Autoimmune Memory T Helper 17 Cell Function and Expansion Are Dependent on Interleukin-23

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Autoimmune Memory T Helper 17 Cell Function and Expansion Are Dependent on Interleukin-23

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SUMMARY

Interleukin-23 (IL-23) is essential for the differentiation of pathogenic effector T helper 17 (Th17) cells, but its role in memory Th17 cell responses is unclear. Using the experimental autoimmune encephalomyelitis (EAE) model, we report that memory Th17 cells rapidly expanded in response to rechallenge and migrated to the CNS in high numbers, resulting in earlier onset and increased severity of clinical disease. Memory Th17 cells were generated from IL-17$^+$ and RORγt$^+$ precursors, and the stability of the Th17 cell phenotype depended on the amount of time allowed for the primary response. IL-23 was required for this enhanced recall response. IL-23 receptor blockade did not directly impact IL-17 production, but did impair the subsequent proliferation and generation of effectors coexpressing the Th1 cell-specific transcription factor T-bet. In addition, many genes required for cell-cycle progression were downregulated in Th17 cells that lacked IL-23 signaling, showing that a major mechanism for IL-23 in primary and memory Th17 cell responses operates via regulation of proliferation-associated pathways.

INTRODUCTION

The generation of memory T cells is an extremely useful event for providing protective immunity to infectious agents. Autoimmunity, on the other hand, represents a situation in which the generation of memory cells contributes to ongoing or recurring disease. T helper 17 (Th17) cells are now strongly associated with many human autoimmune and chronic inflammatory disorders, including psoriasis, Crohn's disease, rheumatoid arthritis, multiple sclerosis, and uveitis (Korn et al., 2009; McGeachy and Cua, 2008). In mouse models of these diseases, Th17 cells have been shown to play a causative role. Many advances have been made in our understanding of how Th17 cell activation is regulated (reviewed extensively elsewhere; Boniface et al., 2008; Korn et al., 2009).

Th17 cell recall responses have been shown to be effective in vaccination models of *Mycobacterium tuberculosis* (Khader et al., 2007), *Coccidioides posadasii*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* (Wüthrich et al., 2011), and *Klebsiella pneumoniae* (Chen et al., 2011), as well as following infection with *Bordetella pertussis* (Warfel and Merkel, 2012), Group A *Streptococcus* (Dileepan et al., 2011), and *Candida albicans* (Hernández-Santos et al., 2012). Th17 cells appear to be inherently plastic and easily convert to Th1, particularly in models of autoimmunity (Bending et al., 2009; Hirota et al., 2011; Lee et al., 2009; Martin-Orozco et al., 2009), which would suggest that the subsequent memory response would be dominated by Th1 cells. However, the characteristics of autoimmune memory Th17 cell responses and requirements for interleukin 23 (IL-23) have so far not been addressed.

Previous work from our laboratory showed that the IL-23 receptor (IL-23R) is required in vivo for effector Th17 cell responses (McGeachy et al., 2009). The requirement for IL-23 in the primary Th17 cell response might suggest that memory responses would be impaired if IL-23 was absent at this stage. In the absence of IL-23R, activated Th17 cells retain low IL-7Rα expression (McGeachy et al., 2009), a receptor that is known to be important for the survival of memory cells in CD8 populations (Kaech et al., 2003) and is also required for susceptibility to the experimental autoimmune encephalomyelitis (EAE) model (Walline et al., 2011). On the other hand, it was recently shown that IL-7Rα does not delineate precursors that form CD4$^+$ T cell memory (Marshall et al., 2011). It remains controversial whether memory cells emerge from effector cell populations or less differentiated cells; a weak effector response could therefore predict either a poor or an enhanced memory response. Finally, IL-23R blockade 1 week after immunization is not effective in blocking the subsequent Th17 effector cell response in EAE, suggesting that memory Th17 cells may also not require IL-23 for their subsequent reactivation in vivo. Therefore, we set out to investigate the requirements for IL-23

in autoimmune Th17 cell memory responses using an adapted model of EAE.

RESULTS AND DISCUSSION

Autoimmune CD4$^+$ Memory Cells Transfer Severe EAE

In order to examine the phenotype and function of autoimmune memory CD4$^+$ T cells, we developed a system in which memory cells can be reactivated and analyzed separately from primary effector cells in vivo. CD45.2$^+$ mice were immunized with MOG(35-55) in complete Freund's adjuvant (CFA) to generate MOG-specific Th17 cells, and CD4$^+$ cells containing this memory population were then transferred into naive congenic recipients. After an additional 2–9 weeks, the recipients were immunized with MOG(35-55) to induce EAE. Cell-transfer recipients consistently showed a faster onset and increased severity of clinical scores compared with untransferred controls (Figures 1A, 1B, and S1), correlating with a memory response. Unimmunized recipients did not develop EAE, showing that the response is dependent on secondary activation of the memory cells.

Analysis of CD45.2$^+$ cells revealed a large expansion by day 4 postimmunization, reaching a peak in the lymph nodes (LNs) at day 7 (Figure 1C), with a large increase in CD44^{hi}CD27^{lo} effectors (Figure 1D) that produce IL-17 (Figure 1E). The proportion of interferon-γ^+ (IFNγ^+) cells also increased over time, as has been reported to occur in the primary Th17 cell response (Hirota et al., 2011). Hence, it appeared that memory for Th17 cells was stable in this system, in the sense that most of the cells initially produced IL-17 and IFNγ emerged later.

The initial expansion in the LNs was followed by a large efflux of cells into the blood (Figure 1F) and infiltration into the CNS (Figure 1G). A comparison of transferred memory cells with endogenous primary activated cells in the CNS revealed that the memory cells were not only present at slightly higher frequencies but also produced more cytokine, particularly IL-17 (Figures 1G and 1H). These results show that memory Th17 cells can be generated and tracked in our system, and these cells strongly contribute to disease after re-exposure to antigen.

Memory Cells Are Generated from IL-17$^+$ and RORγt$^+$ Precursors

Whether memory CD4$^+$ T cells are generated from effectors or noncommitted Th0 cell precursors remains controversial, but for Th1 cells, Yeh et al. (2011) demonstrated that memory cells are generated from IFNγ^+ precursors. Also, Th17 cells have been proposed to be inherently plastic, readily converting to Th1. Therefore, we asked whether the IL-17$^+$ memory cells we observed were derived from the original IL-17-producing cells present in the primary response by using IL-17-GFP reporter mice as donors and sorting GFP$^+$ cells on day 8 postimmunization (Figure S2).

