explain the complexity of why IL-12p35−/− animals are resistant to AIA, Ab-induced arthritis, and hapten-induced colitis (11, 14) and yet exhibit exacerbated autoimmune disease, including CIA (7).

**Discussion**

The discrepancy between studies showing that animals lacking the IL-12p35 subunit can either be protected from or develop exacerbated arthritis and DTH responses led us to investigate how the IL-12p35 subunit contributes, if at all, to inflammation and disease. Our results have shown that in the AIA model of arthritis, mice lacking the IL-12p35 subunit, and hence IL-12 and IL-35, were unable to mount an inflammatory response and were protected from arthritis. We have demonstrated that the lack of the IL-12p35 subunit leads to an increase in the release of IL-27. The upregulation of IL-27 production resulted in further differentiation of immunoregulatory cells and secretion of IL-10, contributing to the generation of an anti-inflammatory environment.

IL-27 was originally reported as cytokine promoting Th1 development and secretion of IFN-γ (39, 40). However, more recently IL-27 has been defined as an important anti-inflammatory mediator that can induce both Treg1 and Foxp3+ Tregs and additionally inhibit the induction of Th17 cells (34, 41, 42). Our results also show that the increase in IL-27 is followed by an upregulation of IL-10 and regulatory cell differentiation, which was inhibited after in vivo neutralization of IL-27. Taken together, our results demonstrate that in the absence of the opposing signals driven by IL-12 and IL-35, the suppressive effect of IL-27 overpowers the pathogenic capacity of IL-23 (3, 43, 44).
FIGURE 6. In IL-12p35−/− mice the dominant cytokine during acute inflammation is IL-27. A. Draining LN cells were isolated from IL-12p35−/− and WT mice at day 3 after disease onset and cultured for 60 h with mBSA. mRNA expression of IL-12p35, IL-27p28, and Ebi-3 were assessed by real-time PCR and normalized to GAPDH. Results are expressed as relative expression calculated using the value obtained from WT LN cell expression. Data are expressed as mean values ± SEM (n = 5). These data are representative of three independent experiments, with similar results. B. Draining LN cells were isolated from IL-12p35−/− and WT mice at day 3 after disease onset. Cells were cultured for 5 h in the presence of PMA, ionomycin, and brefaldin A. Intracellular levels of IL-27 were measured by FACS intracellular staining. Representative histogram shows IL-27 expression in IL-12p35−/− (thick line) or WT mice (dashed line). Bar chart shows the mean percentage of IL-27+ cells ± SEM (n = 5/group). One experiment representative of three is shown. The groups were compared by statistical analysis using an unpaired t test. C. Draining LNs from WT or IL-12p35−/− mice day 3 after disease onset were obtained, cultured for 60 h with mBSA (50 μg/ml), and the production of IL-27 was measured in the supernatant by ELISA. One of three independent experiments is shown and data are expressed as mean values ± SEM (n = 5). The p values were determined by an unpaired t test. D. Representative FACS plots show costaining of CD19 and IL-27p28 in the draining LNs of IL-12p35−/− and WT mice. *p < 0.05, **p < 0.01.

We have also demonstrated that during AIA, IL-27 was increased in the absence of IL-12p35, as compared with WT animals, whereas in CIA IL-27 was reduced. This goes toward explaining why different groups have observed differential responses to inflammatory and infectious disease models (7, 9, 11, 12, 14, 45). We do not claim that the data presented in this study account for all of the differences observed in different mouse model of arthritis. This could be due to the different genetic backgrounds used (C57BL/6 versus DBA/1), animal husbandry practices, different modality of immunizations, differential time of onset, and the capacity to resolve inflammation. The differential production of IL-27 provides a mechanistic explanation of why IL-12p35−/− mice are protected from AIA, Ab-induced arthritis, hapten-induced colitis, and L. major- and L. donovai-induced DTH response (11, 13, 14). More tailored experiments are needed for future studies to unravel the mechanisms that coordinate the differential responses in the various models of arthritis.

