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Compromised Humoral and Delayed-Type Hypersensitivity Responses in IL-23-Deficient Mice¹

Nico Ghilardi,^{2*} Noelyn Kljavin,* Qi Chen,[†] Sophie Lucas,* Austin L. Gurney,*[‡] and Frederic J. de Sauvage*

The heterodimeric cytokine IL-23 consists of a private cytokine-like p19 subunit and a cytokine receptor-like subunit, p40, which is shared with IL-12. Previously reported IL-12p40-deficient mice have profound immune defects resulting from combined deficiency in both IL-12 and IL-23. To address the effects of specific IL-23 deficiency, we generated mice lacking p19 by gene targeting. These mice display no overt abnormalities but mount severely compromised T-dependent humoral immune responses. IL-23p19^{-/-} mice produce strongly reduced levels of Ag-specific Igs of all isotypes, but mount normal T-independent B cell responses. In addition, delayed type hypersensitivity responses are strongly impaired in the absence of IL-23, indicating a defect at the level of memory T cells. T cells stimulated with IL-23-deficient APCs secrete significantly reduced amounts of the proinflammatory cytokine IL-17, and IL-23-deficient mice phenotypically resemble IL-17-deficient animals. Thus, IL-23 plays a critical role in T cell-dependent immune responses, and our data provide further support for the existence of an IL-23/IL-17 axis of communication between the adaptive and innate parts of the immune system. *The Journal of Immunology*, 2004, 172: 2827–2833.

Interleukin-23 is a novel member of an emerging heterodimeric cytokine family (1), which also includes IL-12 (2), IL-27 (3), and cardiotrophin-like cytokine/cytokine-like factor 1 (4). It consists of a helical cytokine subunit, p19, and a soluble receptor subunit, p40. The p40 subunit is promiscuous and can associate with another helical cytokine subunit, p35, to form IL-12. The IL-23R has recently been described and consists of the IL-12Rβ1 (IL-12Rβ1) and the proprietary IL-23R chain (5). In parallel, IL-12 uses IL-12Rβ1 and the p35-specific IL-12Rβ2 chain (6) for signaling. In mice, IL-12p40 is also found as a homodimer (p40₂), which antagonizes IL-12 and likely also IL-23 through nonproductive binding of IL-12Rβ1 (2, 7).

Many of the biological effects of IL-12 have been deduced either from the phenotype of mice that lack p40 or from experiments with p40-specific inhibitory Abs. For example, it was shown that IL-12p40^{-/-} mice have reduced NK cell responses, reduced delayed-type hypersensitivity (DTH)³ responses, and deficient Th-type 1 (Th1) development (8). Through the discovery of IL-23, however, it became apparent that these mice suffer from the combined loss of IL-23 and IL-12. Indeed, IL-12p35^{-/-} mice, which lack only IL-12, but not IL-23, and are less well characterized, display only some, but not all, phenotypes of IL-12p40^{-/-} mice (9, 10). There is growing evidence that loss of p40 results in a more profound

phenotype than loss of p35 in animal models of infectious (11–13) and autoimmune (14, 15) disease. Very recently, it was shown that IL-23p19^{-/-} mice are completely protected against experimental autoimmune encephalitis (EAE) (16); this finding was in accordance with a previous report showing that IL-12 responsiveness is not required in the pathogenesis of this disease (17).

Like IL-12, IL-23 is known to be produced by activated myeloid APCs such as dendritic cells (DC) and macrophages (Mφ) (1, 18, 19). Its production is induced by Toll-like receptor (TLR) 2 agonists (19) and certain viruses (18). The major target population of IL-23 appears to be CD4⁺ T cells of the memory phenotype (1, 5, 20), although increasing evidence suggests that it also has potent stimulatory effects on APC (16, 21). Upon receptor engagement, IL-23 activates a pattern of intracellular signaling molecules that is similar to that activated by IL-12 and includes STAT-4 (1, 5). STAT-4 is essential for Th1 differentiation and was previously not known to be activated by any cytokine but IL-12 (22, 23). Because of its ability to activate STAT-4, as well as its ability to stimulate IFN-γ production by human T cells, IL-23 was initially recognized as similar to IL-12, with the important distinction that its actions appear to be restricted to a subset of CD4⁺ cells bearing markers characteristic for memory and/or regulatory cells (1, 20). More recently, however, we found that IL-23 is a potent inducer of IL-17, but not IFN-γ, in murine T cells (20). Conversely, neither IL-12 nor IL-4 induced IL-17 production by CD4⁺ cells, and thus it seems that IL-23 and IL-17 form a new axis through which the adaptive and the innate parts of the immune system communicate. Indeed, IL-12p40 deficiency, but not IL-12p35 deficiency, resulted in almost complete abrogation of IL-17 production in a *Klebsiella* infection model, supporting a role of IL-23 in inducing IL-17 in vivo (24). Furthermore, IL-17 was overproduced in a model of EAE where IL-12 responsiveness was abrogated through deletion of the IL-12Rβ2 chain (17), in agreement with our observation that IL-12 can suppress IL-23 signaling (20).