Recipients of IL-17-GFP$^+$ cells consistently showed a faster onset and increased severity of clinical scores compared with recipients of IL-17-GFP$^-$ cells (Figure 2A), and IL-17-GFP$^+$ cells were present in high numbers in the CNS, whereas few IL-17-GFP$^-$ transfer cells were present at that site (Figure 2B). By day 11 of the secondary response, we found that most of the originally IL-17-GFP$^+$ cells were no longer producing IL-17 in the CNS and were instead positive for IFNγ (Figure 2C). We hypothesized that early removal of primed Th17 cells from the inflammatory environment might have impaired their ability to form stable Th17 memory. To address this, we waited until day 18 after primary immunization to sort and transfer the IL-17-GFP$^+$ cells. In comparison with the day 8 transfers, day 18 cells showed a greater stability of the Th17 phenotype, although there was still some conversion to IL-17$^+$IFNγ^+ and IFNγ^+ cells (Figures 2D and 2E). Therefore, it appears that Th17 cells become more stable over time during the primary response, and this may be one factor contributing to the reported plasticity of this population.

To investigate the expression of RORγt, we immunized RORγt-GFP reporter mice as donors. Similarly to IL-17-GFP sorted cells, the RORγt-GFP sorted cells initially produced IL-17 with little IFNγ (Figure 2F), followed by a small increase in IFNγ-producing cells. The cells that had maintained RORγt in the secondary response were also the main producers of IL-17, with a small proportion of double producers, whereas cells that had downregulated RORγt produced some IFNγ but little IL-17 (Figure 2G). Corresponding with the increase in IFNγ, many of the previously RORγt$^+$ cells expressed T-bet on day 13 (Figure 2H). However, many RORγt$^+$IL-17$^+$ cells were also T-bet$^+$ (Figure 2I), suggesting that RORγt and T-bet expression are not necessarily mutually exclusive in Th17 cells. Although these data confirm the inherently plastic nature of Th17 cells, they also suggest that the memory population is either generated before the conversion of effectors toward a Th1 phenotype or that Th17 memory cells survive more readily than "ex-Th17" Th1 cells, since the memory response is characterized by the appearance of IL-17 producers before IFNγ producers.

IL-23 Is Required for Activation of the Recall Th17 Memory Response

In order to test whether memory Th17 cells required IL-23 for their activation, we transferred CD4$^+$ T cells from immunized donors as before and administered anti-IL-23R to the recipients at the time of challenge. Anti-IL-23R protected memory transfer recipients from EAE induction with either CFA or incomplete Freund's adjuvant (IFA; Figures 3A, 3B, and S3). The percentage and number of memory cells in the LNs were reduced by anti-IL-23R (Figure 3C), which was particularly apparent at the peak of LN expansion and corresponded with a reduced frequency of CD44^{hi}CD27^{lo} effector cells (Figure 3D) and few transferred memory cells infiltrating the CNS (Figure 3E). Transfer of cells into IL-23R$^{-/-}$ naive recipient mice, in which endogenous T cells do not form effector Th17 responses (McGeachy et al., 2009), resulted in a disease course similar to transfer into wild-type (WT) hosts (Figure S4A), and this was effectively blocked by anti-IL-23R treatment. Furthermore, memory cells induced EAE in RAG-deficient animals that lack endogenous T cells, and these effects were also dependent on IL-23 (Figure S4B), confirming that these transferred Th17 memory cells alone are sufficient to initiate EAE when activated in the presence of IL-23. Given that MOG is a self-antigen, we also tested whether similar IL-23-dependent Th17 memory responses could be induced using the putative nonself immunogenic

