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# IL-22 Is Expressed by Th17 Cells in an IL-23-Dependent Fashion, but Not Required for the Development of Autoimmune Encephalomyelitis<sup>1</sup>

Katharina Kreymborg,\* Ruth Etzensperger,<sup>2,3\*</sup> Laure Dumoutier,<sup>3,†</sup> Stefan Haak,\* Angelita Rebollo,<sup>§</sup> Thorsten Buch,\* Frank L. Heppner,<sup>||</sup> Jean-Christophe Renauld,<sup>4,†</sup> and Burkhard Becher<sup>4,5\*</sup>

Lately, IL-17-secreting Th cells have received an overwhelming amount of attention and are now widely held to be the major pathogenic population in autoimmune diseases. In particular, IL-22-secreting Th17 cells were shown to specifically mark the highly pathogenic population of self-reactive T cells in experimental autoimmune encephalomyelitis (EAE). As IL-17A itself was found to only play a minor role during the development of EAE, IL-22 is now postulated to contribute to the pathogenic function of Th17 cells. The goal of this study was to determine the role and function of IL-22 during the development of CNS autoimmunity in vivo. We found that CNS-invading encephalitogenic Th17 cells coexpress IL-22 and that IL-22 is specifically induced by IL-23 in autoimmune-pathogenic CD4<sup>+</sup> T cells in a time- and dose-dependent manner. We next generated IL-22<sup>-/-</sup> mice, which—in contrast to the prediction that expression of inflammatory cytokines by CNS-invading T cells inevitably confers pathogenic function—turned out to be fully susceptible to EAE. Taken together, we show that self-reactive Th cells coexpress IL-17 and IL-22, but that the latter also does not appear to be directly involved in autoimmune pathogenesis of the CNS. *The Journal of Immunology*, 2007, 179: 8098–8104.

**M**ultiple sclerosis (MS)<sup>6</sup> is the most common inflammatory disease of the CNS and its animal model experimental autoimmune encephalomyelitis (EAE) is mediated by the actions of autoreactive encephalitogenic Th cells. Although Th1 cells were long suspected to be the major pathogenic population, the discovery that IL-23, and not the Th1-induc-

ing cytokines IL-12 and IL-18, is vital for EAE development initiated a major paradigm shift with regards to the role of Th1 cells in inflammation (1–5). The impact of IL-23 on Th cells appears to be restricted to memory cells, which in response to IL-23R engagement secrete IL-17 (5). IL-17 expression by T cells correlates superbly with an autoimmune-pathogenic phenotype and this polarization pattern was coined Th17 (6, 7). Although studying IL-23 under various inflammatory conditions lead to the discovery of Th17 cells, it was later found that the cytokines TGF- $\beta$  and IL-6 are dominant in their capacity to polarize Th17 cells (8, 9). The role and function of IL-23 in maintaining this phenotype remains a subject of debate (5).

Th17 cells have received much attention lately and mice lacking IL-17A were found to be moderately resistant to EAE (10). However, in contrast to IL-17A<sup>-/-</sup> mice, IL-23-deficient mice are completely EAE resistant (1, 2). Thus, we reasoned that IL-17A is unlikely to be the only factor produced by Th17 cells involved in the inflammatory process. To identify the expression signature of IL-23-driven genes, we used high-density transcriptomics and identified IL-22 to be induced by IL-23 in autoimmune-pathogenic CD4<sup>+</sup> T cells in a time- and dose-dependent manner. IL-22 belongs to the IL-10 superfamily of cytokines and exhibits—unlike IL-10—potent proinflammatory properties. Its recently reported role in psoriasis (11–13) combined with our finding that IL-22 is specifically induced by IL-23 points toward a relevant function of IL-22 in autoimmune inflammatory diseases. Bettelli et al. (14) further reported that IL-22 marks a particularly pathogenic population of autoreactive T cells implicating IL-22 as a major pathogenic cytokine during CNS inflammation. In addition the IL-22 gene, together with IL-26 and IFN- $\gamma$  on the human chromosome 12q14, are considered a prominent susceptibility locus for MS (15). We found that following IL-23 stimulation, IL-22 is specifically secreted by pathogenic Th cells. To determine the actual role

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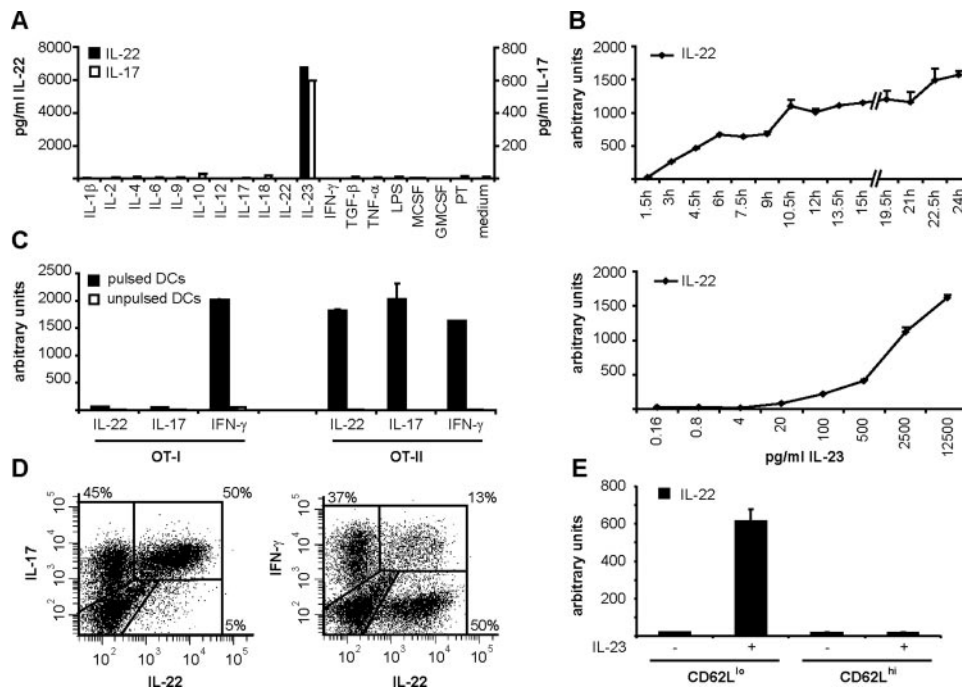
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<sup>4</sup> J.-C.R. and B.B. contributed equally to this work.