To further investigate the relationship between IL-23 and IL-17 in vivo, we compared the phenotype of IL-23-deficient mice to that of IL-17-deficient animals reported recently (25). IL-17-deficient mice display severely impaired DTH responses and strongly reduced humoral immune responses. We found that IL-23 deficiency

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³ Abbreviations used in this paper: DTH, delayed-type hypersensitivity; EAE, experimental autoimmune encephalitis; DC, dendritic cell; TLR, Toll-like receptor; TNP, trinitrophenyl; EGFP, enhanced green fluorescent protein; ES, embryonic stem; KLH, keyhole limpet hemocyanin; CD62L, CD62 ligand; mBSA, methylated BSA; LNC, lymph node cell; TI, T-independent; BLP, bacterial lipopeptide.

results in lowered production of IL-17 *in vivo* and recapitulates the phenotype of IL-17-deficient mice. Therefore, our data give further support to the existence of an IL-23/IL-17 axis *in vivo*.

Materials and Methods

Mice

All mice were housed under specific pathogen-free conditions. IL-12p40^{-/-} mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and C57BL/6 were obtained from Charles River Breeding Laboratories (San Diego, CA).

Reagents

Unless otherwise indicated, reagents were purchased from the following suppliers: Abs and ELISA reagents were obtained from BD Pharmingen (San Diego, CA), cytokines from R&D Systems (Minneapolis, MN), trinitrophenyl (TNP)-coupled Ags from Biosearch Technologies (Novato, CA), and tissue culture reagents from Invitrogen (Carlsbad, CA).

Generation of IL23p19-deficient mice

Genomic DNA encompassing the murine IL23p19 locus was isolated from clone 198a3 of a genomic BAC library by Genome Systems (Incyte Genomics, Palo Alto, CA). A targeting vector designed to replace the entire IL23p19 coding region with an enhanced green fluorescent protein (EGFP) reporter gene was constructed from the following DNA fragments using standard molecular cloning techniques: a thymidine kinase selection cassette; a 5' homology arm of 5403 bp defined by endogenous *SacII* and *BglII* sites on the distal and proximal ends, respectively; an EGFP expression cassette excised from pEGFP-1 (Clontech Laboratories, Palo Alto, CA) using *BamHI* (5' end) and *AflIII* (3' end); a phosphoglycerate kinase-neo resistance cassette; and a 1203-bp short arm defined by an endogenous *XhoI* site at the proximal end and the primer 5'-GCTTGGTGGCCAC CTATGAT-3' at the distal end (see Fig. 1A). This construct was electroporated into 129/SvEv embryonic stem (ES) cells (26), and homologous recombination occurred in 9 of 600 clones following selection with G418 and gancyclovir. To verify correct targeting of the locus, we analyzed genomic DNA from ES cells and animals by Southern blot. Digestion with *BamHI* followed by hybridization of membranes with probe 1 (a 831-bp genomic DNA fragment obtained by PCR with oligos 5'-AGACCCTCAAAGTTCATGAC-3' (sense) and 5'-CTGACGGCGCTTTCTC TACC-3' (antisense)) yielded a 7,027-bp fragment for the wild-type allele and an 11,788-bp fragment for the correctly targeted mutant allele. Similarly, digestion of genomic DNA with *EcoRI* followed by hybridization of membranes with probe 2 (a 390-bp genomic DNA fragment obtained by PCR with oligos 5'-TTTTGCCAGTGGGATACACC-3' (sense) and 5'-AACTGCTGGGGCTGTACAC-3' (antisense)) yielded a 9197-bp fragment for the wild-type allele and a 6211-bp fragment for the correctly targeted mutant allele. Two ES cell clones (1c5 and 3h6) were injected into blastocysts, and chimeric animals that transmitted the mutant allele in their germline were obtained. For routine genotyping, we used a PCR-based method with a common antisense primer (5'-GCCTGGGCTCACTTTT TCTG-3'), and wild type-specific (5'-GCGTGAAGGGCAAGGACACC 3') and knockout-specific (5'-AGGGGGAGGATTGGGAAGAC-3') sense primers. This primer-triplet amplifies a 210-bp fragment for the wild-type allele and a 289-bp fragment for the mutant allele. PCR was conducted in a Robocycler (Stratagene, La Jolla, CA), using the following conditions: 1 cycle of 94°C, 60 s; 35 cycles of 94°C, 30 s, 58°C, 30 s, 72°C, 60 s; 1 cycle of 72°C, 7 min.

FACS analysis of blood cell subsets

Spleens, thymi, and lymph nodes were isolated from 6- to 8-wk-old mice, and single cell suspensions were prepared by standard methods. Peripheral blood was obtained by cardiac puncture and treated with EDTA to prevent coagulation, and erythrocytes were lysed using ACK lysing buffer (BioSource International, Camarillo, CA). All cells were incubated for 30 min on ice in HBSS supplemented with 2% heat inactivated bovine calf serum. Cells were then stained in the same buffer with 1 μ g per million cells of various Abs coupled to PE, biotin, or CyChrome. Where biotinylated Abs were used, streptavidin-coupled PE-Texas Red conjugate (Caltag Laboratories, Burlingame, CA) was used for detection. After two washes with the same buffer, fluorescence was detected using an Epics-XL flow cytometry system (Beckman Coulter, Fullerton, CA).

