Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Nat Commun. Author manuscript; available in PMC 2014 November 06.

Published in final edited form as:

Nat Commun. ; 5: 3753. doi:10.1038/ncomms4753.

IL-21 induces IL-22 production in CD4+ T-cells

Ada Yeste[1,*], **Ivan D. Mascanfroni**[1,*], **Meghan Nadeau**[1], **Evan J. Burns**[1], **Ann-Marcia Tukpah**[1], **Andrezza Santiago**[1], **Chuan Wu**[1], **Bonny Patel**[1], **Deepak Kumar**[2], and **Francisco J. Quintana**[1]

[1]Center for Neurologic Diseases, Brigham and Women's Hospital, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA

[2]Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Avenue, Boston 02115, MA, USA

Abstract

IL-22 produced by innate lymphoid cells (ILCs) and CD4+ T cells plays an important role in host defense and mucosal homeostasis, thus it is important to investigate the mechanisms that regulate IL-22 production. We investigated the regulation IL-22 production by CD4+ T cells. Here we show that IL-21 triggers IL-22, but not IL-17 production by CD4+ T cells. STAT3, activated by IL-21, controls the epigenetic status of the *il22* promoter and its interaction with the aryl hydrocarbon receptor (AhR). Moreover, IL-21 and AhR signaling in T cells control IL-22 production and the development of dextran sodium sulfate-induced colitis in ILC-deficient mice. Thus, we have identified IL-21 as an inducer of IL-22 production in CD4+ T cells *in vitro* and *in vivo*.

Introduction

IL-22 is a member of the IL-10 family of cytokines; cytokines in this family display homologous secondary structures and 20%-30% amino acid identity[1]. IL-22 acts on non-hematopoietic cells in epithelial tissues, which express a functional IL-22 receptor composed of an heterodimer of IL-22R1 and IL-10R2[2, 3]. IL-22 signaling triggers the production of anti-microbial peptides and pro-inflammatory molecules, and it also promotes tissue repair by inducing epithelial cell proliferation and survival[2, 3]. These effects of IL-22 are thought to be responsible for its important role in the control of host defense against bacterial infections, and also in limiting tissue destruction during hepatitis, colitis, graft versus host disease and thymus involution[4-9]. Conversely, dysregulated IL-22 signaling

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use:http://www.nature.com/authors/editorial_policies/license.html#terms

Corresponding author: Francisco J. Quintana fquintana@rics.bwh.harvard.edu, Center for Neurologic Diseases, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.

*These authors contributed equally to the work

Authors' contribution: A.Y., I.D.M, E.J.B., M.N., A-M.T, A.S. and D.K. performed *in vitro* and *in vivo* experiments, B.P. performed bioinformatics analysis, C.W. provided unique reagents, A.Y and F.J.Q. wrote the manuscript and F.J.Q. supervised the study and edited the manuscript.

Competing financial interests. The authors declare no competing financial interests.

Accession codes: Microarray data has been deposited in the GEO database under accession code GSE55509.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

contributes to the pathogenesis of psoriasis, arthritis, inflammatory bowel disease and colon cancer[10-13].

IL-22 is produced by cells of the innate system, particularly innate lymphoid cells (ILCs), and also by CD4+ T cells, CD8+ T cells and γδ T cells[2, 3]. ILCs expressing the transcription factor RORγt are an important source of IL-22 *in vivo*[14]. Following activation with IL-23 or IL-1β, RORγt+ ILCs produce IL-22 through a mechanism controlled by the ligand-activated transcription factor aryl hydrocarbon receptor (AhR)[15-17]. AhR signaling is also critical for the development of ILCs, thus it has been postulated that AhR ligands in the diet or produced by commensal bacteria regulate ILC development and function[15-17]. However, commensal bacteria can also promote IL-22 production through AhR-independent mechanisms, for example by inducing the production of IL-1β and IL-23 by myeloid cells[18, 19].