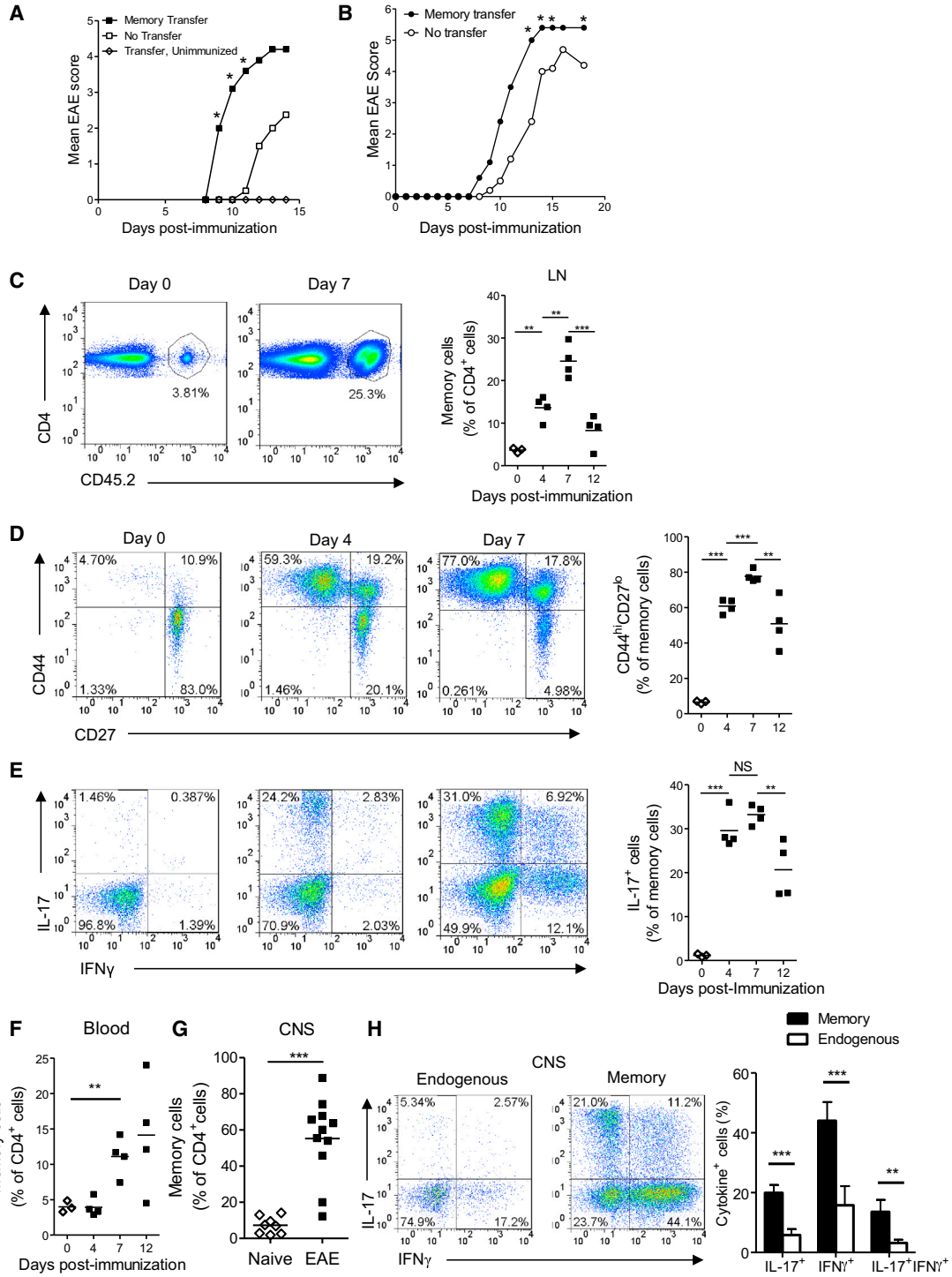

Figure 1. Autoimmune Memory Cells Transfer Severe EAE

Donor C57Bl/6 mice were immunized with MOG(35-55). dLNs and spleens were harvested 30 days later and $CD4^+$ cells (memory cells) were transferred into naive $CD45.1^+$ congenic recipients. The recipients were rested for 2–3 weeks (except for the recipients shown in B, which were rested for 9 weeks posttransfer) and then immunized to induce EAE.

(A) Mean clinical scores of memory transfer recipients that were immunized or not immunized, and immunized controls that did not receive any memory cells.

(B) Mean clinical scores of memory transfer recipients that were rested for 9 weeks before immunization.

(C) Time course of transferred memory cells analyzed by flow cytometry for $CD45.2^+$ surface expression and shown as a percentage of the $CD4^+$ cell population; representative dot plots and pooled data are shown. Day 0 is equivalent to unimmunized recipient of memory cell transfer.

(legend continued on next page)

peptide OVA(323-339) as the immunizing antigen. Indeed, we observed similar expansion and cytokine production by transferred T cells in this OVA-driven recall assay (Figure S5).

Anti-IL-23R Inhibits the Generation of Large Numbers of Cytokine-Producing Effector Cells

We previously reported that IL-23R blockade during the primary response both reduced the percentage of IL-17$^+$ cells in the periphery and impaired the resulting effector response (McGeachy et al., 2009). In contrast, in the present study, IL-23R blockade did not reduce the percentage of IL-17 producers during the memory response (Figure 4A), and instead drastically reduced the absolute number of IL-17$^+$ cells (Figure 4B). The IL-17$^+$IFNγ^+ and IL-17$^-$IFNγ^+ populations were also reduced in both percentage and number at day 7 in the LNs (Figures 4C and 4D). It was recently reported that IL-23 is required for granulocyte-macrophage colony-stimulating factor (GM-CSF) production by Th17 cells in EAE (Codarri et al., 2011; El-Behi et al., 2011). In our memory population, GM-CSF producers were not significantly reduced as a percentage of memory cells (Figure 4E). However, similarly to IL-17, there was a significant decrease in the absolute numbers of GM-CSF$^+$ cells when anti-IL-23R was administered (Figure 4F). In the target tissue (CNS), the percentage of memory cells producing IL-17 was again not different, but IL-17$^+$IFNγ^+ and IL-17$^-$IFNγ^+ cells were reduced with anti-IL-23R (Figure 4G). Corresponding with reduced IFNγ, T-bet expression was also reduced when IL-23 signals were blocked during the secondary response (Figure 4I). Similar reductions in T-bet expression by primary Th17 cells activated in IL-23 knockout mice were recently reported (Hirota et al., 2011). Therefore, induction of T-bet and IFNγ appear to be part of the Th17 program initiated by IL-23 in vivo. Interestingly, Yeh et al. (2011) reported that IL-12 is not required for T-bet expression in EAE.

It remains unclear what the functions of T-bet and IFNγ are in Th17 cells. Although T-bet is thought to be required for autoimmune inflammation (Lazarevic and Glimcher, 2011), IFNγ is not, at least in commonly used models. It was recently proposed that T-bet inhibits RORγt in Th17 cells through binding to the cofactor RUNX1 (Lazarevic et al., 2011), and T-bet-deficient animals do have increased numbers of IL-17$^+$ cells despite their resistance to autoimmunity. However, it is clear that in human chronically inflamed tissues, such as the inflamed colon in Crohn's disease or brain lesions in multiple sclerosis, these double-producing cells are found in high numbers (Kebir et al., 2009; Kleinschek et al., 2009). In our study we also observed a high frequency of double-positive cells expressing RORγt and T-bet as well as IL-17 and IFNγ, and this coexpression was dependent on IL-23, suggesting a proinflammatory role for these cells. Furthermore, a high proportion of RORγt$^+$T-bet$^+$ cells produced IL-17 but not IFNγ in vivo. Therefore, the relationship between T-bet and Th17 cells warrants further investigation.

Anti-IL-23R Reduces the Number of Proliferating Memory Cells

The findings so far suggest that IL-23 is required for the memory Th17 response, but not necessarily for production of IL-17 or other Th17-associated factors. Rather, the biggest defect appeared to be in the number of cells generated, suggesting a proliferation defect. Analysis of Ki67 expression in the memory cells confirmed that proliferation was indeed being affected by IL-23R blockade (Figure 5A).

Since we previously observed that IL-23 drives expansion of effector Th17 cells in the primary response (McGeachy et al., 2009), we turned to a system that allowed more in-depth analysis of the effects of IL-23 on proliferation. Using adoptive transfer of OTII cells, which have a transgenic T cell receptor specific for OVA(323-339), we were able to generate activated cells (all CD44^{hi}) by immunizing recipients as previously described (McGeachy et al., 2009). We then sorted the OTII cells based on IL-17 expression for messenger RNA (mRNA) analysis. By comparing IL-17$^+$ and IL-17$^-$ cells derived from the same antigen-specific activated T cell population, we were able to ask which genes were differentially regulated. *Il17a* was increased in IL-17$^+$ cells, confirming sort purity (Figure 5B). *Ki67* was also upregulated in IL-17$^+$ cells and reduced in IL-23R-deficient cells, as expected (Figure 5C). Furthermore, *Il23ra* expression correlated with *Il17* expression, being increased on IL-17$^+$ cells compared with IL-17$^-$ cells, further confirming that IL-23 signaling drives proliferation in Th17 cells (Figure 5D).

We then investigated the expression of genes that are involved in cell cycle. Many of these genes were indeed upregulated in IL-17$^+$ cells compared with IL-17$^-$ cells (Figure 5E). These included genes for transcription factors that regulate cell-cycle progression (E2F, Tfdp, Myb, cdc, and cyclin families), genes for enzymes required for DNA replication (polymerases, ligases, and primases) as well as DNA repair proteins of the Brca and Chk families, and genes involved in chromosome assembly and segregation during division (Cenp, Kif, Haus, and Ncap families). The expression pattern of *Il23ra*$^{-/-}$ IL-17$^+$ cells showed that there was at least a 2-fold downregulation in almost all cell-cycle genes that were associated with WT IL-17$^+$ cells (Figure 5F). Not all genes were downregulated in the *Il23ra*$^{-/-}$ IL-17$^+$ cells: *Il17a*, *Rorc*, and *Il17f* were unchanged, confirming previous reports that upregulation of *Rorc* does not require IL-23.