Stimulation of allotypic T cells

CD4 and CD62 ligand (CD62L) double-positive T cells were isolated from the spleens of 6- to 8-wk-old BALB/c mice by a two-step isolation pro-

cedure. First, T cells were depleted of other cell types by a negative magnetic selection (Miltenyi Biotec, Auburn, CA). These cells were then labeled with Abs against CD4 and CD62L and sorted by FACS on a MoFlo sorter (DAKO Cytomation, Fort Collins, CO). DCs from wild-type or IL-23p19^{-/-} mice, both in the C57BL/6 background, were also isolated by a two-step protocol. CD11c-positive splenocytes were positive selected by magnetic separation (Miltenyi Biotec) before labeling with Abs against CD11c, MHC class II, and CD8. CD11c⁺/MHC-II⁺/CD8⁻ cells were then sorted by FACS, again using a MoFlo sorter. All populations used in the experiment were at least 98% pure. To elicit allostimulatory responses, increasing amounts of DCs were cocultured with 10⁵ T cells in a total of 200 μ l of IMDM supplemented with penicillin-streptomycin and 10% heat inactivated bovine calf serum (HyClone Laboratories, Logan, UT) in duplicates. In some cases, 100 ng/ml bacterial lipopeptides were added to stimulate cytokine production by DCs. After 5 days of incubation, 120 μ l of supernatant were removed for cytokine measurement by ELISA, and replaced with fresh medium containing 1 μ Ci of [³H]thymidine per well. Thymidine incorporation was determined 16 h later using a Top Count liquid scintillation counter according to the manufacturer's instructions (Packard Instrument, Meriden, CT).

In vivo T cell differentiation

Four male and four female mice per group were immunized in the left hind footpad with 75 μ g of keyhole limpet hemocyanin (KLH) (Sigma-Aldrich, St. Louis, MO) in 30 μ l of a 1:1 emulsion of CFA (BD Biosciences, San Diego, CA) and PBS. Draining inguinal and popliteal lymph nodes were harvested 5 days later and restimulated in IMDM supplemented with penicillin-streptomycin, 10% heat inactivated bovine calf serum (HyClone Laboratories), and 25 μ g/ml KLH. For proliferation assay, 5 \times 10⁵ cells were seeded in 200 μ l in triplicates in 96-well plates and allowed to proliferate for 112 h with addition of 1 μ Ci of [³H]thymidine per well during the last 18 h of the incubation period. Thymidine incorporation was determined using a Top Count liquid scintillation counter according to the manufacturer's instructions (Packard Instrument). For cytokine secretion, 2.5 \times 10⁶ cells were incubated in 1 ml in 48-well plates, and supernatants were harvested after 72 h. Cytokine secretion was determined by ELISA. The data presented is one representative of three total experiments.

DTH responses

Six mice per group were s.c. injected with 200 μ g of methylated BSA (mBSA) (Sigma-Aldrich) at three sites in the abdomen in a combined total of 200 μ l of a 1:1 emulsion of CFA (BD Biosciences, San Diego, CA) and PBS. On day 8 following immunization, the mice were challenged by injection of 20 μ l of 5 mg/ml mBSA in PBS into one rear footpad, while the other rear footpad received 20 μ l of PBS. Measurements of footpad swelling were taken at 18, 42, 66 h after challenge, using a series 7 spring-loaded caliper (Mitutoyo, City of Industry, CA). The magnitude of the DTH responses was determined from differences in footpad thickness between the Ag- and PBS-injected footpads.

T-dependent humoral responses and Ig analysis

For the measurement of total Ig levels, serum was obtained from eight male and eight female, 6- to 9-wk-old, unimmunized mice of either genotype. Total Ig isotype levels were measured by Luminex bead assay (Upstate Biotechnology, Lake Placid, NY). To assess the OVA-specific humoral immune response, groups of seven mice per genotype (four males and three females) were immunized with OVA in CFA on day 0 and received booster immunizations of the same Ag in IFA (Sigma-Aldrich) on days 21 and 42. For serum analysis, blood was obtained by retro-orbital bleeding before immunization and on days 14, 28, and 49 after immunization. OVA-specific Ig isotypes were detected by ELISA, using OVA as a capture agent and isotype-specific secondary Abs for detection. To be in the linear range of the ELISA, serum samples were diluted as follows: 1/3,125,000 for IgG1, 1/25,000 for IgG2a, 1/625,000 for IgG2b, and 1/1,000 for IgG3, IgM, IgA, and IgE. A dilution series of a serum obtained from an OVA-immunized mouse from a previous experiment was used as a standard, because purified, OVA-specific isotypes are not commercially available. Results are expressed as arbitrary units, where the average of the wild-type group in the last bleed was set as 100. To assess the contribution of memory T cells to the humoral response, groups of five to six mice of either genotype were immunized with OVA in CFA on day 0 and received a booster immunization of TNP₁₁-OVA in IFA on day 21. For serum analysis, blood was obtained by retro-orbital bleeding before immunization and on days 14 and 28 after immunization. TNP-specific Ig isotypes were detected by ELISA, using TNP₂₈-BSA as a capture agent and isotype-specific secondary Abs for detection. For TNP-specific IgG1, a commercially available standard was used. For TNP-specific IgG2a, a dilution series of a

serum obtained from a TNP-immunized mouse from a previous experiment was used, and results were calculated as described above. The sample dilutions were 1/31,250 for IgG1 and 1/1,250 for IgG2a.