CD4+ T cells also produce significant amounts of IL-22. We[20] and others[21-23] have shown that AhR signaling regulates the production of IL-22 by Th17 cells differentiated in the presence of IL-6 and TGFβ1. Indeed, the production of IL-22 by IL-17+ CD4+ T cells has been shown to limit tissue destruction during experimental acute hepatitis[7] and inflammatory bowel disease (IBD)[8, 24]. Although IL-22 expression is usually linked to Th17 cells[25], human and murine CD4+ T cells that produce high amounts of IL-22 but not IL-17 have also been identified[13, 25-28]. These CD4+ IL-22+ IL-17- T cells play a protective role in mucosal antimicrobial host defense[9, 29]. Thus, considering the significant role of IL-22 produced by CD4+ T cells in host defense and mucosal barrier integrity it is important to investigate the mechanisms that regulate IL-22 production in T cells.

In vitro, the activation of naïve CD4+ T cells in the presence of cytokines that trigger STAT3 activation, such as IL-6, triggers the production of IL-22 but almost no IL-17[13]. IL-21, a cytokine that belongs to the type I four-α-helical-bundle family of cytokines, can replace IL-6 during the differentiation of Th17 cells[30, 31]. Thus, in this work we investigated the role of IL-21 in the differentiation of IL-22-producing CD4+ T cells. We found that IL-21 induces the differentiation of CD4+ T cells that produce IL-22, but not IL-17, by a mechanism that involves the transcription factors STAT3, AhR and RORγt. Moreover, AhR and IL-21 signaling control IL-22 production by CD4+ T cells that limit mucosal inflammation in ILC-deficient mice. Taken together, these results indicate that IL-21 signaling controls IL-22 production by T cells that limit mucosal inflammation.

Results

IL-21 induces IL-22 expression in CD4+ T cells

IL-6 triggers the production of IL-22 by naïve CD4+ T cells in the absence of IL-17 production[13]. Based on the ability of IL-21 to replace IL-6 during the differentiation Th17 cells[30, 31], we investigated the role of IL-21 in the differentiation of IL-22 producing CD4+ T cells. We found that the activation of naïve CD4+ T cells with antibodies to CD3 and CD28 in the presence of IL-21 results in the production of IL-22 but not IL-17 (Figs. 1a-b); no significant changes in IFNγ expression were detected. The production of IL-22 induced by activation with IL-21 was comparable to that observed following activation with IL-6

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(Supplementary Fig. 1a). Moreover, in agreement with previous reports[31, 32] we found that exogenous IL-21 also up-regulated its own expression in T cells (Fig. 1c), suggesting that a positive feed forward loop stabilizes IL-22 production in IL-21 stimulated T cells. The up-regulation of *il21* expression was reduced by TGFβ1 blockade, suggesting that it is boosted by trace amounts of TGFβ1 provided either by the medium or the T cells (Supplementary Fig. 1b).

The production of IL-22 by T cells activated in the presence of IL-21 was associated to proliferation, peaking at 3 cell divisions as determined by staining with carboxyfluorescein succinimidyl ester (CFSE)(Fig. 1d). To investigate the stability of IL-22 producing T cells induced with IL-21, naïve CD4+ T cells were initially activated in the presence of IL-21, rested, and reactivated in the presence of IL-21, or under Th17 (IL-6 and TGFβ1) or FoxP3 iTreg (TGFβ1) polarizing conditions. The re-stimulation of T cells that have been previously activated in the presence of IL-21 resulted in a significant population of IL-17- IL-22+ T cells, similar results were found upon reactivation in the presence of IL-21 (Supplementary Fig. 2c). Re-stimulation under Th17 polarizing conditions resulted in similar numbers of IL-17- IL-22+ T cells, however under these experimental conditions we also detected the generation of IL-17 producing T cells (IL-17+ IL-22+ and IL-17+ IL-22- T cells) (Supplementary Fig. 2c). Similar results were obtained following re-activation under FoxP3 iTreg polarizing conditions, probably as a result of the promotion of Th17 cell differentiation by exogenous TGFβ1 acting in combination with T-cell produced IL-21[30, 31] (Supplementary Fig. 2c). These results suggest that IL-22 producing T cells induced with IL-21 are relatively stable, and that additional Th17 cells can be differentiated from non-polarized T cells in the culture.