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<sup>6</sup> Abbreviations used in this paper: MS, multiple sclerosis; EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; Tg, transgenic; wt, wild type; LN, lymph node; BM, bone marrow; DC, dendritic cell.

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**FIGURE 1.** IL-22 expression is specifically induced by IL-23. **A**, A total of  $4 \times 10^6$  splenocytes were obtained from C57BL/6 mice, stimulated with 20 ng/ml of the indicated substances, and incubated for 24 h. The supernatant was collected and used for IL-22 and IL-17A protein detection by ELISA. **B**, A total of  $4 \times 10^6$  naive wt splenocytes were stimulated with 20 ng/ml IL-23 and collected at indicated time points or stimulated with indicated concentrations of IL-23 and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22 mRNA expression performed. **C**, A total of  $4 \times 10^6$  CD8<sup>+</sup> or CD4<sup>+</sup> T cells were purified from OT-I or OT-II splenocytes, respectively, and incubated with cognate peptide pulsed or unpulsed BM-derived DCs for 48 h and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22, IL-17, and IFN- $\gamma$  mRNA expression performed. All data shown are representative of at least three individual experiments and SD were calculated from duplicate wells. **D**, Splenocytes from a MOG-immunized C57BL/6 mouse were activated in vitro 7 days postinfection with 15  $\mu$ g/ml MOG peptide, 10 ng/ml IL-23, 20 ng/ml IL-6, 5 ng/ml human TGF- $\beta$ , IL-7, and IL-2 and 5  $\mu$ g/ml anti IFN- $\gamma$  Ab to generate Th17 cells. Intracellular cytokine staining was performed on day 5. The plot is gated on CD45<sup>+</sup>CD4<sup>+</sup> cells. Shown are the percentages only of polarized T cells. **E**, Splenocytes were obtained from C57BL/6 mice and MACS sorted for CD4<sup>+</sup>CD62L<sup>high</sup> and CD4<sup>+</sup>CD62L<sup>low</sup>. A total of  $4 \times 10^6$  cells were stimulated with 20 ng/ml IL-23 and harvested after 24 h. RNA was extracted, and quantitative RT-PCR for IL-22 mRNA expression performed. Data shown are representative of three experiments.

of this cytokine in autoimmune inflammation, we generated IL-22<sup>-/-</sup> mice, which were found to be surprisingly fully susceptible to EAE. We show that self-reactive Th cells coexpress IL-17 and IL-22, but that the latter does not appear to be directly involved in autoimmune pathogenesis of the CNS.

## Materials and Methods

### Peptides, Abs, and recombinant cytokines

Myelin oligodendrocyte glycoprotein (MOG)<sub>35–55</sub> peptide (MEVGWYRSPFSRVVHLYRNGK) and OVA<sub>323–339</sub> (ISQAVHAAHAEINEAGR) were obtained from Research Genetics. All recombinant cytokines were purchased from PeproTech and all Abs were purchased from BD Biosciences. The Ab to murine IL-22 was provided by Genentech and labeled with Alexa 488 (Invitrogen Life Technologies) according to the manufacturer's directions.

### Mice and induction of EAE

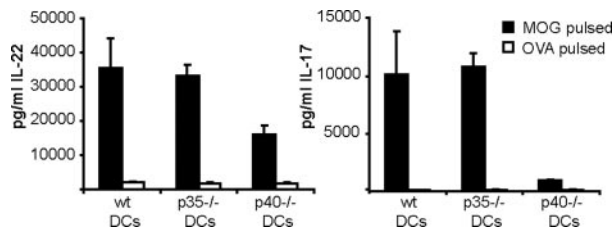
C57BL/6 mice, IL-12 p35<sup>-/-</sup>, and IL-12 p40<sup>-/-</sup> mice on a C57BL/6 background were purchased from The Jackson Laboratory and were bred under specific pathogen-free conditions. The 2D2 (MOG-TCR-transgenic (Tg)) mice were provided by V. Kuchroo (Harvard Medical School, Boston, MA). IL-22<sup>-/-</sup> mice were generated by targeting exons 1–3 and backcrossed onto C57BL/6 for more than eight times. The targeting vector was constructed to replace the exons 1a, 1b, 2, and a part of exon 3 of the IL-22a gene by a neomycin-resistant gene. A 5' arm of 1521 bp was amplified using a mutated sense primer with a XhoI site 5'-CTTCGGCTCGAGATGGCCAC-3' a mutated antisense primer containing also a XhoI site 5'-GCCCTCGAGACACCAGGGT-3' to allow the direct insertion into the pPNT vector. The 3' arm consisted of a 3559-bp KpnI fragment, containing the end of exon 3 and exon 4, and was cloned. For genotyping, the

targeted gene was amplified using a sense primer located upstream the 5' arm: 5'-CTGCTGTCCAACAGAGCTCT-3' and antisense primer on neomycin gene: 5'-CGCCTCCCTACCCCGGTAGA-3', resulting in a 1.7-kb amplified sequence. The wild-type (wt) gene was amplified using a sense primer located into the 5' arm 5'-AATCTATGAAGTTGGTGGGA-3' and an antisense primer located on exon 2 5'-ACTGACTCCTCGGAACAGTT-3', resulting in a 1.2-kb amplified sequence. Mouse IL-22 RT-PCR was performed as previously described (16). EAE was induced and scored as described (17).