T-independent (TI) humoral responses

Groups of six mice per genotype were immunized i.p. with 50 μ g of TNP₁-LPS or 100 μ g of TNP₂₀-aminoethylcarboxymethyl-Ficoll in PBS. Serum was harvested 10 days later, and TNP-specific IgM was analyzed by ELISA, using TNP₂₈-BSA as a capture agent and an IgM-specific secondary Ab for detection. A TNP-specific IgM Ab was used as a standard for the ELISA. The sample dilutions were 1/1280 for Ficoll and 1/5120 for LPS.

Results

Deletion of the IL-23p19 gene

To determine the nonredundant *in vivo* effects of IL-23, we generated mice that are deficient in IL-23 but competent to produce IL-12. We constructed a targeting vector in which the entire coding region of p19, consisting of four exons, is replaced by an EGFP reporter gene, and a neomycin resistance cassette (Fig. 1). Germ-line transmission was obtained from two correctly targeted ES cell clones, 1c5 and 3h6, and the mutation was backcrossed into the C57BL/6 background using speed congenics with three markers per chromosome (27, 28). Based on this analysis, we selected only mice in which the genetic contamination from the 129 background was <5% for experiments. The pattern of EGFP expression was comparable to that of endogenous p19 mRNA (1) (data not shown).

IL-23p19^{-/-} mice have no overt phenotype

As expected from the phenotype of IL-23/IL-12 double-deficient IL-12p40^{-/-} mice, IL-23p19^{-/-} animals did not display any overt

phenotype and were born at Mendelian frequencies. No abnormalities in organs were found upon histopathological examination, and further analysis of clinical chemistry and hematology parameters did not reveal differences between wild-type and knockout animals. Furthermore, IL-23p19^{-/-} mice were normal in size and weight, and both sexes were fully fertile. Flow cytometric analysis of thymocytes, splenocytes, and peripheral blood leukocytes with various cell surface markers did not indicate any major differences between wild-type and IL23p19^{-/-} animals (Table I). Because IL-23 is known to act on memory T cells, we determined the ratio of memory (CD44^{high}CD62L⁻) vs naive (CD62L⁺) cells of each subset, but did not find a difference between wild-type and IL-23p19^{-/-} mice. In the entire analysis, the only noticeable difference between the two genotypes consists in a slight skewing of the DC subpopulations toward a CD8⁺ phenotype. Although the effect was minor, it reached statistical significance due to the tightness of the data, and could be compatible with recent observations that IL-23 has effects on APCs (16, 21). In summary, IL-23 does not appear to be required for normal development, and the introduction of an EGFP cassette does not have a toxic effect on any cell type tested.

Humoral immune responses in IL-23p19^{-/-} mice

To determine the role of IL-23 in the generation of a humoral immune response, we first measured total Ig levels of all isotypes in serum of 16 mice of either genotype. We did not find any statistically significant difference between wild-type and IL-23p19^{-/-} mice (Fig. 2), indicating that IL-23 is not critically required for the maintenance of normal Ig levels. Next, we tested

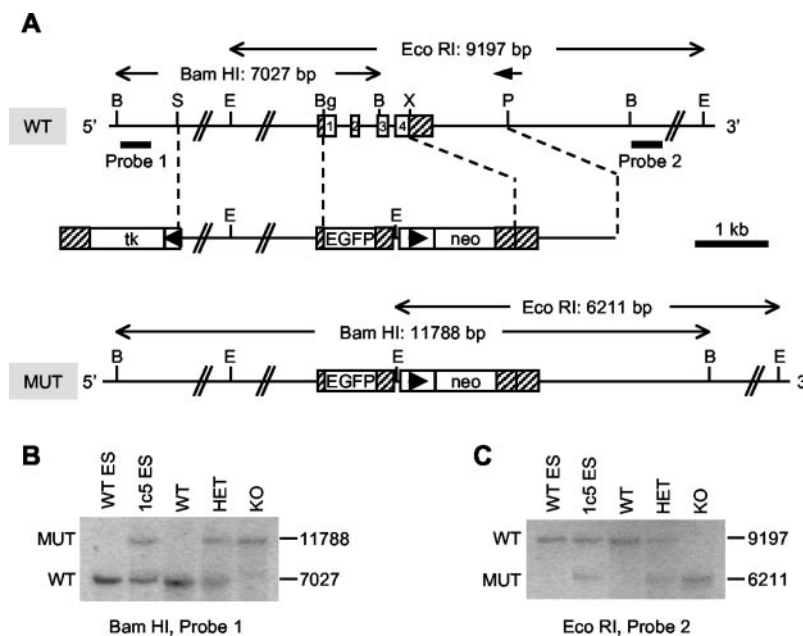


FIGURE 1. Targeting of the IL-23p19 locus. *A*, The native IL-23p19 locus (*top*), the targeting construct (*middle*), and the correctly targeted locus (*bottom*) are depicted to scale unless otherwise indicated by double slashes. Open boxes indicate coding exons, and hatched boxes represent exons encoding 5' and 3' untranslated regions of the resulting mRNA. The four coding exons of the p19 gene are numbered. Boxes with arrows indicate the promoter regions for neomycin (*neo*) and thymidine kinase (*tk*) selection cassettes, and an open box labeled EGFP indicates the location of an EGFP reporter gene. Restriction sites used for cloning and analysis of the arms are labeled as follows: B, *Bam*HI; S, *Sac*II; E, *Eco*RI; Bg, *Bgl*II; X, *Xho*I. The location of an antisense primer used to amplify the short arm is indicated by the letter P and an arrow. The size of restriction fragments resulting from digestion with *Bam*HI and *Eco*RI are indicated in the wild-type (WT) and the mutated (MUT) locus, and the locations of two probes used to detect these fragments by Southern blot are shown by thick lines. *B* and *C*, Southern blot analysis of *Bam*HI digests probed with probe 1, and *Eco*RI digests probed with probe II, respectively. DNA was extracted from wild-type (WT) embryonic stem (ES) cells, from ES clone 1c5, and from a wild-type, a heterozygous (HET), and a knockout (KO) mouse. The identity of the band is indicated at the left side of the blot, while its size is given on the right side.