Since IL-6 and IL-23 have also been shown to trigger the production of IL-22 by CD4+ T cells[9, 13, 28, 29], we studied the effects of IL-21 on the expression of IL-6R, IL-23R and IL-21R. T-cell activation in the presence of IL-21 resulted in a significant up-regulation of *il23r* expression, but did not modify the expression of *il6r* or *il21r* (Fig. 1e), suggesting that IL-21 signaling may also modulate the production of IL-22 triggered in CD4+ T cells by IL-23. Similar levels of *il21r* and *il23r* expression were observed following T-cell stimulation in the presence of IL-21 or IL-6 (Supplementary Fig. 1d). In accordance with these findings, we found a significant synergism between IL-21 and IL-23 in inducing IL-22 expression in CD4+ T cells (Fig. 1f,g). IL-23, however, did not synergize with IL-21 to boost *il21* expression by T cells (Fig. 1h).

IL-1β boosts IL-22 production by Th17 cells[33], thus we investigated the effects of IL-1β in the production of IL-22 triggered by IL-21. We found that IL-21 up-regulated *il1r1* expression in T cells, this up-regulation was partially dependent on TGFβ1 signaling (Fig. 1i and Supplementary Fig. 1d). Moreover, IL-1β synergized with IL-21 in inducing the production of IL-22, but not of IL-17 (Fig. 1j,k). IL-1β, however, did not boost *il21* expression induced by IL-21 (Fig. 1l). Taken together, these results suggest that IL-21, alone or in combination with IL-23 or IL-1β, triggers IL-22 production independently of IL-17 in CD4+ T cells.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Transcriptional profiling of IL-21-stimulated CD4+ T cells

To study the molecular mechanisms mediating the production of IL-22 by CD4+ T cells stimulated with IL-21, we analyzed the mRNA expression profile by whole-genome microarrays. We compared the mRNA expression profiles of naïve CD4+ T cells activated in the presence of IL-21, IL-6 and TGFβ1 (Th17 cells) or without the addition of exogenous cytokines (Th0 cells). We found 869 genes that were up- or down-regulated at least 1.5 fold in CD4+ T cells stimulated in the presence of IL-21 relative to their expression in control Th0 cells (Fig. 2a). Principal-component analysis (PCA) showed that the transcriptional signature of CD4+ T cells treated with IL-21 is significantly different from that of Th17 cells differentiated with IL-6 and TGFβ1 (Fig. 2b). Although there was a partial overlap between genes up-regulated in IL-22+ IL-17- T cells and Th17 cells, a direct comparison of their transcriptional profile identified 179 genes with differences in expression. We found significant differences in the expression of key cytokines, chemokines, receptors and transcription factors recently associated with Th17 cells differentiated *in vitro* with TGFβ1 and IL-6[34] (Fig. 2c). We focused our attention on transcriptional modules recently identified to control Th17 cell differentiation: a module of "Th17-positive factors" that promote Th17 cell differentiation and a module of "Th17-negative factors" that interferes with this process[34]. We found that IL-22 producing T cells induced with IL-21 differed from Th17 cells in the expression of genes belonging to these Th17-positive and Th17-negative transcriptional modules (Fig. 2d).

On the basis of the biological function of their products, we selected a representative subset of genes coding for transcription factors such as *ahr, rora, rorc, tbx21, socs3*; granzymes A and B (*gzma and gzmb*) which have been linked to effector and regulatory T cells[35]; the IBD linked mucin 1 (*muc1*), known to be a transcriptional target of STAT3[36-38], and the inhibitors of IL-10 production metallothioneins 1 and 2 (*mt1, mt2*)[34]and confirmed their differences in expression by qPCR (Fig. 2e). The up-regulation of AhR and RORγt expression was also confirmed at the protein level by western blot (Fig. 2f). However, and in agreement with previous reports on IL-22 producing T cells generated with IL-6[28], this up-regulation was promoted by trace amounts of TGFβ1 provided either by the medium or the T cells, because it could be inhibited with TGFβ1-specific blocking antibodies (Supplementary Fig. 2a). Of note, activation of T cells in the presence of IL-21 did not result in a significant up-regulation of *tbx21*, which has been recently linked to the production of IL-22 triggered in CD4+ T cells IL-6[29]. We also failed to detect *foxp3* expression in T cells activated in the presence of IL-21 (Supplementary Fig. 2b).