### Histology and flow cytometry

Whole mouse brains or spinal columns were fixed in 4% paraformaldehyde in PBS, paraffin-embedded, cut and stained with H&E and Luxol-Nissl according to standard protocols. Immunohistochemical stainings on serial sections using Abs to neurofilament protein (NF, 200 kDa subunit; 1:20; Bio-Science), Iba1 (1:100; Wako Chemicals), CD3 (1:150; Labvision), and B220 (1:1000; BD Biosciences) were conducted on an automated Nexus staining apparatus (Ventana Medical Systems), following the manufacturer's guidelines.

CNS-infiltrating lymphocytes were isolated as described previously (4). For flow cytometry, Abs were incubated with cells for 20 min at 4°C and then cells were analyzed with a FACSCalibur (BD Pharmingen) and FACSDiva software. Postacquisition analysis was done with FACSDiva (BD Pharmingen) or FlowJo7 software (Tree Star). For intracellular cytokine staining, cells were restimulated with 50 ng/ml PMA, 500 ng/ml ionomycin, and GolgiPlug (BD Biosciences) for 5 h. Cells were first stained for surface Ags and then permeabilized with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's recommendations. Intracellular cytokine staining was performed using Abs to IFN- $\gamma$ , IL-17A, or IL-22 as described above.



**FIGURE 2.** IL-22 and IL-17 are produced by MOG-reactive T cells upon encounter with their cognate Ag. T cells from mice Tg for the 2D2 TCR were isolated and stimulated with MOG<sub>35-55</sub> or control peptide (5  $\mu$ g/ml) pulsed mature BM-derived DCs obtained from wt, p35<sup>-/-</sup>, or p40<sup>-/-</sup> mice for 2 days and IL-22 and IL-17A protein expression was measured by ELISA. Shown is a representative of three individual experiments ( $n = 4$ ).

#### Cell culture and in vitro assays

Mice were sacrificed using CO<sub>2</sub>, axillary and inguinal lymph nodes (LN) and spleens were collected and treated with 0.5 mg/ml DNase and 1 mg/ml Liberase (Roche) for 30 min at 37°C. Cells were cultured in RPMI 1640 supplemented with 10% FCS, 50 U/ml penicillin, and 50  $\mu$ g/ml streptomycin (Invitrogen Life Technologies) in the presence or absence of the factors indicated in the figure legends and harvested at indicated time points. Where indicated, T cells were purified from splenocytes by magnetic cell sorting with MACS Beads following the manufacturer's recommendation (Miltenyi Biotec).

Bone marrow (BM)-derived dendritic cells (DCs) were generated as described (4). To mature DCs, 10  $\mu$ g/ml LPS (Fluka) was added to the culture for 24 h. Mature DCs were pulsed with 5  $\mu$ g/ml peptide for 4 h, washed extensively, and incubated with splenocytes at a ratio of 1:4 and harvested after 48 h.

#### Cytokine analysis

ELISA for IL-17A (BD Pharmingen) and IL-22 (Antigenix) were performed according to the manufacturer's instructions. Proliferation of MOG-reactive cells were stimulated in triplicate for 48 h with either 50

$\mu$ g/ml MOG<sub>35-55</sub>, 5  $\mu$ g/ml Con A, or medium, and 0.5  $\mu$ Ci/ml [<sup>3</sup>H]thymidine was added after 24 h for assessment of proliferative responses. Thymidine incorporation was assessed with a Filtermate Collector (Applied Biosystems) and a scintillation and luminescence counter.