Table I. Immune cell populations of IL-23p19^{-/-} mice^a

	Wild Type	Knockout	<i>p</i> (Diff.)
Thymus			
CD4 ⁺	5.7 ± 0.5	5.5 ± 0.0	0.504
CD8 ⁺	3.3 ± 0.1	3.1 ± 0.3	0.397
DN	25.0 ± 4.2	17.0 ± 8.0	0.202
DP	65.9 ± 3.7	74.3 ± 8.0	0.174
Spleen			
CD4 ⁺	24.3 ± 0.8	22.5 ± 2.7	0.342
% Naive	69.0 ± 1.3	67.5 ± 2.1	0.090
% Memory	29.1 ± 1.2	31.0 ± 1.9	0.029
CD8 ⁺	15.2 ± 1.2	12.3 ± 2.0	0.101
% Naive	64.1 ± 5.4	67.0 ± 2.8	0.199
% Memory	18.1 ± 1.8	18.3 ± 1.4	0.084
I-A(b)+/CD11c ⁺	2.0 ± 0.2	2.2 ± 0.2	0.041
%CD8 ⁺	12.8 ± 0.9	16.3 ± 1.7	0.000
%CD8 ⁻	87.2 ± 0.9	83.6 ± 1.8	0.000
CD19 ⁺	52.4 ± 2.0	55.2 ± 6.5	0.512
B220 ⁺	52.0 ± 2.0	55.5 ± 5.3	0.360
NK1.1 ⁺	3.2 ± 0.1	2.8 ± 0.1	0.055
Peripheral blood			
CD3 ⁺	47.9 ± 2.6	44.9 ± 3.6	0.053
CD4 ⁺	28.2 ± 2.3	26.9 ± 2.5	0.270
CD8 ⁺	16.5 ± 0.8	15.6 ± 1.7	0.150
CD19 ⁺	43.2 ± 3.2	45.2 ± 3.6	0.215
B220 ⁺	44.9 ± 3.5	46.4 ± 4.8	0.466
DX5 ⁺	9.9 ± 3.0	9.7 ± 5.0	0.929
CD16 ⁺	8.0 ± 0.9	8.6 ± 1.5	0.302
I-A(b) ⁺	44.0 ± 1.9	45.4 ± 4.9	0.428

^a Thymocytes, splenocytes, and peripheral blood leukocytes were harvested from three male and three female mice of either genotype. Average percentages and SD of cells staining positive for each marker are shown. A Student's *t*-test was used to calculate the probability (*p*) values of whether differences between the genotypes are statistically significant. DN, double negative; DP, double positive.

whether IL-23 is involved in the generation of a T-dependent humoral response against a protein Ag delivered in adjuvant. To this end, we immunized groups of seven mice each with OVA, and assessed OVA-specific Ig isotypes in preserum (all negative, data not shown), and after each of two consecutive immunizations (Fig. 3). After primary immunization, none of the groups differed from each other significantly for OVA-specific IgG1, IgG2b, IgG3, and IgE. However, we observed significantly reduced levels of OVA-specific IgG2a and IgA in IL-23p19^{-/-} and IL-12p40^{-/-} animals after primary immunization. As expected, the levels of all isotypes were increased dramatically after the second immunization. At this point, both IL-23p19^{-/-} and IL-12p40^{-/-} mice displayed marked reduction of all isotypes tested. The difference between these two genotypes was generally not significant, indicating that endogenous IL-12 does not play a major role in the humoral response in the absence of IL-23.

Because humoral immune responses depend on the proper function of both B and T cells, we next sought to determine by what mechanism IL-23 exerts its stimulatory effects. To test whether B cell function is directly affected by the lack of IL-23, we tested the ability of IL-23-deficient mice to mount B cell responses against TI Ags. The TI-1 Ag TNP-LPS leads to B cell activation via CD14 and TLR4, while the TI-2 Ag TNP-Ficoll activates B cells through clustering of surface B cell receptors. IL-23p19^{-/-} mice mounted normal B cell responses to both types of Ags (Fig. 4), indicating that IL-23 does not play a role in TI B cell responses. Furthermore, B cells from IL-23p19^{-/-} mice proliferated normally in vitro in response to LPS, anti-IgM, and anti-CD40 and underwent normal isotype switching in response to IL-4 (not shown). IL-23 stimulation of B cells did not lead to increased proliferation or isotype

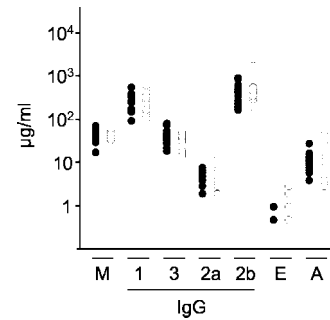


FIGURE 2. Total serum Ig levels, IL-23p19^{-/-} mice. Serum levels of Ig isotypes were determined by isotype-specific ELISA from groups of 16 wild-type (●) and IL-23p19^{-/-} (○) mice. Ig isotypes are indicated at the bottom of the graph.

switching (not shown), and thus we conclude that IL-23 does not directly affect B cell function.

