IL-22 Fate Reporter Reveals Origin and Control of IL-22 Production in Homeostasis and Infection

Helena Ahlfors, Peter J. Morrison, João H. Duarte, Ying Li, Judit Biro, Mauro Tolaini, Paola Di Meglio, Alexandre J. Potocnik and Brigitta Stockinger

*J Immunol* 2014; 193:4602-4613; Prepublished online 26 September 2014;
doi: 10.4049/jimmunol.1401244
http://www.jimmunol.org/content/193/9/4602

**References**
This article cites 37 articles, 9 of which you can access for free at:
http://www.jimmunol.org/content/193/9/4602.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-22 Fate Reporter Reveals Origin and Control of IL-22 Production in Homeostasis and Infection

Helena Ahlfors, Peter J. Morrison, João H. Duarte, Ying Li, Judit Biro, Mauro Tolaini, Paola Di Meglio, Alexandre J. Potocnik, and Brigitta Stockinger

IL-22 is a cytokine that regulates tissue homeostasis at barrier surfaces. A variety of IL-22–producing cell types is known, but identification on the single-cell level remains difficult. Therefore, we generated a fate reporter mouse that would allow the identification of IL-22–producing cells and their fate mapping in vivo. To trace IL-22–expressing cells, a sequence encoding Cre recombinase was cloned into the Il22 locus, and IL22Cre mice were crossed with reporter mice expressing enhanced yellow fluorescence protein (eYFP) under control of the endogenous Rosa26 promotor. In IL22CreR26R/eYFP mice, the fluorescent reporter permanently labels cells that have switched on Il22 expression, irrespective of cytokine production. Despite a degree of under-reporting, eYFP expression was detectable in nonimmune mice and restricted to group 3 innate lymphoid cells (ILC3) in the gut and γδ T cells in skin or lung. Upon skin challenge with imiquimod, eYFP+ γδ and CD4 T cells expanded in the skin. Infection with Citrobacter rodentium initially was controlled by ILC3, followed by expansion of eYFP+ CD4 T cells, which were induced in innate lymphoid follicles in the colon. No eYFP expression was detected in small intestinal Th17 cells, and they did not expand in the immune response. Colonic eYFP+ CD4 T cells exhibited plasticity during infection with expression of additional cytokines, in contrast to ILC3, which remained largely stable. Single-cell quantitative PCR analysis of eYFP+ CD4 T cells confirmed their heterogeneity, suggesting that IL-22 expression is not confined to particular subsets or a dedicated Th22 subset.


Interleukin-22 is a cytokine expressed by immune cells but acting on nonhemopoietic cells. The receptor for IL-22 is expressed in barrier sites, such as skin, intestine, and lung, as well as in liver, pancreas, and kidney (1, 2). IL-22 production is attributed to many immune cell types, such as CD4, CD8, and γδ T cells, NK cells, and subsets of innate lymphoid cells (ILC) (3). Thus, the expression pattern of IL-22 and its receptor creates signaling directionality from the immune system to the tissues, in line with the important function that IL-22 has in maintaining tissue integrity. IL-22 plays an important role in the homeostasis of mucosal surfaces. During inflammation, IL-22 induces the expression of acute-phase proteins, antimicrobial peptides, and chemokines (4), which support resolution of the local inflammation, repair of injured tissue, and re-establishment of homeostasis. IL-22 is required for protective immune responses against certain extracellular bacteria (5–9), and it prevents the dissemination of intestinal microbiota (10). In contrast, dysregulated production of IL-22 is associated with certain human autoimmune inflammatory diseases, including rheumatoid arthritis, inflammatory bowel disease, and psoriasis (2, 11–13).

Despite the undisputed biological importance of IL-22, it remains difficult to follow its expression in vivo either in the steady-state or during inflammatory responses because of technical problems with intracellular staining. An additional complication is the issue of effector cell plasticity, which makes it difficult to unequivocally assign IL-22 production to different subsets. We (14) addressed this issue for IL-17–producing cells by generating a fate reporter that marked cells that had initiated the IL-17 program with an easy identification of such cells ex vivo and further determination of their effector program, irrespective of ongoing IL-17 production.

In this study, we used the same strategy to generate a knock-in mouse strain bearing a gene encoding Cre recombinase in the Il22 locus and bred those mice with reporter mice expressing eYFP from the Rosa26 promoter to monitor expression of IL-22 in the steady-state and during infection with Citrobacter rodentium. Our data demonstrate a substantial expansion of eYFP+ CD4 T cells in the large intestinal lamina propria (LI LP) from day 5 postinoculation (p.i.), whereas eYFP+ ILCs are present in uninfected mice and do not expand substantially on infection. IL-22–expressing CD4 T cells predominantly associate with a Th17 profile but show pronounced plasticity in the course of infection, in contrast to ILCs that remain committed to IL-22 production. Single-cell quantitative PCR (qPCR) analysis of gene expression for CD4 T cell subsets indicates substantial heterogeneity, which suggests that IL-22 expression is not confined to particular subsets or a dedicated Th22 subset.