Finally, we used Ingenuity Pathways Analysis to analyze the genes found to be differentially expressed between Th0 and IL-22+ IL-17- T cells. The resulting model identified important roles for STAT3, RORγt and AhR in the control of IL-22+ IL-17- T cell differentiation triggered by IL-21 (Fig. 2g). In conclusion, these data suggest that IL-21 triggers a transcriptional program in CD4+ T cells that differs from the one of Th17 cells and is controlled by STAT3, RORγt and AhR.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

STAT3 is required for IL-21 induced IL-22 expression

Based on our transcriptional analysis and the reported activation of STAT3 by IL-21 signaling, we studied the role of STAT3 in the production of IL-22 triggered by IL-21. T-cell activation in the presence of IL-21 led to a significant phosphorylation of STAT3, and also to a significant increase in *stat3* expression (Fig 3a-b). T-cell activation in the presence of IL-6, with or without TGFβ1-blocking antibodies, resulted in similar amounts of STAT3 phosphorylation (Supplementary Fig. 3a).

To investigate the function of STAT3 in the regulation of IL-22 production, we activated wild type (WT) and STAT3-deficient CD4+ T cells in the presence of IL-21. The production of IL-22 in response to IL-21 was significantly decreased in STAT3-deficient CD4+ T cells (Fig. 3c). Moreover, in agreement with our bioinformatic analysis STAT3 was also required for the up-regulation of *il21*, *ahr* and *rorc* expression in response to IL-21 (Fig. 3d,e).

Of note, we also found that STAT3 mediated the upregulation of *il23r* and *il1r1*expression in response to IL-21 (Fig. 3f). However, IL-23 or IL-1β did not up-regulate *stat3* expression when their effects on CD4+ T cells were analyzed alone or in combination with IL-21 (Fig. 3g). Taken together, these data suggest that STAT3 plays a central role in the differentiation of IL-22 producing CD4+ T cells in response to IL-21.

RORγt and AhR control IL-22 expression induced by IL-21

The up-regulation of *rorc* expression was linked to the induction of IL-22 expression in CD4+ T cells triggered by IL-21 (Fig. 2). Based on our studies on the transcriptional effects of IL-21 on CD4+ T cells and the reported role of RORγt in the control of *il22* expression in Th17 cells and ILCs[16, 39] we investigated the role of RORγt in the production of IL-22 triggered by IL-21. We found that RORγt deficiency led to a significant reduction in the production of IL-22 induced by IL-21 in naïve CD4+ T cells (Fig. 4a). RORγt-deficiency also decreased the expression of *il23r* but not of *il1r1*, and abrogated the ability of IL-23 to boost IL-22 production triggered by IL-21 (Fig 4b-c). Conversely, activation of naïve T cells in the presence of IL-23 or IL-1β alone did not result in a significant upregulation of *rorc* expression, but IL-23 synergized with IL-21 to boost *rorc* expression (Fig. 4d). Thus, similarly to what has been described for Th17 cells and ILCs, RORγt controls the production of IL-22 by CD4+ T cells treated with IL-21. Moreover, these data suggest that in addition to its effects on the production of IL-22 triggered by IL-21, RORγt also controls the ability of IL-23 to synergize with IL-21 and boost IL-22 expression in T cells.