Because the humoral immune response was mainly compromised at the stage of the secondary immunization, and because B cell function appeared normal in IL-23p19^{-/-} mice, we hypothesized that inefficient reactivation of Ag-specific Th cells might cause the phenotype. To address this question more directly, we immunized groups of five to six mice with OVA on day 0, followed by a secondary immunization with TNP-conjugated OVA on day 14. By using this immunization regimen, memory T cells specific for OVA are reactivated by the secondary immunization, but a novel set of B cells with specificity for TNP is activated at the secondary time point only. Therefore, the OVA-specific memory B cell subset does not contribute to the formation of TNP-specific Igs. Seven days after the booster, we tested for TNP-specific IgG1 and IgG2a in the serum, and found both isotypes to be significantly reduced in IL-23p19^{-/-} mice (Fig. 5). This result is compatible with a defect in memory T cell activation in IL-23p19^{-/-} mice (1, 20) and further underlines the importance of IL-23 in T-dependent B cell responses.

DTH responses in IL-23p19^{-/-} mice

To further investigate the function of memory CD4⁺ cells in IL-23p19^{-/-} mice, we evaluated the ability of these animals to mount DTH responses. DTH responses are strongly T cell dependent and were reported to be defective in IL-12p40^{-/-} mice (8), but appear to be normal in mice lacking IL-12p35 (11), suggesting that they might be mediated by IL-23. To address this question, we sensitized groups of six wild-type, IL-23p19^{-/-}, and IL-12p40^{-/-} animals each with mBSA in CFA and elicited DTH responses 7 days later by injection of mBSA into footpads. To control for nonspecific swelling, we also challenged a group of wild-type mice that had not been sensitized. Specific footpad swelling was measured 18, 42, and 66 h after the challenge and found to be inhibited to a similar degree in both IL-12p40^{-/-} and IL-23p19^{-/-} mice compared with wild-type mice (Fig. 6). The kinetics was also similar, with both IL-12p40^{-/-} and IL-23p19^{-/-} mice showing strongly reduced swelling at the 42 and 66 but not at the 18 h time point. Therefore, IL-23 is a principal mediator of DTH responses, and lack of IL-23 leads to inefficient responses by memory CD4⁺ T cells.

Capacity of IL-23p19^{-/-} DCs to stimulate T cells

To rule out the possibility that the defects observed in IL-23p19^{-/-} mice are due to inefficient T cell priming by IL-23-deficient APCs, we next investigated the potential of IL-23p19^{-/-} DC to stimulate allotypic naive CD4⁺ T cells isolated from the

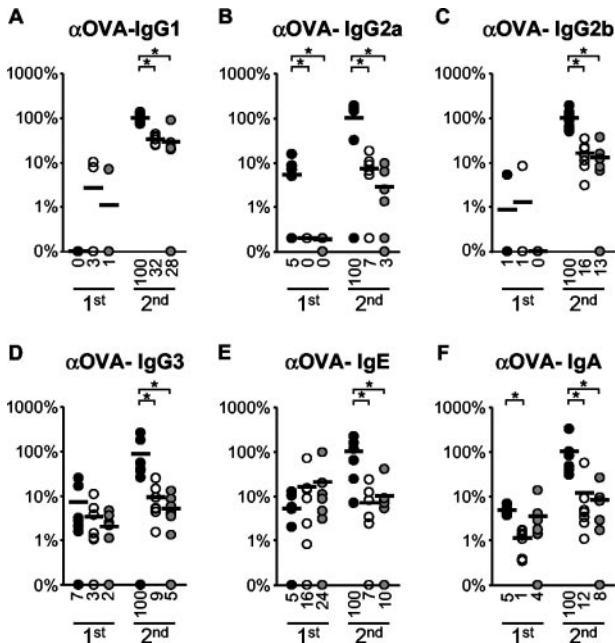


FIGURE 3. Humoral immune response in IL-23p19^{-/-} mice. *A–F*, OVA-specific levels of IgG1 (*A*), IgG2a (*B*), IgG2b (*C*), IgG3 (*D*), IgE (*E*), and IgA (*F*) after one (1st) and two (2nd) immunizations with OVA. ●, Wild-type mice; ○, IL-23p19^{-/-} mice; ◐, IL-12p40^{-/-} mice. Arbitrary units were calculated as described in *Materials and Methods*. The average of each group is indicated by both a black horizontal bar and a numeric value at the bottom of the graph. *, Statistically significant *p* values of <0.05.

spleens of *BALB/c* mice. In the absence of DC, these T cells did not proliferate nor secrete appreciable amounts of cytokines (Fig. 7*A*). Addition of increasing amounts of DC of either genotype resulted in dose-dependent proliferation. Because we have shown previously that IL-23 is a potent inducer of IL-17 (20), we next induced IL-23 production by DC using bacterial lipopeptides (29), a potent TLR-2 agonist and inducer of IL-23 production (19). Wild-type DC potently induced IL-17 production by the T cells in the presence of bacterial lipopeptides, whereas T cells stimulated with IL-23p19^{-/-} DC produced significantly less IL-17 under these conditions (Fig. 7*A*, middle panel). This difference is not due to a general defect of IL-23p19^{-/-} DC in promoting cytokine secretion, because similar amounts of IL-2 were induced by DC of either genotype (Fig. 7*A*, bottom panel). Addition of bacterial lipopeptide (BLP) also did not have an effect on proliferation (not shown). To confirm these observations in a more physiological setting, we next elicited T cell responses in vivo by immunizing