Materials and Methods

Mice

Codon-improved Cre recombinase (iCre) (15) was inserted into the first exon of the Il22 locus by homologous recombination in B6/N mouse embryonic stem cells. The neocassette was removed via FLPe-mediated recombination. To visualize Cre-mediated recombination, IL22Cre mice were intercrossed with R26R/eYFP reporter mice (expressing eYFP from the Rosa26 promoter) (16), generating IL22Cre×R26R/eYFP reporter mice.
C57BL/6 (B6), B6.Rag2−/−/CD45.1, and IL22CreR26R<sup>YFP</sup> reporter mice were bred in the animal facility of the Medical Research Council National Institute for Medical Research. All mice were kept under specific pathogen-free conditions. All animal experiments were approved by the local Ethical Review panel at the National Institute for Medical Research in accordance with the Institutional Committees on Animal Welfare of the UK Home Office (the Home Office Animals Scientific Procedures Act, 1986).

**Generation of bone marrow chimeras**

Bone marrow chimeras were generated by i.v. injection of 10<sup>7</sup> T cell–depleted bone marrow cells from homozygous or heterozygous R26R<sup>YFP</sup> reporter mice into B6.Rag2<sup>−/−</sup>/CD45.1 hosts that were sublethally irradiated (5 Gy). Mice were used in experiments from 7 wk after reconstitution.

**Citrobacter rodentium infection**

*C. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection) was cultured in Luria–Bertani broth overnight, diluted 1:400 with fresh Luria–Bertani broth, and cultured for additional 3–4 h. Bacterial concentration was determined by measuring the OD at 600 nm. The cultures were serially diluted and plated to confirm the inoculum. IL-22 fate reporter, IJ22<sup>wt</sup> or wild-type (wt) mice were gavaged with 2 × 10<sup>8</sup> CFU in 200 μl PBS. Postinfection, mice were weighed daily, and weight loss was determined. Distal colon pieces were weighed and homogenized in sterile PBS, and serial dilutions were plated in duplicates on Brilliant E. coli/colliform Selective Agar (Fisher Scientific) plates for measurement of CFU. *C. rodentium* colonies were identified based on morphology and color after 18–24 h of incubation at 37°C.

**Imiquimod-induced psoriasiform-like skin inflammation**

Mouse dorsal skin was shaved, and Aldara cream containing 5% imiquimod (Meda; 50 mg/mouse) was applied daily for five consecutive days to induce psoriasiform skin inflammation. On day 5, an 8-mm biopsy punch (Kai medical) was used to collect full-thickness skin biopsies of the treated area. Two skin biopsies were minced, digested with a mixture containing 400 μg/ml Liberase TL and 1 mg/ml collagenase D (both from Roche) in IMDM for 2 h at 37°C with shaking, and mashed through a 70-μm cell strainer to obtain single-cell suspensions. Leukocytes were enriched for intraepithelial cells using 36.5% Percoll (Amersham) density gradient centrifugation. The remaining tissue was digested further with a mixture containing 400 μg/ml Liberase TL (Roche) and DNase I (10 U/ml; Sigma) for 30 min at 37°C. For flow cytometry analysis, cells were stained for surface molecules (see list of Abs in Table I) in the presence of 10% Fc block and acquired on a FACSCount II (BD).

**In vitro T cell differentiation**

Naive T cells (CD4<sup>+</sup>CD25<sup>−</sup>CD44<sup>+</sup>) were purified using flow cytometry from spleen and lymph nodes of fate reporter mice and activated with plate-bound anti-CD3 (clone 2C11; eBioscience) and anti-CD28 (clone 37.51; BioLegend) (coated with 0.5 and 5 μg/ml, respectively) in the presence of lineage-polarizing cytokines as follows: IL-12 (5 ng/ml) for Th1 cells; IL-4 (10 ng/ml) for Th2 cells; IFN-γ (10 ng/ml) and TGF-β (5 ng/ml) for Th9 cells; TGF-β (10 ng/ml) for inducible regulatory T cells (tTregs); and IL-6 (20 ng/ml), TGF-β (1 ng/ml), IL-1β (10 ng/ml), IL-23 (20 ng/ml) (all from R&D Systems), or FICZ (250 nM; Enzo) for Th17 cells. Alternatively, CD4<sup>+</sup> cells were purified using an EasySep Mouse CD4+ T Cell Enrichment Kit (STEMCELL Technologies) from spleen of fate reporter mice and activated as described above. Cells were cultured with anti-IFN-γ (10 μg/ml, clone XMG1.2; eBioscience), anti–IL-4 (10 μg/ml, clone 11B11), IL-6, IL-1β, and FICZ, as well as with TGF-β for Th17 cells or with anti–TGF-β (10 μg/ml, clone 1D11) for “Th22” cells. All cytokines were of mouse origin. Cells were cultured in IMDM (Sigma) supplemented with 5% FCS, 0.002 M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10<sup>−5</sup> M 2-ME and analyzed for intracellular cytokines on day 4.