#### Real-time RT-PCR

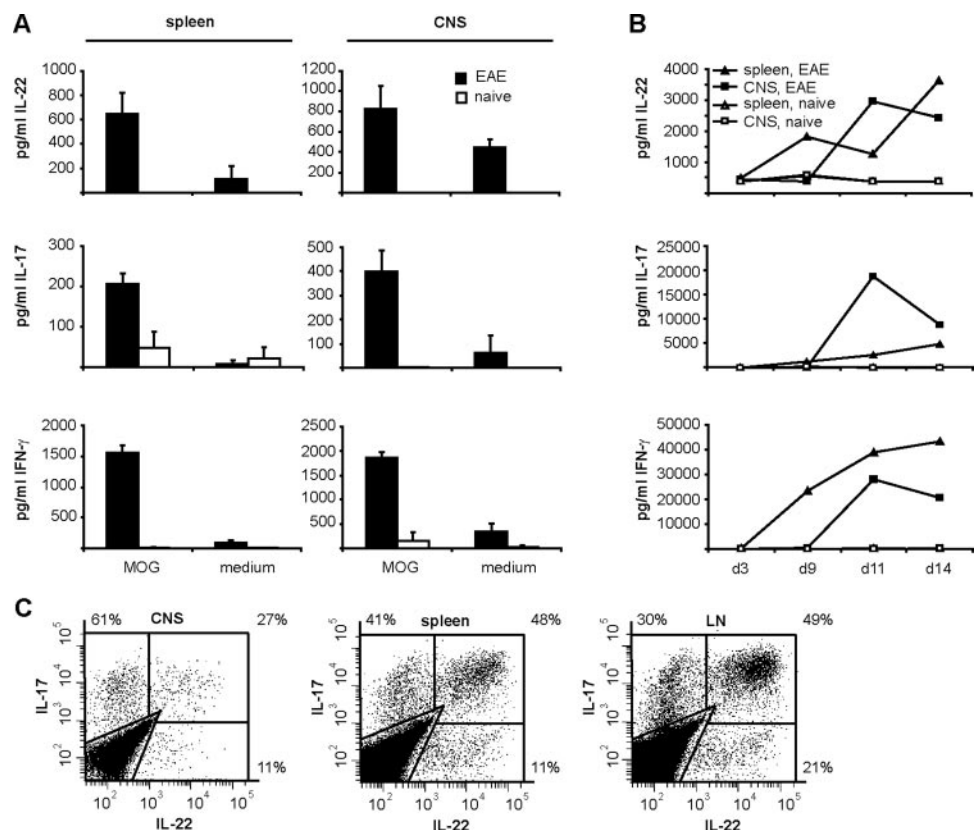
Cells or tissues were homogenized in 1 ml of TRIzol reagent (Invitrogen Life Technologies). Total RNA was extracted and reverse transcription was performed using random hexamer primer and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies). After PCR amplification using SYBR Green PCR master mix (Invitrogen Life Technologies), quantitative values of each sample were normalized to its  $\beta$ -actin content and converted to relative cDNA quantities by comparison to a standard curve generated with dilutions of  $\beta$ -actin plasmid. Primers were purchased from Operon Technologies. The primers used were: (5'-3')  $\beta$ -actin forward (fw): agagggaatctgtcgtgac,  $\beta$ -actin reverse (rev): caatagtgatgacctggcgt; IL-22 fw: ttgaggtgtccaactccagca, IL-22 rev: agccggagctctgtgttgta; IL-17 fw: atcaggacgcgcaacatga, IL-17 rev: ttggacacgctgacttga.

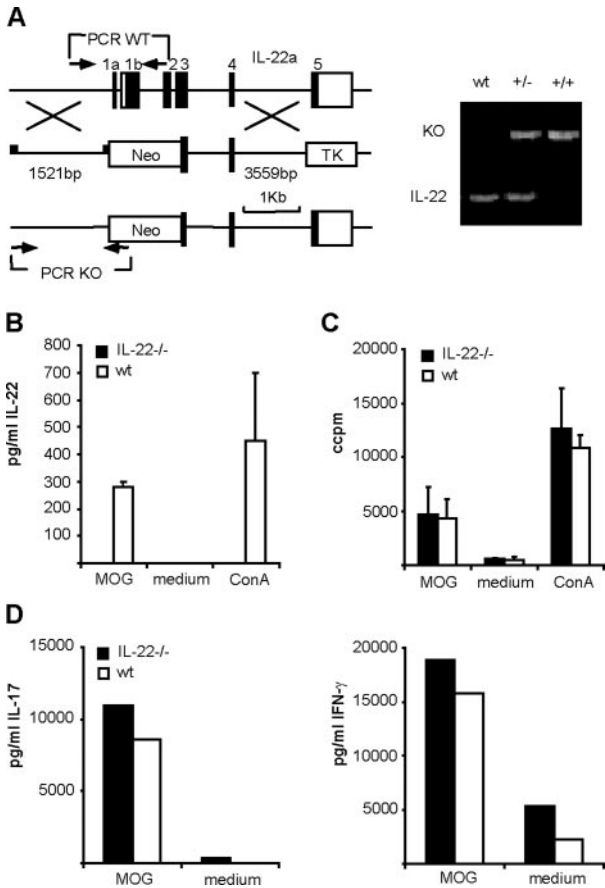
## Results

### IL-23 induces IL-22 gene expression

To elucidate the identity of IL-23-driven gene transcripts, we devised two reciprocal approaches for whole genome transcriptomics. We compared gene expression induced by IL-23 stimulation with those absent in Ag-driven IL-23-deficient (p40<sup>-/-</sup>) lymphocytes. In the first approach, genes up-regulated (>4-fold) by IL-23 were identified by stimulating splenocytes obtained from an unmanipulated mouse with recombinant IL-23 or IL-12 as a control. In the second approach, we immunized wt, IL-12p35<sup>-/-</sup>, and IL-12/23p40<sup>-/-</sup> mice with keyhole limpet hemocyanin and 7 days postinfection harvested lymphocytes and rechallenged them in vitro with keyhole limpet hemocyanin before harvesting the mRNA for microchip analysis (Affymetrix chip MOE430A). We used IL-12 as a control, first because IL-12-induced gene expression is well-characterized, and second to eliminate IL-12-induced

**FIGURE 3.** CNS-infiltrating Th17 cells express IL-22. Wild-type mice were immunized with MOG<sub>35-55</sub> emulsified in CFA and sacrificed either (A and C) at the peak of disease (day 21) or (B) at indicated time points after immunization. Lymphocytes were isolated out of CNS and spleen or LN of naive or MOG<sub>35-55</sub>-immunized wt mice, restimulated with 50  $\mu$ g/ml of their cognate Ag MOG<sub>35-55</sub>, and IL-22, IL-17A, and IFN- $\gamma$  expression was analyzed by (A) ELISA or (C) intracellular cytokine staining. A, A representative ELISA of four individual experiments ( $n = 2$ /group/experiment). B, Pre-onset disease was performed with  $n = 3$ . C, The percentages only of polarized T cells. Shown is a representative of three individual experiments,  $n = 3$ .