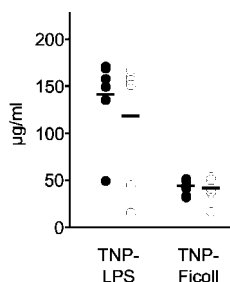


FIGURE 4. T1B cell responses are normal in IL-23p19^{-/-} mice. Serum levels of TNP-specific IgM were determined by ELISA from mice immunized with TNP-LPS (type I, left) or TNP-Ficoll (type II, right). ●, Wild-type mice; ○, IL-23p19^{-/-} mice.

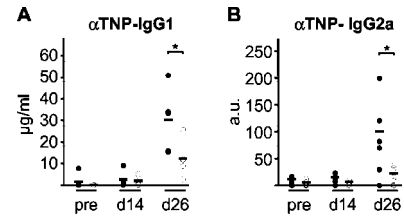


FIGURE 5. Memory T cell function. Wild-type (●) and IL-23p19^{-/-} mice (○) were immunized on day 0 with OVA and challenged on day 21 with TNP-OVA. Serum was harvested on days 0, 14, and 26 and tested by ELISA for the presence of TNP-specific IgG1 (*A*) and IgG2a (*B*). For IgG1, a commercially available standard was used. For IgG2a, arbitrary units were calculated as described in *Materials and Methods*.

groups of eight mice with KLH in CFA. Draining lymph node cells (LNC) were harvested 5 days later and restimulated with KLH in vitro. Again, we observed that LNC harvested from IL-23p19^{-/-} mice produced significantly less IL-17 (Fig. 7*B*, bottom panel). LNC proliferation was comparable in both genotypes (Fig. 7*B*, top panel), indicating that both wild-type and IL-23p19^{-/-} mice mounted robust T cell responses against the Ag. Thus, IL-23 deficiency does not grossly impair the stimulatory potential of DCs, but results in attenuated IL-17 production by T cells.

Discussion

Using IL-23p19-deficient mice, we assessed the nonredundant in vivo functions of IL-23, and found that IL-23 deficiency results in compromised T cell-dependent immune responses, such as humoral immune responses and DTH reactions.

We observed profoundly reduced humoral immune responses in IL-23p19^{-/-} mice, affecting all Ig isotypes. In parallel, responses of IL-12p40^{-/-} mice were inhibited to a similar or slightly higher degree. To our knowledge, this phenotype of IL-12p40^{-/-} mice has not previously been characterized, even though it was shown that these mice display reduced humoral responses against *Plasmodium chabaudi* (30). The differences in OVA-specific Ig levels between IL-23p19^{-/-} mice and IL-12p40^{-/-} mice did not reach statistical significance, indicating that endogenous IL-12 is unable to compensate for the lack of IL-23. This is somewhat surprising in light of the well characterized, potent adjuvant effects that IL-12 exerts when administered in pharmacological doses (31–33). Our results support the conclusion that IL-23 is absolutely required for an efficient humoral response, while it remains to be determined, through the use of IL-12p35^{-/-} mice, whether IL-23 is sufficient for normal humoral responses in the absence of IL-12.

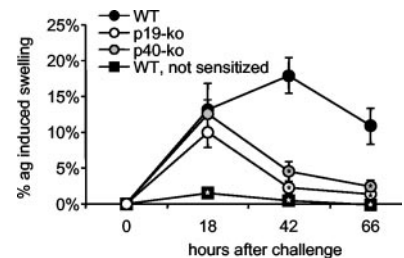


FIGURE 6. DTH reactions. Ag-specific swelling is calculated as percent increase in footpad thickness over the value measured just before the challenge. The results were averaged over all six mice in each group, and error bars represent the SDs. A second wild-type group that was not sensitized is used as a control for swelling induced by the Ag alone. An asterisk inside a symbol indicates that the difference between the corresponding group and wild-type mice is statistically significant (*p* < 0.05). WT, wild type; p19-ko, IL-23p19^{-/-} mice; p40-ko, IL-12p40^{-/-} mice.