**Cytokine measurement**

Cells were stimulated for 4 h with PdBU (500 ng/ml) and ionomycin (500 ng/ml) in the presence of brefeldin A (1 μg/ml), fixed with 4% paraformaldehyde, permeabilized with 0.1% Nonidet P-40 or 0.1% saponin, and stained for intracellular cytokines or transcription factors (Abs in Table I). Cells were acquired on a FACSCanto II (BD). The Th cell culture supernatants (Fig. 1D) were analyzed for IL-22 using FlowCytomix, according to the manufacturer’s instructions (Bender MedSystems, Vienna, Austria). eYFP<sup>+</sup> and eYFP<sup>−</sup> CD4<sup>+</sup> T cells (CD4<sup>+</sup>CD44<sup>+</sup>TCR<sup>β</sup><sup>+</sup>) were FACS sorted from LI LP of *C. rodentium*-infected fate reporter mice on day 14 p.i., and 25,000 cells were cultured on anti-CD3–coated wells for 18 h. Medium-only

---

Table I. Abs used in flow cytometry analysis

<table>
<thead>
<tr>
<th>Ag</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3e</td>
<td>145-2C11</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD4</td>
<td>GK.1</td>
<td>Allophyocyanin, V500</td>
<td>BioLegend, BD Biosciences</td>
</tr>
<tr>
<td>CD8a</td>
<td>53-6.7</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD11c</td>
<td>N418</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD19</td>
<td>6D5</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD45.2</td>
<td>104</td>
<td>PE/Cy7, allophyocyanin/Cy7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>eKit</td>
<td>2B8</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD49b</td>
<td>DX5</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Foxp3</td>
<td>150D</td>
<td>Alexa Fluor 647</td>
<td>BioLegend</td>
</tr>
<tr>
<td>y8TCR</td>
<td>UC7-13D5</td>
<td>PE/Cy7, BV421, allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Ly-6G/Ly-6C (Grl1)</td>
<td>RB6-8C5</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>IFNγ</td>
<td>PE</td>
<td>XMG1.2</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IL-17A</td>
<td>TC11-18H10.1</td>
<td>Alexa Fluor 647, Pacific Blue</td>
<td>BioLegend</td>
</tr>
<tr>
<td>IL-22</td>
<td>AM-22</td>
<td>Allophyocyanin</td>
<td>Gift from J-C. Renauld</td>
</tr>
<tr>
<td>IL-4</td>
<td>11B11</td>
<td>PE</td>
<td>BioLegend</td>
</tr>
<tr>
<td>IL-7R</td>
<td>A7R34</td>
<td>PE, PE-Cy5</td>
<td>BioLegend, eBioscience</td>
</tr>
<tr>
<td>IL-9</td>
<td>RM9A4</td>
<td>PE</td>
<td>BioLegend</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>PE, PerCP/Cy5.5</td>
<td>BioLegend</td>
</tr>
<tr>
<td>NKP46</td>
<td>29A1.4</td>
<td>Brilliant Violet 421</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Sca-1</td>
<td>Pacific Blue</td>
<td></td>
<td>BioLegend</td>
</tr>
<tr>
<td>TCRβ</td>
<td>H57-597</td>
<td>Allophyocyanin/Cy7, PerCP/Cy5.5, allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Ter119</td>
<td>TER-119</td>
<td>Allophyocyanin</td>
<td>BioLegend</td>
</tr>
<tr>
<td>CD90.2 (Th1.2)</td>
<td>53-2.1</td>
<td>PE/Cy7</td>
<td>BioLegend</td>
</tr>
<tr>
<td>Rorγt</td>
<td>O31-378</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>GATA-3</td>
<td>TWA1</td>
<td>PerCP-eFluos 710</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>
wells were included as controls. The culture supernatants were analyzed for IL-22, IL-17A, and IFN-γ, as described above.

For serum IL-22 measurement, blood was collected from both Citrobacter-infected animals and steady-state control mice and processed by centrifugation to isolate the serum. The concentration of IL-22 in the serum was determined by capture ELISA, according to the manufacturer’s instructions (eBioscience).