**FIGURE 4.** Generation and characterization of IL-22<sup>-/-</sup> mice. *A*, The structure of the *IL-22a* locus, the targeting vector, and the predicted homologous recombination are shown. The exons are shown as boxes, with white and black boxes for noncoding and coding regions, respectively. The size of the 5' and 3' arms, as well as the location of the primers used for genotyping are indicated. Neo, neomycin-resistance cassette; TK, thymidine kinase cassette. For genotyping, the wt and targeted alleles were amplified from F<sub>2</sub> tail genomic DNA as described in *Materials and Methods*. Lymphocytes were isolated out of spleen of MOG<sub>35-55</sub> immunized wt or IL-22<sup>-/-</sup> mice, restimulated with 50 μg/ml of their cognate Ag MOG<sub>35-55</sub>, or 5 μg/ml ConA. *B*, IL-22 expression was analyzed by ELISA. *C*, Proliferation was measured by thymidine uptake (shown is a representative of three individual experiments *n* = 3). *D*, IL-17A and IFN-γ expression was analyzed by ELISA. Data shown are representative of at least three individual experiments and SD were calculated from duplicate wells.

target genes from our analysis. By combining both data sets, we found IL-22 to be specifically and strongly induced by IL-23 (data not shown).

To verify that IL-22 expression is specifically induced by IL-23, we treated splenocytes derived from unmanipulated C57BL/6 mice with an array of different stimuli, harvested the mRNA and measured IL-22 and IL-17A protein expression by ELISA (Fig. 1A). Other than IL-23, none of the used substances elicited significant levels of IL-22 and IL-17 expression in splenocytes after 24 h of stimulation. Different concentrations of the different stimuli were used (data not shown). Our data show that a population of splenocytes present in naive pathogen-free C57BL/6 mice respond to IL-23R engagement with IL-22 and IL-17 expression. To further characterize the kinetics and dose dependence of IL-23-induced IL-22 production, we stimulated wt lymphocytes obtained from an untreated mouse with IL-23 for different periods of time or in the presence of different concentrations of IL-23 and observed that IL-22 expression is induced in a time- and dose-dependent manner

**Table I.** Distribution of cell types of the immune system in C57BL/6 and IL-22<sup>-/-</sup> mice: spleen<sup>a</sup>

| Spleen                                                       | Percentage |                      |
|--------------------------------------------------------------|------------|----------------------|
|                                                              | wt         | IL-22 <sup>-/-</sup> |
| Th cells (CD4 <sup>+</sup> )                                 | 18         | 16                   |
| CTLs (CD8 <sup>+</sup> )                                     | 11         | 10                   |
| Double-positive T cells (CD4 <sup>+</sup> CD8 <sup>+</sup> ) | 0.5        | 0.5                  |
| Regulatory T cells (FoxP3 <sup>+</sup> )                     | 0.5        | 0.4                  |
| NK cells (CD3 <sup>-</sup> NK1.1 <sup>+</sup> )              | 2          | 1.9                  |
| NKT cells (CD3 <sup>+</sup> NK1.1 <sup>+</sup> )             | 2.1        | 1.9                  |
| B cells (B220 <sup>+</sup> )                                 | 62         | 65                   |
| Macrophages (CD11b <sup>+</sup> )                            | 4          | 3                    |
| DCs (CD11c <sup>+</sup> )                                    | 3          | 3                    |
| Naive T cells (CD3 <sup>+</sup> CD62L <sup>+</sup> )         | 25         | 23                   |
| Memory T cells (CD3 <sup>+</sup> CD62L <sup>-</sup> )        | 5          | 6                    |

<sup>a</sup> Spleens were obtained from unmanipulated C57BL/6 and IL-22<sup>-/-</sup> mice; cells were isolated and stained with fluorochrome-conjugated Abs against the indicated cell-type-specific surface markers. Data shown are representative of three individual experiments.

(Fig. 1B). We observed a similar expression pattern with IL-17 (data not shown). To verify the notion that Th cells and not CTLs are the main source of IL-22, we stimulated purified CD4<sup>+</sup> as well as CD8<sup>+</sup> T cells obtained from OVA TCR-Tg mice (OT-II and OT-I, respectively) with cognate peptide pulsed DCs for 24 h and found that only TCR-Tg CD4<sup>+</sup> T cells made IL-22 (Fig. 1C). By intracellular cytokine staining of Th17 cells, we could show that >90% of IL-22-secreting cells also produce IL-17, while fewer IFN-γ-secreting cells coexpress IL-22 (20%) (Fig. 1D). To identify whether IL-23 stimulates the secretion of IL-22 by naive or memory T cells, we purified memory T cells (CD62L<sup>low</sup>) and naive T cells (CD62L<sup>high</sup>) followed by an overnight stimulation with IL-23. Our data confirm that IL-23 primarily drives the memory T cell pool and does not influence the naive pool in regards to cytokine secretion measured (Fig. 1E).