We[20] and others[21-23] found that AhR activation modulates IL-22 production in Th17 cells. Moreover, AhR also participates in the production of IL-22 triggered by IL-6[28]. Based on these observations and the results of our transcriptional profiling of CD4+ T cells activated in the presence of IL-21 (Fig. 2), we investigated the role of AhR in the control of IL-22 production triggered by IL-21. The addition of the AhR agonist 6-formylindolo[3,2-b]carbazole (FICZ) led to a significant increase in the production of IL-22 triggered by IL-21 (Fig. 4e). Conversely, the use of the AhR specific antagonist CH-223191 resulted in a significant reduction in IL-22 production triggered by IL-21 (Fig. 4e). A similar reduction in IL-22 production was detected when we used T cells from AhR-d mice, which carry an *ahr*

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

allele coding for a mutant AhR with reduced affinity for its ligands[40] (Fig. 4f). Of note, *ahr* expression levels were not significantly up-regulated in response to T-cell activation in the presence of IL-23 or IL-1β alone, and IL-23 or IL-1β did not synergize with IL-21 to up-regulate *ahr* expression (Fig. 4g). These findings suggest that AhR plays a more important role in the regulation of IL-22 production by CD4+ T cells activated by IL-21 than in response to IL-23 or IL-1β.

AhR has been shown to interact with STAT proteins and modulate their activity[21, 22, 41]. Considering the important role of STAT3 in the production of IL-22 triggered by IL-21, we analyzed whether STAT3 activation in response to IL-21 is modulated by AhR. We found that modulation of AhR signaling with FICZ or CH-223191 had no significant effect in the phosphorylation of STAT3 in T cells in response to IL-21 (Fig. 4h). Similarly, we found no significant differences in STAT3 phosphorylation in response to IL-21 between WT and AhR-d T cells (Supplementary Fig. 3b). Taken together, these data suggest that RORγt and AhR control the IL-22 production triggered by IL-21, and these effects do not involve the modulation of STAT3 activation.

STAT3 controls the accessibility of the il22 promoter

We recently reported that AhR and STAT3 co-operate to regulate *ikzf3* expression in Th17 cells[42]. A bioinformatic analysis of the *il22* promoter revealed 2 putative STAT3 responsive elements (SRE-1 and SRE-2) upstream of the transcription start site and 3 putative binding sites for AhR (xenobiotic response elements, XRE-1, XRE-2 and XRE-3) (Fig. 5a). Thus, to investigate whether STAT3 and AhR interact with these potential binding sites in the *il22* locus we performed chromatin immunoprecipitation (ChIP) assays. We detected a significant interaction of AhR and STAT3 with the *il22* promoter in CD4+ T cells activated in the presence of IL-21 (Fig. 5b-c).

We then analyzed the functional effects of the interaction of STAT3 and AhR with its DNA responsive elements using a reporter plasmid carrying the firefly luciferase under the control of the *il22* promoter (il22:Luc). Transfection with vectors coding for AhR or constitutively activated STAT3 did not result in a significant induction of luciferase activity (Fig. 5d). However, co-transfection with constructs coding for constitutively activated STAT3 and AhR induced a significant up-regulation of luciferase activity, suggesting a synergistic effect of STAT3 and AhR in the production of IL-22 (Fig. 5d).

c-Maf interacts with AhR to transactivate the *il10* promoter[35, 43], but it suppresses *il22* expression induced with IL-6[28]. Although we detected a small up-regulation of *maf* in response to IL-21 (Supplementary Fig. 4a), we found no significant binding of c-Maf to its potential binding sites in the *il22* promoter (Supplementary Fig. 4b). These data suggest that, similarly to what has been described for IL-22+ IL-17- T cells induced with IL-6, c-Maf does not promote *il22* expression in T cells activated in the presence of IL-21[28].

Both AhR and RORγt have been shown to control IL-22 expression in Th17 cells. Indeed, AhR and RORγt physically interact, and in over-expression experiments in EL4 cells it was shown that a constitutively active mutant AhR is recruited to the *il22* locus in a RORγt-dependent manner[16]. Since we found that RORγt-deficient T cells show a significant

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

decrease in IL-22 production following activation in the presence of IL-21, we investigated whether RORγt was needed for the recruitment of endogenous AhR to its target sequences in the *il22* locus. We found that RORγt deficiency results in a significant decrease in the recruitment of AhR to the *il22* promoter (Fig. 5e). We also detected a decrease in the acetylation of histones 3 and 4 and the trimethylation of histone 3 at Lys4, concomitant with increased histone trimethylation at Lys9 and Lys 27, supporting our findings on the lack of *il22* expression in the absence of RORγt (Supplementary Fig. 4c).