To confirm that the same defect is responsible for the reduced proliferation in memory Th17 cells, IL-17$^+$ cells were

(D) Surface expression of CD44 and CD27 analyzed by flow cytometry at the indicated time points.
(E) Ex vivo intracellular cytokine analysis of IL-17 and IFNγ in memory cells from dLNs after 4 hr of stimulation in vitro with PMA and ionomycin.
(F) Memory cells in blood, analyzed by expression of CD45.2 at the indicated time points.
(G) Memory cells in the CNS in an unimmunized transfer recipient (naive) and on day 12 postimmunization (EAE).
(H) Ex vivo intracellular cytokine analysis of IL-17 and IFNγ in CD4$^+$ cells, gating on CD45.1$^+$ host endogenous cells and CD45.2$^+$ transferred memory cell populations taken from the CNS on day 12. Data shown are representative of at least three independent experiments with at least four mice per group; bars on graphs show mean and SD.
See also Figure S1.

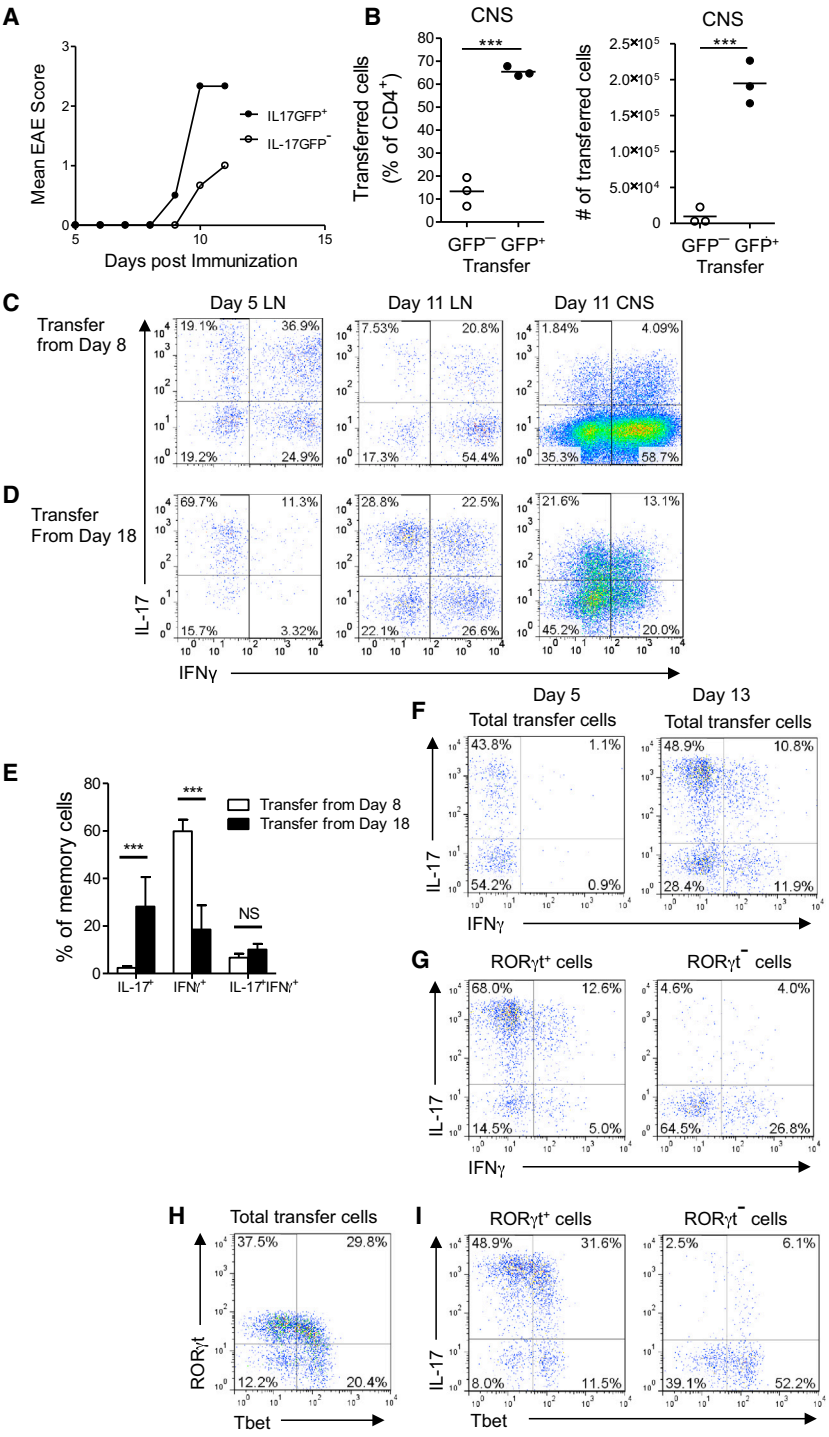

Figure 2. Th17 Memory Cells Are Generated from Primary IL-17$^+$ and RORγt$^+$ Precursors

(A–E) Donor IL-17-GFP reporter mice were immunized with MOG(35-55) in CFA, and dLNs and spleens were harvested 8 days (A–C) or 18 days (D) later. CD4$^+$CD44$^+$GFP$^+$ or GFP$^-$ cells were FACS sorted and transferred into naive CD45.1$^+$ congenic recipients. After resting for 3 weeks, recipients were immunized to induce EAE.

(A) Mean clinical scores of immunized recipients of IL-17-GFP$^+$ or IL-17-GFP$^-$.

(B) Percentage and number of transferred IL-17-GFP$^+$ and IL-17-GFP$^-$ cells in the CNS on day 11 post-immunization.

(C and D) Intracellular cytokine analysis, following stimulation with PMA and ionomycin, of IL-17 and IFNγ gating on transferred CD45.2$^+$ cells in recipient dLNs on day 5 and day 11 postimmunization, and from the CNS on day 11.

(E) Comparison of cytokine expression in CD4$^+$CD45.2$^+$ day 8 and day 18 transfer cells taken from the CNS of immunized recipients on day 11.

(F–I) Donor RORγtGFP reporter mice were immunized with MOG(35-55), and dLNs and spleens were harvested 18 days later. CD4$^+$CD44$^+$GFP$^+$ or GFP$^-$ cells were FACS sorted and transferred into naive CD45.1$^+$ congenic recipients. After resting for 3 weeks, recipients were immunized to induce EAE.