*IL-22 is expressed by encephalitogenic Th cells*

To determine the induction of IL-22 and IL-17A expression in a more physiologic manner in response to cognate Ag, we isolated T cells from MOG-reactive TCR-Tg (2D2) mice and stimulated them with MOG<sub>35-55</sub> or control peptide-pulsed mature BM-derived DCs obtained from wt, IL-12p35<sup>-/-</sup>, or IL-12/23p40<sup>-/-</sup> mice. IL-22 and IL-17A expression was subsequently measured by

**Table II.** Distribution of cell types of the immune system in C57BL/6 and IL-22<sup>-/-</sup> mice: lymph node<sup>a</sup>

| LN                                                           | Percentage |                      |
|--------------------------------------------------------------|------------|----------------------|
|                                                              | wt         | IL-22 <sup>-/-</sup> |
| Th cells (CD4 <sup>+</sup> )                                 | 37         | 37                   |
| CTLs (CD8 <sup>+</sup> )                                     | 27         | 27                   |
| Double-positive T cells (CD4 <sup>+</sup> CD8 <sup>+</sup> ) | 1.2        | 1.7                  |
| Regulatory T cells (FoxP3 <sup>+</sup> )                     | 0.3        | 0.2                  |
| NK cells (CD3 <sup>-</sup> NK1.1 <sup>+</sup> )              | 0.5        | 0.8                  |
| NKT cells (CD3 <sup>+</sup> NK1.1 <sup>+</sup> )             | 0.4        | 0.6                  |
| B cells (B220 <sup>+</sup> )                                 | 33         | 35                   |
| Macrophages (CD11b <sup>+</sup> )                            | 3          | 3                    |
| DCs (CD11c <sup>+</sup> )                                    | 1          | 1                    |
| Naive T cells (CD3 <sup>+</sup> CD62L <sup>+</sup> )         | 64         | 62                   |
| Memory T cells (CD3 <sup>+</sup> CD62L <sup>-</sup> )        | 9          | 9                    |

<sup>a</sup> LNs were obtained from unmanipulated C57BL/6 and IL-22<sup>-/-</sup> mice; cells were isolated and stained with fluorochrome-conjugated Abs against the indicated cell-type-specific surface markers. Data shown are representative of three individual experiments.

Table III. Distribution of cell types of the immune system in C57BL/6 and IL-22<sup>-/-</sup> mice: thymus<sup>a</sup>

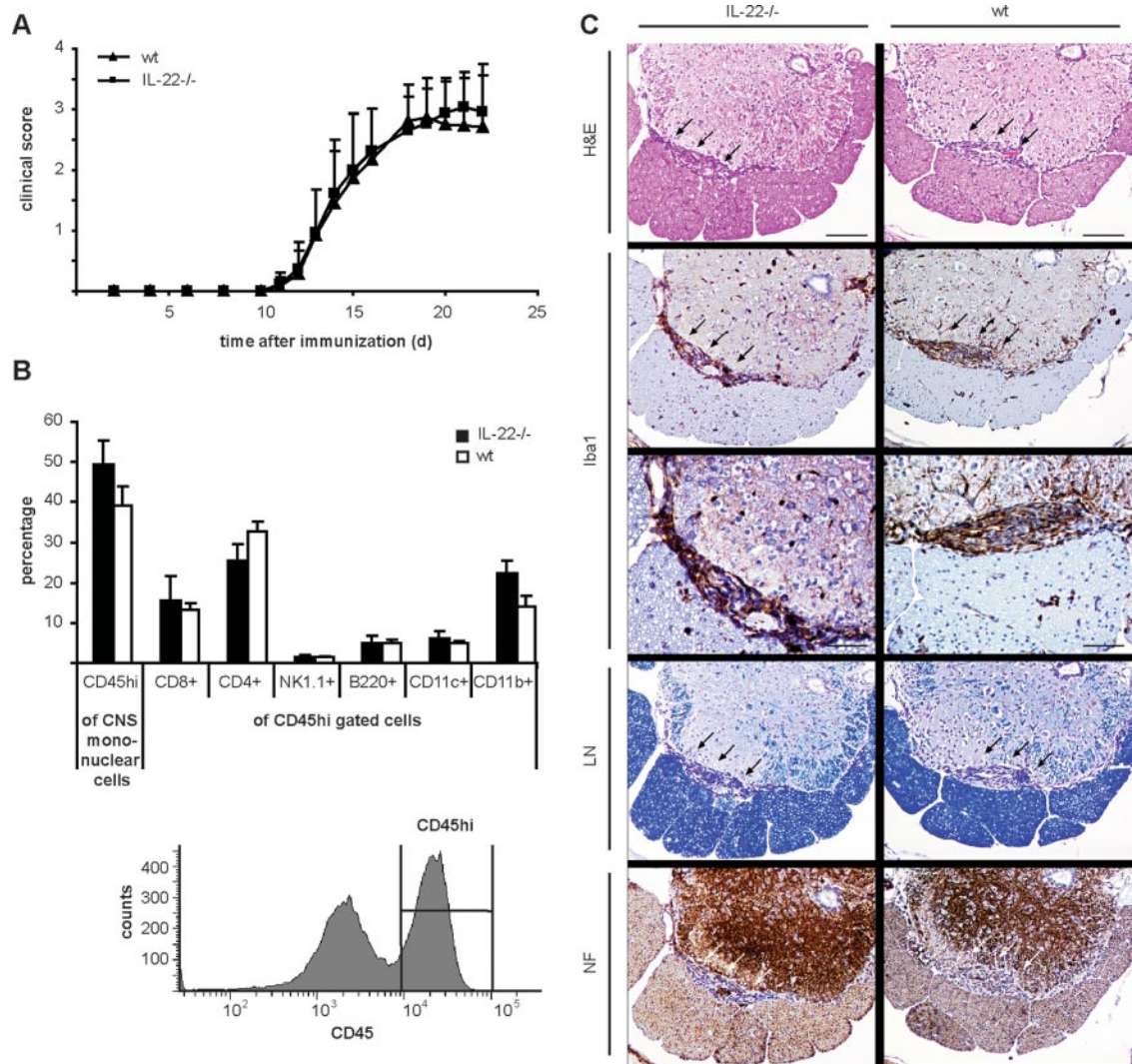
| Thymus                                                       | Percentage |                      |
|--------------------------------------------------------------|------------|----------------------|
|                                                              | wt         | IL-22 <sup>-/-</sup> |
| Th cells (CD4 <sup>+</sup> )                                 | 7          | 7                    |
| CTLs (CD8 <sup>+</sup> )                                     | 3          | 3                    |
| Double-positive T cells (CD4 <sup>+</sup> CD8 <sup>+</sup> ) | 87         | 83                   |

<sup>a</sup> Thymi were obtained from unmanipulated C57BL/6 and IL-22<sup>-/-</sup> mice; cells were isolated and stained with fluorochrome-conjugated Abs against the indicated cell-type-specific surface markers. Data shown are representative of three individual experiments.