Overexpression of AhR and RORγt in CD4+ T cells activated under non-polarizing Th0 conditions, however, does not result in IL-22 expression[22]. The ability of transcription factors to bind regulatory regions on target genes is affected by the structure of the chromatin, which is regulated by several processes including post-translational histone modifications[44]. Thus, we reasoned that the lack of IL-22 expression observed in CD4+ T cells overexpressing AhR and RORγt[22]might reflect the need for epigenetic mechanisms that increase the accessibility to regulatory elements in the *il22* promoter. First, we analyzed the acetylation status of histone H3 and H4 in the *il22* promoter of naive T cells activated in the presence of IL-21. We found a significant increase in the acetylation of histones H3 and H4 in CD4+ T cells activated in the presence of IL-21 (Fig 5f), which is usually associated with a relaxed chromatin structure that facilitates the interaction of transcription factors with target sequences. We also found that T-cell activation in the presence of IL-21 results in increased histone 3 trimethylation in Lys4, associated to actively transcribed genes, concomitant with decreased trimethylation in Lys9 and Lys27, which have been associated with the silencing of transcription[45] (Fig. 5f). Of note, we detected no changes in the methylation status of the *il22* promoter (Supplementary Fig. 4d).

STAT3 binds target sequences in the *il22* promoter and is required for the production of IL-22 triggered by IL-21 (Figs. 3c and 5c). Given the reported role of STAT3 in regulating the epigenetic status of target genes[46], we investigated the role of STAT3 in regulating the epigenetic status of the *il22* locus using WT and STAT3-deficient T cells activated in the presence of IL-21. We found that the lack of STAT3 recruitment to the *il22* promoter in STAT3-deficient T cells resulted in a significant decrease in H3 and H4 acetylation in STAT3 and AhR binding sites (Figs. 5g-i). We also detected a decrease in histone 3 trimethylation at Lys4 and an increase in trimethylation at Lys9 and Lys27 in STAT3-deficient T cells (Supplementary Fig. 4e). Moreover, the reduced histone acetylation observed in STAT3-deficient T cells was concomitant with a reduced recruitment of AhR to the *il22* promoter (Fig. 5j). Taken together, these results demonstrate that in addition to its effects on the expression of AhR and RORγt, STAT3 activated in response to IL-21 controls the accessibility to regulatory elements in the *il22* promoter recognized by AhR and probably additional transcription factors.

IL-21 and AhR control IL-22 expression in vivo

IL-22 maintains the integrity of the colonic epithelium during inflammation[8, 24]. Dextran sodium sulfate (DSS)-induced colitis is thought to result from the disruption of the epithelial barrier in the colon, resulting in the translocation of commensal bacteria, the activation of innate immunity and up-regulation of IL-23 and IL-6[47].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

IL-22 plays a central role in the control of intestinal inflammation[2, 3]. Although both ILCs and CD4+ T cells produce IL-22 during the course of DSS-induced colitis, ILCs constitute a more abundant source of IL-22 in WT mice (Supplementary Fig. 5). The development of ILCs is regulated by IL-7 signaling through the IL-2 receptor common gamma chain (IL-2RG)[48]. Thus, to focus our studies on the role of IL-22 producing CD4+ T cells during gut inflammation, we used mice deficient in both RAG2 and IL-2RG (RAG2/IL2RG mice); these mice lack T cells and also NK cells and ILCs[3, 17]. To study the role of AhR and IL-21 in the regulation of IL-22 production by T cells *in vivo*, we reconstituted RAG2/IL2RG mice with WT CD4+ T cells, IL-21R-deficient (IL-21R KO) T cells or AhR-d T cells. Following confirmation of successful T-cell reconstitution by flow cytometry from blood samples, we induced DSS colitis in the recipient mice and monitored weight change daily. We found a significant worsening of DSS-induced colitis in mice reconstituted with AhR-d or IL-21R-deficient T cells, manifested as increased weight loss (Fig. 6a) and colon shortening (Fig. 6b) suggestive of augmented colon inflammation. Accordingly, IL-21R KO and AhR-d mice showed increased colon inflammation upon blind histological examination (Fig. 6c).