Single-cell gene-expression analysis
eYFP+ CD4 T cells from LILP were FACs sorted and subsequently deposited into 96-well PCR plates using an automated cell deposition unit on a MoFlo sorter (Becton Dickinson, Franklin Lakes, NJ) containing 10 μl lysis buffer. The sorter provided single cells in >99% of the wells, and no well contained more than one cell, as assessed by routinely sorting fluorescent beads or cells prior to and after single-cell sorting. Cells were processed for quantitative real-time PCR analysis using a Single Cell-to-CT qRT-PCR Kit (Ambion), according to the manufacturer’s protocol. A total of 175 single cells was analyzed (day 0 = 75, day 5 = 44, day 25 = 56).

The resultant cDNA served as a template for the amplification of the genes of interest by real-time PCR with TaqMan Gene Expression assays and universal PCR Master Mix on an ABI PRISM 7900 Sequence Analyzer (all from Applied Biosystems).

Immunohistolgy
Frozen sections of large intestine were prepared at 8 μm thickness and fixed in 4% paraformaldehyde. Sections were counterstained with DAPI and mounted using Fluoromount-G (Southern Biotechnology). mAbs used are listed in Table I. Confocal images were obtained using a Leica TCS-SP2 microscope equipped with 405-, 488-, 594-, and 647-nm lasers, and image analysis was done in Adobe Photoshop.

Skin was fixed in neutral buffered formalin (Sigma), and tissue sections were stained with H&E for histopathology analysis. Epidermal and scale thickness were quantified as previously described (17).

Statistical analysis
Statistical analysis was performed using Prism version 6.0 (GraphPad Software). If two groups were compared, significance was calculated using the unpaired t test. If more than two groups were assessed, significance was calculated using one-way ANOVA and the Dunnnett or Sidak corrected p value for multiple comparisons. For weight loss data, significance was calculated using two-way ANOVA and the Tukey multiple-comparisons test.

Results
Generating an IL-22 fate reporter mouse
To generate a reporter system that would allow for the identification of IL-22 cytokine–producing cells, as well as mapping the fate of these cells in vivo, we cloned a sequence encoding Cre recombinase into the Il22 locus (Il22Cre). To visualize Cre activity, IL22Cre mice were crossed with reporter mice expressing eYFP under the control of the endogenous Rosa26 promoter (R26ReYFP). In IL22Cre/R26ReYFP mice, the fluorescent reporter permanently labels cells that have switched on expression of IL-22, regardless of the present production status of this cytokine. The endogenous Il22 locus, the gene-targeting vector, the targeted Il22 allele, including the Neo resistance gene cassette (Il22Cre-Neo), and the final targeted allele (Il22Cre) after FLPe-mediated recombination are shown in Fig. 1A. Fig. 1B shows the sequence of Il22 exon 1, with a linker consisting of 6 aa placed between the original Il22 ATG site and the respective start of the iCre minigene.

We first tested reporter activity in vitro following stimulation of FACs-selected naive CD4 T cells for 3 d under Th17, Th1, Th2, Th9, or iTreg conditions. IL-22 was previously identified as a Th17 cytokine, whereas IL-23 had no enhancing effect on IL-22/eYFP expression. Optimal expression was achieved by combining FICZ and IL-1β (Fig. 1E, 1F). There was only marginal induction of eYFP or IL-22 under “Th22” conditions using IL-6 and anti–TGF-β (Fig. 1G).

Only a small proportion of cells that stained for intracellular IL-22 coexpressed eYFP; in addition, a fraction of eYFP* cells did not stain for IL-22 (Table I). This suggests that there is a disconnect between the production of IL-22 cytokine and induction of the reporter. We also had seen a degree, although less pronounced, of “underreporting” in the IL-17Cre fate reporter model. A likely explanation for incomplete reporting is the delay between cytokine induction and production of Cre, which is necessary to reveal the fluorescent reporter. In the case of IL-22, cytokine production may be of shorter duration, thus compromising effective Cre induction. Furthermore, IL-22 expression, like that of the closely related cytokine IL-10, may be mononucleic and, therefore, more severely compromised when combined with the disruption of one allele by Cre.

Detecting IL22-eYFP* cells in vivo under steady-state conditions
To assess which cells express IL-22 in unchallenged mice, different organs, including gut, lung, skin, lymph nodes, spleen, thymus, liver, pancreas, kidney, and bone marrow, were analyzed for the presence of eYFP* cells. In concordance with the predominant function of IL-22 in maintaining epithelial barriers, eYFP* cells were found only in tissues involved in barrier function, namely gut, mesenteric lymph nodes (mLN), lung, and skin in 2 mice and lung of the majority of eYFP* cells were γδ T cells. We did not detect eYFP* cells in the resident Th17 population of the small intestinal lamina propria (SI LP) with the caveat that a small number of such cells might have escaped detection because of underreporting. However, there were eYFP+ CD4 T cells in the intraepithelial lymphocyte population and Peyer’s patches, as well as CD8αα and γδ T cells and ILCs (Fig. 2B, 2C). Among the eYFP+ ILC fraction in the SI LP and LILP (see gating strategy in Fig. 3A), the majority was NKP46+ CD4+ T cells, but CD4+ (LTI) and NKP46+ ILCs also were present (Fig. 3C–E). All eYFP+ ILCs expressed RORγt and, therefore, can be assigned to the ILC3 subset (Fig. 3B).