Several studies have identified IL-23 among the potent inducers of IL-17 and critical in the development of several autoimmune diseases (46–49). We showed that the levels of IL-23 were not significantly affected by the lack of IL-12p35. Whereas our data do not dispute the involvement of IL-23 in the pathogenesis of arthritis, or in other autoimmune disorders, they show that in the absence of IL-12/IL-35 cytokines, IL-27 promotes a tolerogenic environment despite the presence of IL-23. Our results showing higher expression of both p28 and Ebi-3 early after the onset of the inflammatory reaction, in the absence of the IL-12p35 subunit, may suggest two scenarios. First, IL-12 may directly suppress IL-27 production. Second, the Ebi-3 chain (which couples with IL-12p35 to give rise to IL-35) is more available to react with p28, thus explaining an increase in IL-27. Our results demonstrating that the administration of IL-12 fails to inhibit IL-27 (data not shown) and to restore disease support the latter explanation. However, our findings also show that IL-27 is only upregulated following immunization, demonstrating that this cascade of events takes place following activation. It has been shown that the TLR4 agonist LPS upregulates the transcription of p28 and Ebi-3 in a MyD88-dependent fashion (50, 51). Therefore, we cannot exclude that the increase in the expression of p28 and Ebi-3 genes is due to the effect of mycobacteria, a component of CFA.

Previous results have demonstrated that IL-35 mediates the suppressive function of regulatory CD4+CD25+Foxp3+ Tregs (22, 24). However, whereas Foxp3+ Tregs isolated from IL-12p35−/− mice are unable to suppress Teff responses in vitro following anti-CD3 stimulation (Supplemental Fig. 1C and Ref. 24), they have retained the capacity to suppress in response to autotaxigen stimulation. These results suggest that IL-35 produced by Tregs may downregulate nonantigen-specific responses, but that during autoreactive response this is left to other components of the “immunoregulatory machinery.” In support of this hypothesis, our studies have shown that despite being unable to produce IL-35, Tregs accumulate in the synovia of IL-12p35−/− mice where they are likely to contribute to the control of inflammatory arthritis. Additionally, these Tregs express higher levels of Foxp3, previously shown to be “proportional” to the strength of suppression (21). Furthermore, IL-12p35−/− CD25+CD4+ Tregs obtained from the draining LN secreted higher levels of TGF-β than did WT Tregs. Previous work has shown that IL-12p35−/− mice are resistant to developing Ab-induced arthritis and that the mechanism for this was attributed to an increase of TGF-β levels in the joint.
as compared with WT mice (14). Collectively, we suggest that IL-10 and TGF-β not only compensate for the lack of IL-35, but they are more (or equally) effective in controlling acute inflammatory arthritis. It would be very interesting to investigate the in vivo effects of adding rIL-35 to the IL-12p35−/− mice once this agent is easily and cheaply available.

In addition to affecting Treg homeostasis and function, the lack of IL-12p35 also altered the numerical balance of different B cell subsets involved in the maintenance of tolerance versus pathogenicity. The lack of the IL-12p35 subunit increased the number of T2-MZP Bregs, previously shown to be the major subset of B cells producing IL-10 in the context of CIA and the subset that efficiently suppresses arthritis and other arthritic disorders (20, 26, 27). Interestingly, the number of pathogenic MZB cells was reduced. MZB cells are implicated in autoreactive B cells responses (52, 53), and autoreactive MZB cells are considered as a pathogenic hallmark in various models for arthritis (29, 54). Therefore, the reduction of autoreactive MZB cells in favor of T2-MZP Bregs is likely to contribute to the generation of a tolerogenic environment. Our findings in this study also demonstrate that both IL-12p35 and Ebi-3 are expressed at high levels in B cells; however, our results suggest that IL-35 is not essential for the suppressive function of T2-MZP Bregs in arthritis, unlike colitis (25). Furthermore, Vignali and colleagues (24) demonstrated that both IL-12p35 and Ebi-3 are expressed at high levels in B cells; however, our results suggest that IL-35 is not necessary for Breg suppressive function. We have shown that IL-12p35−/− animals have a complex phenotype where neither the lack of IL-12 nor the lack of IL-35 seems to be as important as the increase in both the levels and dominance of IL-27 following induction of CIA. Our data, although at odds with some of the current literature describing
a redundant function for the IL-12 p35 chain in inflammation, have highlighted an important function of IL-27 and show that even in the presence of an intact IL-23 pathway IL-27 has an overpowering anti-inflammatory effect. The long-term ability of patients treated with these drugs, for example with ustekinumab, an anti–IL-12/IL-23p40 mAb currently used as a treatment for psoriasis with some success (55), to combat infection, cancer, and allergy remain to be seen. This poses a note of warning for therapy aimed at the neutralization of shared cytokine chains, as it may result in unpredictable and wide-reaching immunological effects, including skewing the immune response and modulation of Treg and Breg numbers.

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Disclosures

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