To directly investigate the impact of IL-21 and AhR signaling in T cells on the immune response during DSS-induced colitis, we analyzed cytokine production by excised colon cultures prepared from mice reconstituted with WT, AhR-d or IL-21R-deficient T cells. We found a significant reduction in the production of IL-22 by colon explants isolated from AhR-d or IL-21R deficient mice (Fig. 6d) and a concomitant increase in the levels of the pro-inflammatory cytokines IL-6 and IL-23 (Fig. 6e). The decreased production of IL-22 correlated with a decrease in the frequency of IL-22 producing T cells in AhR-d and IL21R-deficient mice compared to WT (Fig. 6f). Of note, we did not detect significant changes in *il10* expression nor in the frequency of FoxP3+ T cells (Fig. 6g). Taken together, these results demonstrate that IL-21 and AhR signaling in T cells limit gut inflammation.

Discussion

IL-22 regulates tissue homeostasis and inflammation at mucosal surfaces[2, 3]. ILCs have been identified as an important source of IL-22 *in vivo*, but the importance of IL-22 producing T cells and the mechanisms regulating their differentiation and function are largely unknown. Human and murine γδ T cells, Th1 and Th17 cells produce IL-22[2, 3]. In addition, IL-22+ IL-17- cells named Th22 cells have been described in humans[26, 27] but it has proven difficult to identify the murine equivalent of this population. CD4+ T-cell activation in the presence of IL-6, IL-21 or IL-23 induces IL-22+ IL-17- T cells that have been linked to early host defense against *Citrobacter rodentium*[9, 13, 28, 29, 49]. Activation of Notch signaling has also been shown to induce IL-22 production by CD4+ T cells in a STAT3-independent manner[50]. These data suggest that, similarly to what is observed for human Th22 cells, IL-22 can be expressed independently of IL-17 in mouse T cells. However, until specific transcription factors are identified to act as master regulators of IL-22+ IL-17- T cells, it cannot be determined whether these cells constitute a separate CD4+ T cell lineage or simply represent an activation stage of CD4+ T cells.

IL-21 is produced by NKT cells, γδ T cells, follicular helper T cells, Th17 cells, type 1 regulatory T cells and a recently described subpopulation of mucosal CCR9+ Th cells[43, 51].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Thus, these cell populations may regulate the production of IL-21 dependent IL-22 by T cells *in vivo*. We found that IL-21 up-regulates *rorc* expression, and also *il1r1* and *il23r* in a RORγt-dependent manner. Indeed, IL-21 synergized with IL-1β and IL-23 to induce IL-22 production by CD4+ T cells. These data suggest that by increasing *il1r1* and *il23r* expression, IL-21 decreases the activation threshold for IL-1β and IL-23-triggered IL-22 production. In addition, IL-6 has also been shown to induce IL-21 production by T cells[32]. Thus, it is likely that similarly to their reported effects on Th17 cell differentiation[52], IL-21 acts in combination with IL-23, IL-1β and IL-6 to stabilize and boost the production of IL-22 by T cells *in vivo*. IL-21 can replace IL-6 during the differentiation of Th17 cells, and IL-21 and IL-6 exhibit redundancy during the differentiation of follicular T helper cells[30, 31, 51], however that redundancy is not complete *in vivo*[53]. Future studies should determine the relative contribution of IL-6 and IL-21, and also IL-1β and IL-23 during the regulation of IL-22 production by T cells and other cell populations.