Induction of eYFP expression during inflammatory responses
IL-22 expression has been documented in inflammatory skin conditions, such as psoriasis, and in the mouse model of psoriasiform inflammation induced by the TLR7 ligand imiquimod (20, 21). Therefore, we treated IL22CreR26ReYFP mice with imiquimod to induce inflammation induced by the TLR7 ligand imiquimod (20, 21). We did not detect any CD8 T cells or ILCs expressing eYFP in this model (20, 21).

We next investigated the induction of eYFP during the course of infection with C. rodentium, an effacing attaching bacterial pathogen that causes severe inflammation of the large intestine and...
FIGURE 1. Fate-reporter eYFP+ cells are induced among IL-22–producing cells. (A) Schematic presentation of the endogenous Il22 locus, the targeting vector, the targeted Il22 allele, including the Neo resistance cassette (Il22<sup>Cre</sup>-Neo), and the final targeted allele (Il22<sup>Cre</sup>) after FLPe-mediated recombination. The iCre component is a minigene composed of a codon improved Cre, followed by an intron and polyadenylation site derived from SV40. (B) Sequence of Il22 exon 1 after targeting showing the original 6 aa after the Il22 ATG-site and the respective start of the iCre minigene. (C) FACS plots showing the expression of eYFP and intracellular cytokines or Foxp3 in Th1, Th2, Th9, or Th17 cells or iTregs after 4 d of in vitro differentiation. (D) Concentration of IL-22 in day-4 supernatants of in vitro–differentiated Th1, Th2, Th9, and Th17 cells or iTregs. Data are mean ± SEM and are representative of three independent experiments. (E) FACS plots showing the expression of eYFP and intracellular IL-22 in naive CD4<sup>+</sup>CD44<sup>lo</sup>CD25<sup>+</sup> (Figure legend continues)
The percentage of eYFP+ cells cultured for 4 d in modified Th17 conditions (as indicated on the surface markers as follows: CD4+ T cells: TCRgd+CD4+; CD8a+ T cells: TCRgd+CD8a+; γδ T cells: γδTCR+; NK cells: NK1.1+; and ILCs: lineage mixture: CD11c, CD11b, DX5, Gr1, Ter119, CD69, CD122, and CD19). Data shown are mean ± SEM and are representative of two to nine independent experiments. (B) FACS plots showing the expression of eYFP and CD45 in various organs of untreated fate reporter mice. Data are representative of two to nine independent experiments. (C) Relative number of eYFP+ cell types in intestinal tissues, skin, and lung. Cells were gated first for lymphocytes (forward scatter–area versus side scatter–area) and singlets (forward scatter–area versus forward scatter–width). These cells were analyzed for their CD45 expression. CD45+ cells were analyzed for their CD45+ cell types (CD45+CD4+; CD45+CD8a+; CD45+γδTCR+; CD45+NK cells). Data shown are mean and are representative of two to nine independent experiments. BM, bone marrow; pLN, peripheral lymph nodes; PP, Peyer’s patches; SI IEL, small intestinal intraepithelial lymphocytes.

requires IL-22 for restoration of the intestinal barrier following infection (7–9). It was noticeable that IL22CreR26ReYFP mice showed a more pronounced transient weight loss following infection than did wt B6 mice and even heterozygous IL-22+/− mice (Fig. 5A). This is indicative of stochastic monoallelic expression of IL-22, which, in combination with only one functional allele, results in lower IL-22 expression than that of heterozygous IL-22+/− mice. However, at the time points these mice were analyzed (from day 28) they had all cleared the infection and the kinetics of Citrobacter clearance in IL22CreR26ReYFP followed those reported for wt mice (23) (data not shown).

Kinetic analysis, between days 2 and 82 p.i., for appearance and expansion of eYFP+ expressing CD4 T cells (Fig. 5B) and other cell types showed that cellular expansion was prominent in the CD4 cell population in the LI LP, whereas eYFP+ cells in the SI LP increased only in the later stages, presumably due to overspill from the LI LP (Fig. 4C, 4D). eYFP+ ILCs or γδ T cells did not expand significantly throughout the course of infection. IL-22 protein was detectable in serum from day 5 p.i. (Fig. 5E). Histological analysis of colon from IL22CreR26ReYFP mice showed eYFP+ cells in structures resembling isolated lymphoid follicles (ILFs) and their expansion throughout the colon following infection. Initially, eYFP+ cells were CD3-ve, indicative of ILCs, whereas eYFP expression was extended to CD3+ cells p.i. (Fig. 6).