ELISA. MOG-reactive T cells clearly expressed high levels of IL-22 and IL-17A after encounter with their cognate Ag (Fig. 2). This response is dependent on IL-23 as reduced levels of IL-22 and IL-17 were detectable when T cells were cocultured with DCs

obtained from IL-12/23p40<sup>-/-</sup> mice. We have also performed this restimulation experiment using polyclonal effector T cells isolated from MOG-immunized wt mice and confirmed that the expression of IL-22 and IL-17 is dependent on the secretion of IL-23 by APCs (data not shown).

To analyze the expression of IL-22 in mice with autoimmune inflammation, we induced EAE in wt C57BL/6 mice through immunization with MOG<sub>35-55</sub> emulsified in CFA and harvested brain and spinal cord as well as splenocytes at the peak of disease (day 21). Lymphocytes were isolated as described, challenged with MOG<sub>35-55</sub>, and analyzed for cytokine expression after 48 h by ELISA (Fig. 3A). As expected, we found IFN-γ and IL-17 to be expressed by splenocytes and CNS-invading lymphocytes. Most importantly, we detected a significant production of IL-22 by encephalitogenic, CNS-infiltrating lymphocytes after re-encounter with their cognate MOG Ag in vitro. Kinetic analysis of IL-22 secretion was performed by sacrificing mice at different time



**FIGURE 5.** IL-22<sup>-/-</sup> mice are fully susceptible to EAE. *A*, IL-22<sup>+/+</sup> and IL-22<sup>-/-</sup> mice were immunized with MOG<sub>35-55</sub> emulsified in CFA and the clinical score recorded as described. Two merged representative experiment ( $n \geq 12$ ) of five individual experiments are shown. Error bars represent the average deviation. Statistical significance was determined using an unpaired Student *t* test. *B*, Mice were sacrificed at the peak of disease (day 22), lymphocytes were isolated out of CNS, stained for the indicated surface markers and analyzed by flow cytometry. The histogram represents a staining for CD45 to distinguish CNS-resident microglia (CD45<sup>low</sup>) from CNS-invading leukocytes (CD45<sup>high</sup>). Shown is an average of five individually analyzed mice  $\pm$  average deviation. *C*, Spinal cord cross-sections of wt and IL-22<sup>-/-</sup> mice displayed similar inflammatory lesions (arrows), which often appeared to be accentuated around vessels. They consisted of many activated Iba1<sup>+</sup> macrophages/microglia (second and, at higher magnification, third row) and caused an impairment of myelinated and axonal structures, as depicted by a Luxol-Nissl (LN; fourth row) as well as a neurofilament (NF) stain (fifth row). Scale bars: 100  $\mu$ m for first, third, fourth, and fifth column; 100  $\mu$ m for second, fourth, and sixth column.

Table IV. *IL-22<sup>-/-</sup> are fully susceptible to EAE<sup>a</sup>*

| Genotype               | wt          | IL-22 <sup>-/-</sup> |
|------------------------|-------------|----------------------|
| Incidence              | 21 of 22    | 19 of 19             |
| Day of disease onset   | 13.3 ± 1.78 | 13.7 ± 2.22          |
| Maximum clinical score | 2.93 ± 0.44 | 3.17 ± 0.46          |

<sup>a</sup> IL-22<sup>+/+</sup> and IL-22<sup>-/-</sup> mice were immunized with MOG<sub>35-55</sub> emulsified in CFA and the clinical score was recorded as described. Three merged representative experiments (*n* ≥ 19) of five individual experiments are shown. Onset and maximum clinical score were calculated ± average deviation. Statistical significance was determined using an unpaired Student's *t* test.

points after immunization with MOG/CFA. Similar to IFN- $\gamma$  and IL-17, IL-22 expression by CNS-infiltrating lymphocytes increased with disease severity (Fig. 3*B*). To study which population of polarized Th cells secrete IL-22 in peripheral organs and the inflamed CNS, we immunized C57BL/6 mice with MOG/CFA and harvested spleen, LNs, and CNS at the peak of clinical EAE (average score of 3). The mononuclear cells were then restimulated with MOG<sub>35-55</sub> followed by intracellular cytokine staining. Cytofluorometric analysis revealed that in spleen and LN, there is a high overlap of IL-17- and IL-22-secreting T cells, while in the inflamed CNS, IL-17-secreting cells dominate over IL-22 and IL-17/22-secreting T cells (Fig. 3*C*).