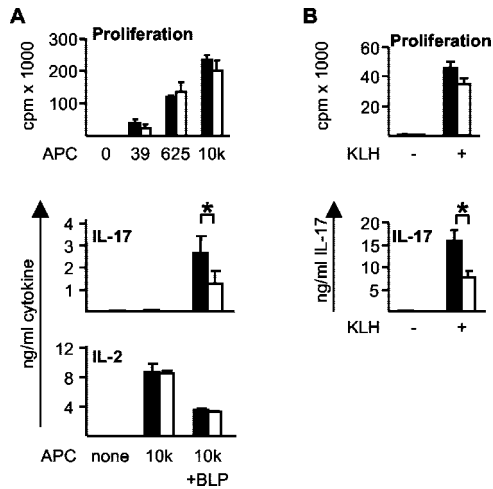


FIGURE 7. Normal T cell priming yet reduced induction of T cell IL-17 production by IL-23p19^{-/-} APCs. *A*, In vitro allostimulation experiment of BALB/c T cells in combination with wild-type (■) or IL-23p19^{-/-} (□) DCs. Naive CD4⁺ T cells and CD8⁻/CD11c⁺/MHC-II⁺ cells were isolated by FACS, and 10⁵ cells per well were incubated in the presence or absence of increasing numbers of APCs and/or BLP, as indicated. Proliferation and cytokine levels in the supernatants were determined after a 5-day incubation period. *B*, In vivo T cell response. LNC suspensions from wild-type (□) or IL-23p19^{-/-} mice (■) immunized with KLH were isolated and restimulated in vitro with 25 μg/ml KLH. Proliferation and IL-17 levels were measured after 5 days in culture.

Several lines of evidence suggest that the humoral immune response in IL23p19^{-/-} mice is compromised at the level of Ag-specific memory CD4⁺ T cells. First, humoral responses to both type I and type II TI Ags were normal in IL23p19^{-/-} mice. Second, we did not find any appreciable direct effects of IL-23 on B cells in a series of in vitro experiments. And third, we observed the most dramatic effects of IL-23p19 or IL-12p40 deficiency after a booster immunization but saw little to no difference after the primary immunization, providing evidence for reduced memory cell function in this experiment. Although we cannot rule out the possibility that memory B cell function is compromised by IL-23 deficiency, we present two independent indications that this phenotype is due to a defect at the memory T cell level: first, when we reimmunized OVA-primed mice with TNP-OVA, we observed a decrease in TNP-specific Igs in IL-23-deficient mice, suggesting that OVA-specific memory T cells are inefficient in supporting activation of a naive set of TNP-specific B cells in IL23p19^{-/-} mice. Second, we observed strongly attenuated DTH responses in an independent experiment. Because DTH responses to an exogenous protein Ag are mainly mediated by Ag-specific CD4⁺ memory T cells, this result further indicates that T cell responses are attenuated in IL-23-deficient animals.

Because IL-23 is a cytokine produced by APC, it remains possible that T cell priming is inefficient in its absence. This hypothesis is contradicted by several experimental observations: first, IL-23p19^{-/-} animals had normal levels of memory T cells. Second, the kinetics of the DTH reaction suggests that an Ag-dependent, normal swelling response takes place during the first 18 h even in the absence of IL-23, indicating efficient T cell priming. Third, the evidence presented in Fig. 7 suggests that IL-23p19^{-/-} APC are generally equivalent to wild-type APC in their ability to prime T cells. We did not notice any significant differences between the two genotypes in T cell proliferation or the stimulation of IL-2, IFN-γ, IL-4, IL-10, and GM-CSF production (Fig. 7 and data not shown). However, T cells stimulated by IL-23-deficient APC se-

creted lower amounts of IL-17. Last, Cua et al. (16) recently reported normal T cell proliferation and cytokine production when EAE was induced in IL-23-deficient mice. Together, these observations suggest that there is no defect at the priming stage of the T cell response, but rather that a downstream effect of T cell reactivation is compromised.

The defects we observed in IL-23p19^{-/-} mice are accompanied by reduced production of IL-17, and are remarkably similar to the defects observed in IL-17^{-/-} mice (25). IL-17 deficiency results in both compromised DTH responses as well as attenuated T-dependent humoral immune responses and thus mimics the effects of IL-23 deficiency described here. We observed significantly reduced production of IL-17 by in vivo activated T cells isolated from IL-23-deficient mice (Fig. 7*B*), or by T cells primed in vitro with IL-23-deficient APC in the presence of TLR-2 agonists (Fig. 7*A*), which can potentially induce IL-23 production (19, 34). Because IL-17 induces formation and recruitment of myeloid cells to the challenge site through induction of various inflammatory factors such as G-CSF, GM-CSF, IL-6, IL-8, and others (35), it appears likely that lower production of IL-17 is, at least in part, responsible for attenuated DTH responses in IL-23p19^{-/-} mice. The mechanism through which IL-17 deficiency results in compromised humoral responses has not yet been determined, but is likely to be indirect, because neither we (data not shown) nor others (25) have found any direct effects of IL-17 on B cells.

Despite a clear reduction in IL-17 production in IL-23p19^{-/-} animals, lower amounts of this cytokine are still produced. Thus, it is possible IL-23 has effects other than IL-17 induction in T cells, which may mediate its role in the induction of humoral immune and DTH responses. It has been shown that IL-23 can also induce GM-CSF (20) and TNF-α (our unpublished results) in CD4⁺ T cells, and that it can exert adjuvant effects through induction of IL-1β, TNF-α, IL-12, and IFN-γ in APC (16, 21). All of these additional cytokines can contribute to T-dependent immune responses in various ways (36–40). However, they can all be induced by factors other than IL-23 as well, and we have not found consistent and significant reduction of GM-CSF, TNF-α, and IFN-γ in IL-23p19^{-/-} mice in serum and tissue culture supernatants from the experiments described.

In summary, IL-23p19^{-/-} mice have attenuated in vivo T cell responses manifesting in reduced DTH and humoral immune responses, and phenotypically resemble IL-17-deficient mice. Our results indicate that clinical administration of IL-23 might be beneficial to support T cell function in immunization regimens and in immunocompromised patients.

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