Homozygous IL22CreR26ReYFP cells expand in infection but fail to control Citrobacter

We next investigated the fate of eYFP+ CD4 T cells in IL22CreR26ReYFP mice with homozygous expression of Cre. Such mice are IL-22 deficient but still allow identification of the cell type via eYFP reporting (Fig. 7B). IL22CreCre reporter mice were unable to clear Citrobacter, as indicated by a log higher CFU count on day 5 p.i. (the mice succumbed to the infection by day 8) (Fig. 7A). Interestingly, the number of eYFP+ CD4 T cells, but not ILCs, in LI LP and mLNs was substantially higher (Fig. 7C). This could indicate either continuous stimulation of CD4 T cells to curb bacterial expansion or that homozygous Cre expression improved the reporter intensity. To avoid complications due to the differential outgrowth of Citrobacter, we generated chimeric mice in which we cotransferred wt B6 bone marrow with bone marrow from either IL22CreR26ReYFP mice (homozygous for Cre, IL22wt/Cre) or Cre homozygous reporter mice (IL22Cre/Cre) into B6.Rag2−/−CD45.1 hosts. This allowed us to monitor the extent of eYFP induction in CD4 T cells from heterozygous or homozygous donors in mice with comparable bacterial burden. As shown in Fig. 7D, there was no difference in eYFP+ CD4 T cells in recipients of homozygous versus heterozygous Cre reporter bone marrow, and the bacterial burden was similar (Fig. 7E). This makes it less likely that the hyperexpansion of eYFP+ cells in homozygous reporter mice (Fig. 7B) was simply the result of a higher reporting efficiency due to the presence of two Cre alleles; rather, it suggests an ongoing stimulatory drive to CD4 T cells to combat the outgrowth of Citrobacter, which is thwarted because of the lack of IL-22.

Functional plasticity of cytokine expression in eYFP+ cells is prominent in T cells but limited in ILCs

To investigate the cytokine profile of eYFP+ cells, T cells (gated on TCRβγ+ expression) and ILCs (TCRβ−ve) were analyzed throughout the course of infection for the single expression, as well as the coexpression of, IL-22, IL-17, and IFN-γ by intracellular staining. First, we investigated cytokine production of FACS-purified eYFP+ and eYFP− CD4 T cells from Citrobacter-infected mice (day 14) that were restimulated with anti-CD3 in vitro. Interestingly, IL-22, IL-17, and IFN-γ all were predominantly produced by eYFP+ CD4 T cells, indicating the substantial plasticity of these cells.

T cells cultured for 4 d in Th17-differentiating conditions (TGF-β+IL-6) supplemented with FICZ, IL-1β, or IL-23 or their combination. Fold induction in the percentage of eYFP+ cells cultured for 4 d in modified Th17 conditions (as indicated on the x-axis) compared with Th17 cells cultured in the presence of TGF-β and IL-6 only (F) or in the presence of IL-6 and anti–TGF-β (G). Data in (C) and (E) are representative of three independent experiments.
FIGURE 3. eYFP+ ILC3 cells in untreated fate reporter mice. (A) Gating strategy for flow cytometry analysis. Cells were gated first for lymphocytes (forward scatter–area versus side scatter–area) and singlets (forward scatter–area versus forward scatter–width). These cells were analyzed for their CD45 expression. CD45+ cells were analyzed for their Thy1.2 and lineage marker (CD11c, CD11b, DX5, Gr1, Ter119, γδTCR, TCRβ, CD3, CD8α, and CD19) expression. Thy1.2+ lineage2 cells were further analyzed for eYFP, IL-7R, c-Kit, Sca-1, and NK1.1 expression. (B) Thy1.2+ lineage2 cells (left panels) and eYFP+ Thy1.2+ lineage2 cells (middle panels) were analyzed for Rorγt and Gata3 expression. eYFP expression on gated Thy1.2+ RORγt+ lineage2 cells (right panels). (C) Thy1.2+ lineage2 cells analyzed for eYFP and NKp46 expression (left panels). NKp46− cells analyzed for (Figure legend continues)
during the course of infection (Fig. 8A). Fig. 8B shows the concordance of eYFP and IL-22 expression in CD45+ cells, which is higher than what was observed for the in vitro culture (Fig. 1).