#### Gene targeting of IL-22 does not prevent EAE development

Given the clear expression pattern of IL-22 associated with pathogenic Th17 cells, we sought to investigate whether IL-22 plays a role in inflammation of the CNS. To do so, we generated IL-22<sup>-/-</sup> mice by replacing the coding exons 1a, 1b, 2, and a part of exon 3 of the *IL-22* gene with a neomycin-resistant gene (Fig. 4*A*) and verified the absence of IL-22 by genomic PCR, RT-PCR, and ELISA (Fig. 4, *A* and *B*, and data not shown). The mice do not display any obvious malformation of the hemopoietic system and developed normally (Tables I–III). When we analyzed the proliferating capacity as well as the cytokine expression profile of IL-22<sup>-/-</sup> cells after re-encounter with MOG<sub>35-55</sub> by thymidine incorporation or ELISA, respectively, we observed that they behaved similar to wt cells (Fig. 4, *C* and *D*).

We induced EAE in the IL-22<sup>-/-</sup> mice through immunization with MOG/CFA and recorded the clinical disease development. Despite our expectations that IL-22 would display proinflammatory encephalitogenic properties, we observed that IL-22<sup>-/-</sup> mice developed disease similar to wt controls (Fig. 5*A* and Table IV). Cytofluorometric analysis of CNS invading mononucleated cells revealed that their numbers and subset distributions are indistinguishable between the inflamed CNS of wt and IL-22KO mice (Fig. 5*B*). Histological analysis of spinal cord cross-sections further displayed similar inflammatory lesions consisting of activated Iba1<sup>+</sup> macrophages/microglia, CD3<sup>+</sup> T cells, and few B220<sup>+</sup> B cells resulting in an impairment of myelinated and axonal structures (Fig. 5*C* and data not shown). Therefore, while IL-22 is clearly expressed by encephalitogenic Th17 cells, it does not appear to be crucial for the development of autoimmune inflammation of the CNS.

## Discussion

In the recent past, the function, origin, and regulation of IL-17-expressing Th cells received much attention by the immunology community. The discovery of this Th-polarization profile (Th17) finally does resolve a number of conflicting findings regarding the Th1/2 paradigm of tissue-directed autoimmune disease (5). The

expression of IL-17 by Th cells correlates extremely well with their pathogenicity during autoimmunity.

However, despite this close correlation (3, 6, 7), several questions regarding their actual effector function remain unanswered. Foremost, the fact that IL-23 is absolutely vital for the development of autoimmune disease, whereas IL-17A alone has only a moderate impact (10), raises the question whether additional thus far unidentified IL-23-driven cytokines have pathogenic properties. We sought to resolve this question by the global analysis of IL-23-induced genes in lymphocytes. We discovered IL-22 to be the most prominent gene expressed by Th cells after IL-23 treatment. We further found that self-reactive Th cells required the presence of IL-23 for IL-22 production and that IL-23-deficient APCs were not able to properly induce IL-22 by stimulation of a population of MOG-reactive T cells. In agreement with Liang et al. (18), we found IL-22 to be highly expressed by Th17 cells. This suggested that IL-22 could potentially serve a pathogenic function during EAE. To this end, we performed a longitudinal analysis of IL-22 expression during EAE and found a strong correlation between T cell pathogenicity and IL-22 secretion. Bettelli et al. (14) recently claimed that IL-22 expression “marks” a highly pathogenic and proinflammatory population of autoaggressive T cells, heavily implicating IL-22 to exert a pathogenic function during EAE. Also, the receptor for IL-22, a heterodimer of the IL-10R2 and IL-22R1, like the IL-17A receptor is found primarily on stroma cells including endothelial cells, epithelial cells, and CNS-resident astrocytes (12, 14, 19). The close association of IL-22 and IL-17 in pathogenic Th cells, their inducibility by IL-23, and the fact that their receptors are expressed by similar cell types, implies that IL-22 too serves a proinflammatory pathogenic role in CNS inflammatory disease.

To determine whether IL-22 actually contributes to the development of EAE or whether the crisp correlation between IL-22 expression and encephalitogenicity is only an epiphenomenon, we generated IL-22<sup>-/-</sup> mice by gene targeting. To our surprise, we discovered that IL-22<sup>-/-</sup> mice develop EAE with the same severity, day of onset, and clinical manifestations as wt mice. This finding clearly dismisses IL-22 as a major pathogenic player in the development of autoimmune CNS inflammation. The function of IL-22 in autoimmunity, however, cannot be dismissed altogether. Wolk et al. (11) reported that elevated levels of IL-22 can be found in the blood of psoriatic patients and ear-skin acanthosis and inflammation induced by the application of IL-23 is slightly decreased when IL-22 is absent (11, 13).

Taken together, the notion that a cytokine is considered to have pathogenic functions cannot be based on its mere presence in a potentially pathogenic population of T cells. This line of thought had lead to a biased interpretation of the role and function of Th1 and Th2 cells in the context of autoimmune disease (5, 20–22). We were able to identify such a proinflammatory factor, namely IL-22, which is like IL-17A closely associated with an encephalitogenic phenotype. However, the fact that IL-22<sup>-/-</sup> mice develop severe EAE indicates that IL-22, just like IFN- $\gamma$ , is not among the proinflammatory factors mediating the tissue damage seen in EAE. The requirement of the transcription factors which drive Th1 and Th17 polarization (T-BET and ROR- $\gamma$ T, respectively) indicates that features, other than the main cytokines produced, are responsible for their pathogenic behavior. Although Th17 cells secrete IL-17 as well as IL-22, the report by Kebir et al. (23) suggests that cytolytic enzymes and factors that alter the integrity of the blood-brain barrier may be responsible for the encephalitogenicity of human Th17/22 cells. It is however likely that among the genes expressed by Th17 cells, a number of them may turn out to serve as biomarkers if not

therapeutic targets in the treatment of autoimmune diseases in general and MS in particular.

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## Disclosures

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