(F) Intracellular cytokine analysis of transferred cells in LNs of immunized recipients of RORγtGFP$^+$ cells, gated on CD4$^+$CD54.2$^+$ cells.

(G) Intracellular cytokine analysis, gating on CD4$^+$CD45.2$^+$ RORγt$^+$ (GFP$^+$) and RORγt$^-$ (GFP$^-$) cells in LNs of immunized recipients of RORγtGFP$^+$ cells.

(H) T-bet and RORγt (analyzed by GFP) expression in CD4$^+$CD45.2$^+$ cells from LNs of immunized recipients of RORγtGFP$^+$ transfers.

(I) Expression of IL-17 and T-bet following PMA and ionomycin stimulation, gating on CD4$^+$CD45.2$^+$ RORγt$^+$ (GFP$^+$) and RORγt$^-$ (GFP$^-$) cells in LNs on day 13 postimmunization from recipients of RORγtGFP$^+$ cells. Data shown are representative of at least two independent experiments with three to four mice per group; bars on graphs show mean and SD. See also Figure S2.

sorted from memory populations activated in vivo in the presence of anti-IL-23R or an isotype control antibody. Again, gene expression for *Il-17a* was not different between the two populations, confirming the sort integrity (Figure 5G). *Il21* and *Il1r1* expression were not significantly altered by IL-23R blockade (Figures 5H and 5I), suggesting that IL-23 is not absolutely required for production of IL-21 or responsiveness to IL-1R1, two factors known to be required for Th17 development in vivo (Korn et al., 2007; Nurieva et al., 2007; Sutton et al., 2006). A similar pattern of cell-cycle-associated gene downregulation was observed in memory IL-17$^+$ cells that had been activated in vivo in the absence of IL-23 signals (Figure 5J).

IL-23 could regulate these proliferation-associated genes directly or indirectly by activating "intermediary" transcription factors. We therefore asked which of the genes we had identified were upregulated within a short time frame and therefore likely to be directly regulated by IL-23. IL-17$^+$ cells were isolated from LNs on day 4 postimmunization with MOG(35-55)-CFA and stimulated in vitro with IL-23 only, for 2 hr. *Tfdp1*, *Tfdp2*,

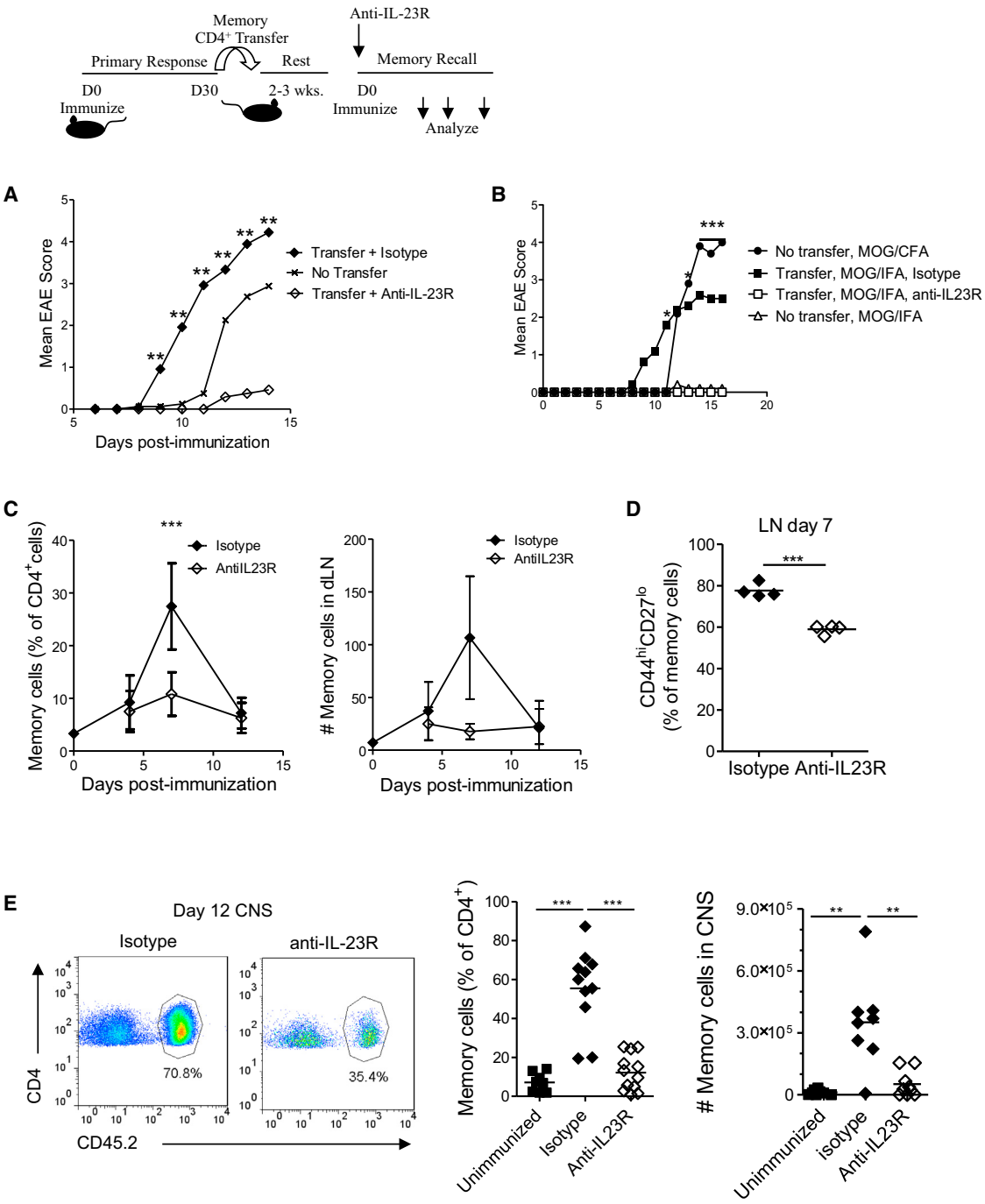

Figure 3. IL-23 Is Required for Secondary Activation of the Th17 Memory Response

C57Bl/6 mice were immunized with MOG(35-55), dLN and spleen were harvested 30 days later, and $CD4^+$ cells were transferred into naive $CD45.1^+$ congenic recipients. After resting for 2–3 weeks, recipients were immunized to induce EAE and either anti-IL-23R or isotype control antibody was administered on the day of EAE induction.

(A) Mean EAE scores of memory transfer recipients after immunization.

(B) EAE scores of mice transferred as in (A) and immunized with MOG(35-55) emulsified in IFA.