eYFP+ ILCs remained predominantly IL-22+ throughout infection, with only a proportion expressing IL-17 and/or IFN-γ. In contrast, the predominant cytokine expressed in eYFP+ T cells was IL-17, alone or together with IL-22 in the early phase of infection, with a gradual increase in IFN-γ expression, alone or together with the other cytokines (Fig. 8C). This confirmed the predominant association of IL-22 with the Th17 subset on the one hand and indicated substantial plasticity on the other hand, as previously seen with Th17 cells in intestinal inflammation (24). In an attempt to identify the developmental origin and fate of eYFP-expressing CD4+ T cells in Citrobacter infection, we performed single-cell qPCR for a range of markers associated with different T cell subsets, including transcription factors and cytokines. The expression changes for these markers were determined from the uninfected basal state to day 5 (the onset of CD4+ T cell expansion)

eYFP and CD4 expression (right panels). (D) Numbers of eYFP+ and eYFP− total ILCs and ILC3 subpopulations. (E) Relative number of eYFP+ ILC3 subpopulations in SI LP and LI LP. Data in (C) and (D) represent the mean and are representative of at least four independent experiments.

FIGURE 4. Fate reporter eYFP+ cells in skin inflammation. (A) Representative images of H&E staining of skin sections from untreated (d0) and imiquimod-treated (d5) IL-22 fate reporter mice at day 5. Scale bars, 100 μm. (B) Quantification of epidermal (left panel) and scale (right panel) thickness of untreated (d0) fate reporter mice and mice at day 5 after imiquimod treatment (d5). Data are mean ± SEM (n = 4 mice/group). (C) Number of eYFP+ γδTCR+ and eYFP+ TCRβ+ cells in dorsal skin of untreated fate reporter mouse (d0) and after 5 d of imiquimod treatment (d5). Data are mean ± SEM and are representative of five independent experiments. (D) FACS plot of eYFP+ CD45+ cells from the dorsal skin of untreated (d0) and imiquimod-treated (d5) fate reporter mice analyzed for their γδTCR and TCRβ expression. (E) FACS plot of γδTCR+ and TCRβ+ cells analyzed for their eYFP expression in dorsal skin of untreated (d0) and imiquimod-treated (d5) fate reporter mice. (F) FACS plot of CD45+ γδTCR+ and TCRβ+ cells from the dorsal skin of imiquimod-treated fate reporter mice analyzed for their eYFP, IL-22, and IL-17A expression. Data in (C–F) are representative of five independent experiments. **p < 0.01, ****p < 0.001.
FIGURE 5. Response of fate reporter mice to infection with *Citrobacter*. (A) Weight curve of B6 (wt/wt), IL-22−/− (wt−/−), and IL-22 fate reporter (wt/cre) mice infected with *C. rodentium* (*n* = 6/group). Data are mean ± SEM. (B) FACS plot of CD45+ cells from LI LP of untreated (d0) or *C. rodentium*-infected fate reporter mice at days 12 (d12) and 28 (d28) analyzed for their eYFP and CD4 expression. Data are representative of two to eight independent experiments. (C) Kinetics of expansion of eYFP+ CD4 T cells, CD8 T cells, ILCs, and γδT cells in LI LP and SI LP of fate reporter mice during the course of infection with *C. rodentium*. Data are mean ± SEM are representative of 2–12 independent experiments. *(Figure legend continues)*
T cells, and ILCs in LI LP and SI LP of C. rodentium–infected fate reporter mice at day 5 (d5) and day 25 (d25) p.i. were stained with anti-GFP (green), anti-CD45 (blue), and anti-CD3 (red) (original magnification ×20). Representative results from one of at least three experiments/time point are shown.

The figure demonstrates the expression of eYFP in CD45+ and CD3+ T cells, with a notable reduction in the CD3+ eYFP+ population over time. This suggests a shift towards Th17 cells as the infection progresses, which is consistent with the expression of Rorc and Tbet in these cells.

In vivo, the fate reporter revealed IL-22 producers in the steady-state and under inflammatory conditions, consistent with previous reports that emphasized the importance of this cytokine at barrier sites of intestine, lung, and skin. The expression of eYFP was restricted to tissue-resident cell types at these locations, such as γδ T cells in skin and lung and ILC3 in the intestine, indicating their important role in homeostatic maintenance of epithelial barrier integrity. Interestingly, the resident Th17 population present in the SI LP of nonimmune mice did not show eYFP expression, which instead was restricted to ILCs that were characteristic of the ILC3 subset (29) in this location, with the caveat that low numbers of eYFP+ cells might have been missed as a result of underreporting.

In line with earlier reports, IL-22–producing intestinal ILC3 control C. rodentium infection in the early phase for ~5 d until the adaptive T cell response ensues (30). This could involve the action of retinoic acid, which was shown to attenuate colon inflammation via increased IL-22 production by ILC3 (31). The induction of IL-22/eYFP expression in CD4 T cells was noticeable first in the colon lamina propria and, to a lesser degree, in the mLNs, suggesting that the adaptive CD4 response was induced locally. Histologically, it appeared that structures resembling colonic patches and ILFs were the main sites of eYFP expression. These structures are induced by the lymphotoxin pathway upstream of IL-22, and both coop-
erate in the organization and maintenance of colonic patches and ILFs during infection with *C. rodentium* (32).

CD4 T cells from the small intestine did not seem to be involved in the immune response to *Citrobacter*, and eYFP+ cells in this location could only be detected at later stages when they might have spilled over from the colon. To some extent, this may be due to the fact that *Citrobacter* infection targets the distal colon (33) and, thus, does not directly involve the small intestinal Th17 cell population. In addition, however, it may indicate that SI LP Th17 cells are not participating in inflammatory immune responses.

The ILC3 response to *Citrobacter* infection remained largely focused on IL-22, with only a minor contribution of IFN-γ and IL-17 production. In contrast, the T cell response was highly plastic, with a large fraction of IL-17 producers and a gradual shift toward IFN-γ, as well as dual cytokine production. Cytokine analysis of FACS-purified CD4 cell populations confirmed that production of IL-22, IL-17, and IFN-γ was restricted to eYFP+ cells, emphasizing their plasticity, as well as the fidelity, of this reporter mouse.

IL-22 was detected maximally around day 2 of infection, prior to the main expansion of CD4 T cells, and it appeared transient compared with IL-17, which remained dominant throughout the infection period. It is conceivable that tight control of IL-22 secretion is necessary to avoid negative side effects associated with this cytokine, such as the promotion of colonization by pathogens (e.g., *Salmonella*), through suppression of related commensal bacteria (34), or the perpetuation of bacteria-induced colon cancer (35).

*C. rodentium* was cleared around 21 d p.i. (data not shown), but elevated numbers of CD4 T cells persisted in the colon (see Fig. 6 for day 25) up to ≈82 d, although most were no longer producing cytokines (data not shown). Detection of cells that had initiated the IL-22 program via expression of eYFP allowed the analysis of single cells via qPCR (36) and confirmed the considerable heterogeneity of eYFP+ CD4 T cells throughout the course of infection.

Single-cell data indicated a gradual shift in profile from a more Th17-like phenotype toward a Th1-like phenotype at later stages. Expression of *Rorα* was quite consistent throughout, whereas *Rorc* expression was not prominent and decreased further at the expense of *Tbet* on day 25; *Ahr* expression was reduced at the later time points. *Runx3* was uniformly expressed in eYFP+ CD4 T cells at day 25 p.i. and, together with *Tbet*, promoted the generation of pathogenic IFN-γ–producing Th17 cells (37). Overall, the emerging picture from the IL-22 fate reporter suggests that, in CD4 T cells, IL-22 is transiently associated with a Th17 population that is highly plastic and prone to adopt a more Th1-like profile during the course of infection. The shortcomings of this reporter, resulting in underreporting of IL-22, make it impossible to unequivocally state that there is no Th22 subset dedicated to producing this cytokine; however, the substantial plasticity of inflammatory CD4 T cells in this setting makes such a scenario less likely.
FIGURE 8. Cytokine and transcription factor expression profile of eYFP+ fate reporter mice. (A) eYFP+ and eYFP− CD4 T cells purified from LI LP of C. rodentium–infected mice at day 14 p.i. were stimulated with anti-CD3 or medium for 18 h. Bar graphs show the amount of IL-22, IL-17A, and IFN-γ produced after stimulation. (B) FACS plot of CD45+ cells from LI LP of C. rodentium–infected fate reporter mice at day 2 analyzed for eYFP and IL-22 expression. Data are representative of four independent experiments. (C) Relative numbers of eYFP+/TCRβ+ and TCRβ− cytokine-expressing cells, based on intracellular cytokine staining, in LI LP of untreated (d0) fate reporter mice and of mice at different time points p.i. with C. rodentium. Data are mean and are representative of two to seven independent experiments. (D) Heat map presentation for single-cell gene expression of selected transcription factors, cytokines, or cytokine receptors, as well as eYFP, in FACS-purified eYFP+ CD4 T cells from LI LP of untreated (d0) and C. rodentium–infected fate reporter mice at day 5 (d5) and day 25 (d25) p.i. Expression levels are shown as Ct values. (E) Summary of data in (B). Heat map represents quantitative values of gene expression (Ct < 35) at different time points. Missing data are shown in gray.
Acknowledgments
The authors have no financial conflicts of interest.

Disclosures
We thank J.-C. Renaud (Ludwig Institute for Cancer Research, Brussels, Belgium) for the AM.3 anti-IL-22 Ab. We gratefully acknowledge the contributions of our Biological Services Facility, the Flow Cytometry Laboratory, and the Histology Laboratory.

References