(C) Time course of the percentage and number of transferred memory cells in LNs, analyzed by flow cytometry for $CD45.2^+$ surface expression.

(D) Percentage of transferred memory cells that were effector cells ($CD44^{hi}CD27^{lo}$), analyzed by flow cytometry from dLNs on day 7.

(E) Percentage and number of transferred memory cells in CNS on day 12 postimmunization. Data shown are representative of at least three independent experiments with four mice per group (in some plots, data are pooled from multiple experiments); bars on graphs show mean and SD.

See also Figures S1, S3, S4, and S5.

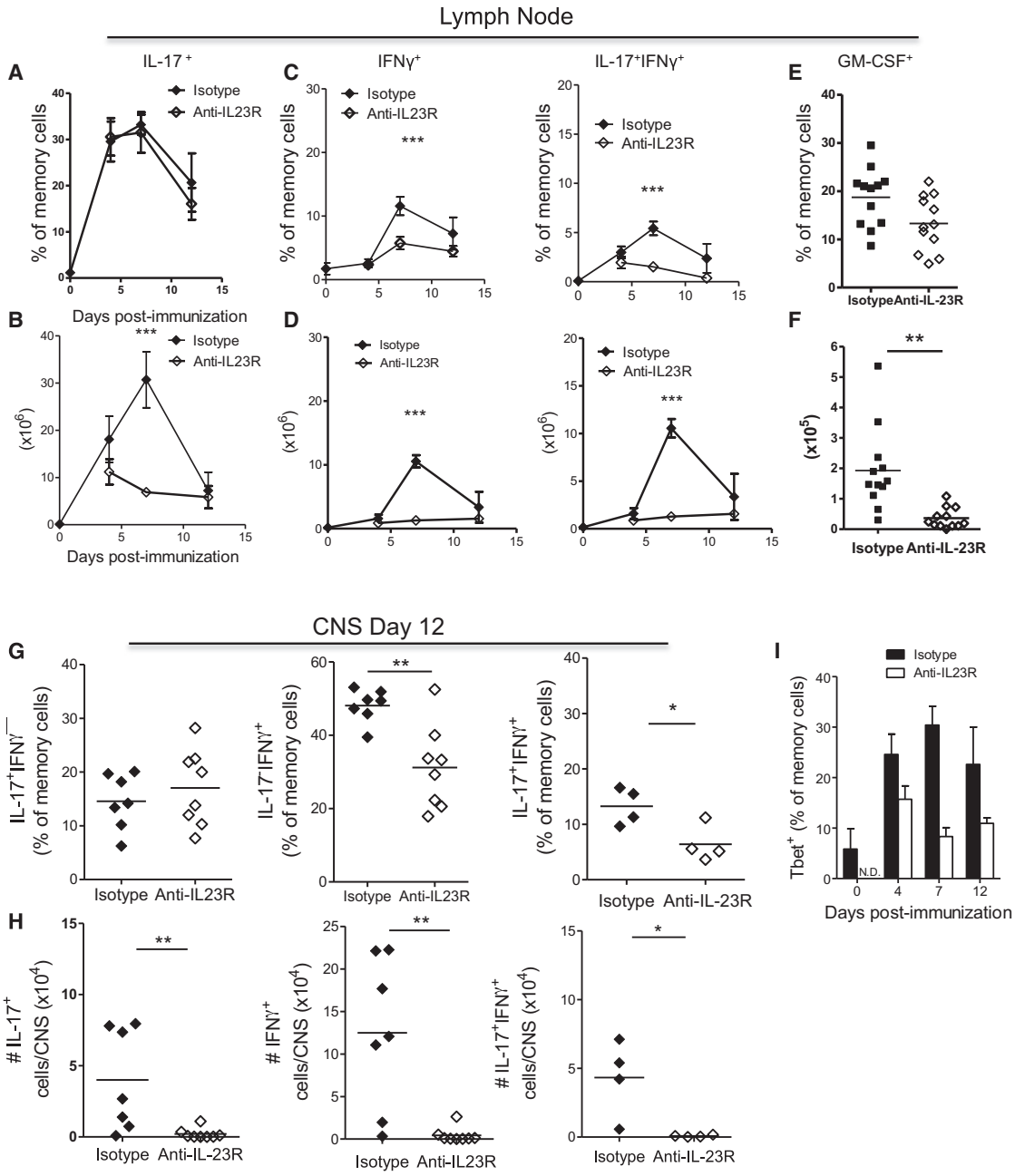

Figure 4. Anti-IL-23R Inhibits the Generation of Large Numbers of Cytokine-Producing Effectors

Memory cells were transferred and recipients immunized as in Figure 3.

(A and B) Time course of IL-17$^+$ memory cells (transferred and activated as in Figure 3) from LNs, analyzed following 4 hr stimulation ex vivo with PMA and ionomycin at the indicated time points.

(C and D) Time course of percentage and number of IFNγ$^+$ and IL-17$^+$IFNγ$^+$ cells cells in LN.

(E and F) Time course of percentage and number of GM-CSF$^+$ transferred memory cells from LN.

(G and H) Percentage and number of IL-17$^+$, IFNγ$^+$, and IL-17$^+$IFNγ$^+$ cells in CNS on day 12, analyzed by intracellular cytokine staining.

(I) Percentage of T-bet$^+$ transferred memory cells in LNs on day 13. Data shown are representative of at least three independent experiments with at least four mice per group (except for E and F, which show two independent experiments). Bars on graphs show mean and SD.

See also Figure S1.

and *E2f2* were rapidly upregulated in response to IL-23 (Figures 5K–5M). The function of these genes remains to be tested, but these data strongly suggest that many of the genes that require IL-23R expression are activated indirectly, and that IL-23 activates key transcription factors in addition to STAT3 to mediate its downstream proliferation effects.

When we compare the primary and secondary requirements for activation of Th17 cells, it is clear that IL-23 is important for both stages. Blockade of IL-23 resulted in fewer effector cells migrating to the blood and tissues, as well as fewer IFNγ^+ cells, and this was associated with reduced proliferation in both the primary and recall responses, although the effects on proliferation were most pronounced in the memory response. Although we did not find regulation of the antiapoptotic factor Bcl2, the observed reduction of multiple proteins required for DNA repair pathways, such as BRCA1 and BRCA2, would increase the susceptibility of dividing cells to apoptosis, further reducing the number of cycling cells when IL-23 is absent. In this regard, it is interesting that IL-1, another cytokine that is critical for Th17 development in both mice and humans, has been shown to promote proliferation of Th17 cells through activation of the mTOR pathway (Gulen et al., 2010). IL-1 is required early in Th17 development (Chung et al., 2009), whereas the effects of IL-23 become more apparent later (McGeachy et al., 2009). Whether IL-1 and IL-23 cooperate directly to promote proliferation or act at different stages of development remains unclear.

Kryczek et al. (2011) recently characterized memory Th17 cells found in three types of chronic inflammation of the digestive system in human patients: chronic graft-versus-host disease, ulcerative colitis, and colon cancer. They found that Th17 cells were a more stable population than was previously reported, in agreement with our finding that the IL-17-producing Th17 phenotype was recapitulated in the memory response. Supporting the role of human Th17 memory cells in chronic disease, Kryczek et al. also reported that these cells appear to be a robust population that is resistant to apoptosis and is associated with high expression of the antiapoptotic genes BCLXL and Bcl2. In their experiments, viability was not influenced by IL-23; rather, HIF1 was proposed to regulate Bcl2. Importantly, human Th17 memory cells were also found to have greater proliferative capacity than other subsets. In our system, we also found that autoimmune IL-17$^+$ cells had increased proliferation compared with their IL-17$^-$ counterparts, and further demonstrated that multiple cell-cycle-associated genes were strongly regulated by IL-23. Therefore, our results correspond with and extend findings that are emerging from studies of human samples, and highlight the potential therapeutic utility of neutralizing IL-23 in chronic inflammatory and autoimmune diseases associated with memory Th17 cells.

EXPERIMENTAL PROCEDURES

Mice

Il23ra$^{-/-}$OTII$^+$CD45.1$^+$ mice (Chan et al., 2006) were generated at Merck (Palo Alto, CA, USA). C57Bl/6 and CD45.1$^+$ mice purchased from The Jackson Laboratory (Bar Harbor, ME, USA). IL-17-GFP reporter mice were purchased from Biocytogen (Worcester, MA, USA). RORγt-GFP reporter mice were a kind gift from Dr. Gerard Eberl (Institut Pasteur, Paris). The animals were housed in an AAALAC-approved facility and procedures were approved by the Merck Palo Alto IACUC committee.

Memory Cell Transfers

Donor C57Bl/6 mice (or IL-23R-GFP reporter mice where indicated) were immunized with 100 μg MOG(35-55) in 200 μl CFA containing 100 μg heat-killed *M. tuberculosis* H37Ra (Difco, Detroit, MI, USA) in four sites on the back. For IL-23R neutralization, a single dose of 800 μg anti-IL-23R (21A4) or isotype control was delivered intraperitoneally at the indicated time points to either donor or recipient mice. Draining LNs (dLNs) and spleens were harvested 30 days postimmunization, and CD4$^+$ cells were isolated by magnetic separation using CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Seven million CD4$^+$ cells were transferred intravenously into naive CD45.1 congenic recipients. For IL-17-GFP and RORγt-GFP transfers, donors were immunized as before, and dLNs were harvested on either day 8 or day 18 as indicated. Cells were labeled with fluorescent antibodies for CD4/CD44 and were sorted into CD44^{hi}GFP$^+$ and CD44^{hi}GFP$^-$ populations. A total of 200,000 cells were transferred into naive congenic recipients.

EAE Induction

EAE was induced 2–3 weeks following transfer of memory cells. Recipient mice or untransferred controls were immunized with 100 μg MOG(35-55) in 200 μl CFA containing 100 μg heat-killed *M. tuberculosis* H37Ra in four sites on the back and received 100 ng pertussis toxin (List Biological Laboratories, Campbell, CA, USA) intraperitoneally on days 0 and 2. EAE was assessed according to the following clinical grades: 1 = flaccid tail; 2 = impaired righting reflex and hindlimb weakness; 3 = partial hindlimb paralysis; 4 = complete hindlimb paralysis; 5 = hindlimb paralysis with partial forelimb paralysis; 6 = moribund/dead. Mouse anti-mouse IL-23R monoclonal antibody (21A4) and isotype control (27F11, mouse IgG1) were generated at Merck, and 700 μg/mouse was administered intraperitoneally.

Flow Cytometry

LNs, blood, and CNS mononuclear cells were isolated as previously described (Cua et al., 2003; Langrish et al., 2005) from mice with EAE and single-cell suspensions were obtained. For cytokine analysis, cells were cultured in complete medium (RPMI media containing 10% fetal calf serum supplemented with Pen-Strep, L-Glutamine, HEPES, sodium pyruvate, and 2-ME) with 50 ng/ml phorbol myristate acetate (PMA) and 500 ng/ml ionomycin (both Sigma-Aldrich, St. Louis, MO, USA) in the presence of Golgiplug (BD Biosciences, San Jose, CA, USA) for 3–4 hr, followed by fluorescence-activated cell sorting (FACS) staining and analysis. The following FACS antibodies were purchased from BD Biosciences: CD4 (RM4-5), CD45.1 (A20), CD45.2(104) CD44 (IM7), CD27 (LG.3A10), CCR6 (140706), Ki67 (B56), IFN-γ (XMG1.2), IL-17 (TC11-18H10), and IL-2 (JES6-5H4). Ki67 and intracellular cytokine staining were performed using the Cytofix/Cytoperm kit (BD) according to the manufacturer's instructions. T-bet (eBio4B10) and GM-CSF (MP1-22E9) were purchased from eBioscience.

OTII Cell Transfer and Sorting

Recipient mice (CD45.2$^+$) received 10^5 CD45.1$^+$ *Il23ra*$^{-/-}$, *Il23ra*$^{+/+}$ OTII CD4$^+$ T cells intravenously 1 day before immunization with 100 μg OVA(323-339) in CFA subcutaneously in the flank. On day 7 postimmunization, dLNs were harvested and cells were stimulated with PMA and ionomycin for 2.5 hr. Cells were stained for CD4, CD45.1, and intracellular IL-17, with the addition of ribonucleoside vanadyl complexes (Sigma) before they were sorted on a FACSAria cell sorter based on IL-17 expression. The sorted cells were snap-frozen at −80°C and then processed for RNA analysis.

Real-Time PCR

Total RNA was isolated using the High Pure FFPE RNA Micro Kit (Roche, Mannheim, Germany) per the manufacturer's protocol. DNase-treated total RNA was amplified using the NuGen WT-Ovation Pico RNA Amplification System (NuGen Technologies, San Carlos, CA, USA) per the manufacturer's instructions. Primers were designed using Primer Express (Applied Biosystems/Life Technologies, Foster City, CA, USA) or obtained commercially from Applied Biosystems. Real-time quantitative PCR on 10 ng of amplified complementary DNA (cDNA) from each sample was performed on the Fluidigm BioMark system (Fluidigm, Foster City, CA, USA). Ubiquitin levels were measured in a separate reaction and used to normalize the data by the δ-δ Ct method.

Statistics

When appropriate, a one-way ANOVA (for multiple groups) or Student's t test was performed for experiments with parametric values (such as FACS percentage). The Mann-Whitney test or Kruskal-Wallis test was performed for

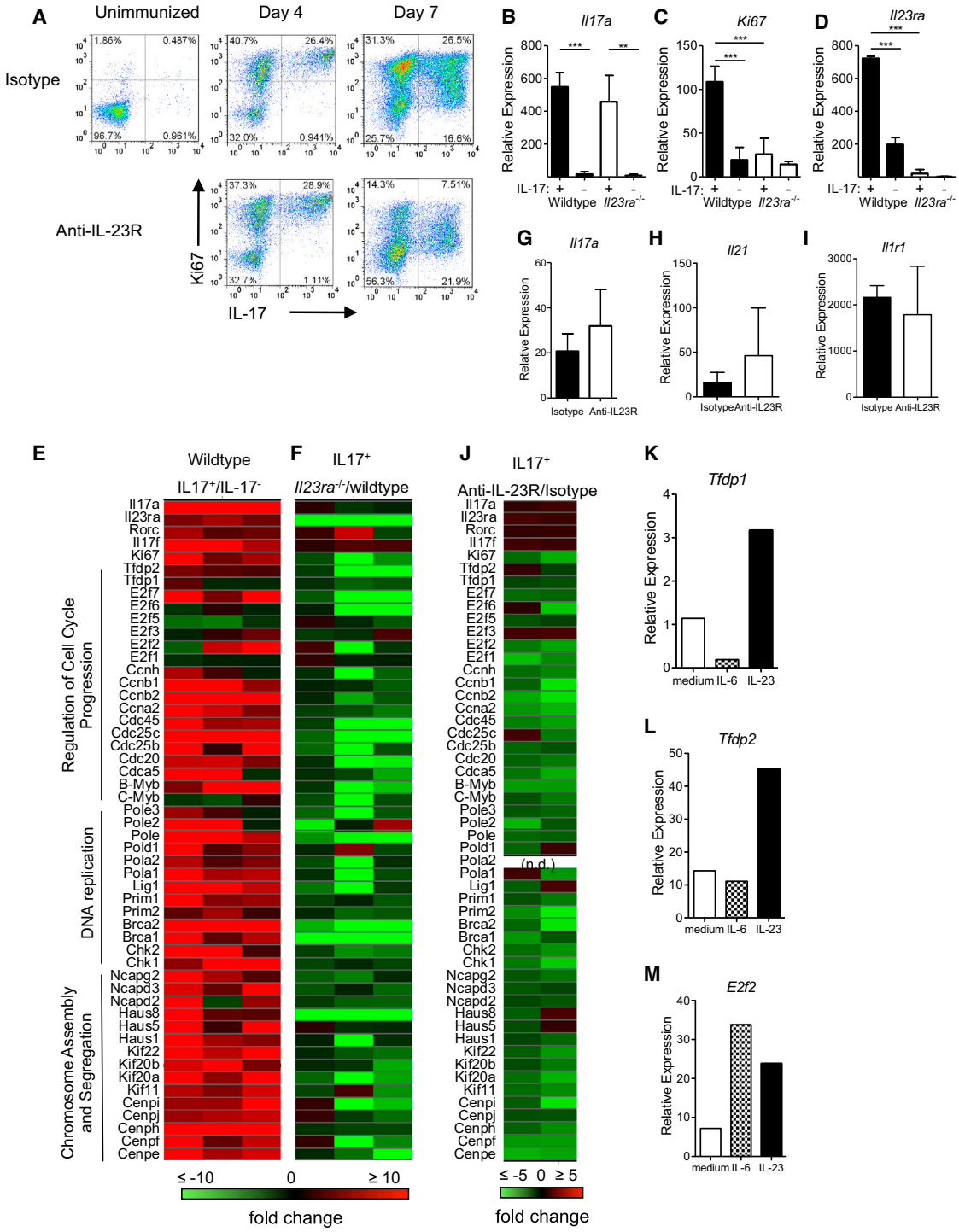

Figure 5. IL-23 Regulates the Cell Cycle to Expand Both Primary and Memory Th17 Cells

(A) Memory cells were transferred and recipients immunized as in Figure 3. Expression of Ki67 and IL-17 was analyzed in LNs by flow cytometry at the indicated time points.

(B–F) C57Bl/6 mice received either WT or *Il23ra*$^{-/-}$ OTII^{+}CD45.1^{+}CD4^{+} T cells 1 day before immunization with OVA(323-339) in CFA. On day 7 postimmunization, OTII cells from dLNs were sorted based on IL-17 expression, and gene-expression analysis was performed. The relative expression of (B) *Il17a*, (C) *Ki67*, and (D) *Il23ra* is shown.

(E) Heatmap of values for the indicated genes expressed as fold change between IL-17^{+} and IL-17^{-} WT OTII cells.

(F) Heatmap of values for the indicated genes, expressed as fold change between WT IL-17^{+} and *Il-23ra*$^{-/-}$ IL-17^{+} OTII cells. An arbitrary maximum threshold range of −10 to +10 was selected, with any values falling outside of that range designated as the maximum value.

(legend continued on next page)

EAE experiments, with scores for each day analyzed separately; p values are shown as *p < 0.05, **p < 0.01, and ***p < 0.001 where statistical significance was found, and all data are represented as means ± SD.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.03.035.

LICENSING INFORMATION

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(G–M) Donor IL-17-GFP reporter mice were immunized with MOG(35-55) as before, and 30 days later $CD4^+$ cells were transferred into naive $CD45.1^+$ congenic recipients. After resting for 2–3 weeks, recipients were immunized to induce EAE and given either isotype or anti-IL-23R on day 0 of immunization. On day 7 postimmunization, dLNs were harvested and $IL\text{-}17^+$ and $IL\text{-}17^-$ fractions were FACS sorted for gene-expression analysis.
(G–I) Relative expression of *Il17a*, *Il21*, and *Il1r1*.
(J) Heatmap of gene expression in $IL\text{-}17^+$ memory cells, with values expressed as fold change between $IL\text{-}17^+$ memory cells from isotype control-treated and $IL\text{-}17^+$ memory cells from anti-IL-23R-treated recipients.
(K–M) IL-17-GFP reporter mice were immunized with MOG(35-55) in CFA and dLNs were harvested on day 4. Cells were stimulated with IL-23 or IL-6 for 2 hr and FACS sorted based on GFP; expression of the indicated cell-cycle genes was analyzed by RT-PCR.
Data shown are representative of three independent experiments (A–F) or from two independent experiments (J–M) with at least four mice per group; bars on graphs show mean and SD. See also Figure S1.

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