Group 3 ILCs comprises an heterogeneous group of cell populations identified as a major source of IL-22 *in vivo*[2, 14, 24]. Interestingly, AhR and RORγt are reported to control IL-22 production by ILCs in response to IL-23, a known activator of STAT3 signaling[15, 16]. These reports and the data presented in this manuscript highlight the transcriptional similarities existing between CD4 T cells activated in the presence of IL-21 and group 3 ILCs, and suggest that additional transcriptional elements involved in the control of IL-22 expression might be shared by these two sources of IL-22. Of note, it has been recently shown that T-bet controls the production of IL-22 production triggered with IL-6[29]. In our experiments we did not detect an up-regulation of *tbx21* following T-cell activation in the presence of IL-21. This finding suggests that similarly to the heterogeneity that characterizes the RORγt+ IL-22+ cells included in group 3 ILCs, distinct IL-22+ IL-17- CD4+ T cell populations might be induced in response to different environmental, microbial and cytokine stimuli.

Because of the dominant role of ILCs and γδ T cells in mucosal IL-22 production we used T-cell reconstituted RAG2/IL-2RG mice to study the role of T-cell produced IL-22 in mucosal inflammation. We found that IL-21 and AhR modulate the production of IL-22 by intestinal CD4+ T cells *in vivo*. In addition, it has been reported that the protective effects of some AhR dietary ligands in the DSS colitis model are T-cell dependent, suggesting that IL-22 production by T cells plays a physiological role in the modulation of mucosal inflammation[54]. Additional support for this interpretation was recently provided by Basu *et al*, who described sequential waves of IL-22 production by ILCs and T cells responsible for host defense against *Citrobacter rodentium* infection[29]. Indeed, it is possible that specialized ILCs with antigen-presenting cell activity participate in the induction and regulation of IL-22 producing mucosal T cells in vivo[55]. The study of the factors that control IL-22 production by different cell types is likely to identify appropriate targets for the therapeutic modulation of IL-22 production.

A partial protection from DSS-induced colitis has been previously reported in complete, non-tissue specific IL-21 deficient mice[56]. We believe that the differences on the role of IL-21 on DSS-induced colitis (protective in our work versus pathogenic in[56]) might reflect differential effects of IL-21 on ILCs and CD4+ T cells and the absence of ILCs in RAG2/IL2RG mice. Alternatively, these different outcomes might result from the use of IL-21

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

deficient mice in the work of Araki *et al*[56], instead of the IL-21R KO mice used in our studies.

We found that AhR modulates IL-22 production by IL-22+ IL-17- T cells induced with IL-21, but AhR inhibition does not completely abrogate IL-22 production *in vitro*. Similar results have been recently reported for the role of AhR in the regulation of IL-22 production triggered in CD4+ T cells by IL-6[28]. Our data, however, suggest that AhR plays a more prominent role in the regulation of IL-22 production *in vivo*. A large proportion of intestinal IL-22+ T cells co-express IL-17, probably as a result of the local abundance of TGFβ1 which in combination with IL-6 and IL-21 favors the differentiation of Th17 cells that may control intestinal inflammation[8, 57]. Indeed, our experiments using blocking antibodies indicate that TGFβ1 promotes AhR expression in IL-22 producing T cells. Th17 cells differentiated in the presence of TGFβ1 express higher amounts of AhR, and consequently AhR is a stronger regulator of IL-22 expression in Th17 cells than in IL-22+ IL-17- T cells[28]. Thus, the more prominent effects of AhR in the regulation of IL-22 *in vivo* might reflect the increased role of Th17 cells as a source of intestinal IL-22. It is also possible that local AhR ligands provided by the host, the diet or the commensal flora are more potent inducers of IL-22 expression in intestinal T cells.

We and others have shown that AhR controls IL-22 and IL-10 production by Th17[20, 22, 23] and Tr1[35, 43, 58] cells. Considering the importance of IL-10 and IL-22 for the control of mucosal homeostasis and inflammation[2, 3, 59], AhR seems to be closely linked to a transcriptional program that limits immunopathology at environmental interfaces by the combined effects of IL-10 on the adaptive and the innate immune response, and the effects of IL-22 on epithelial cells. The regulation of AhR activity by endogenous, microbial, dietary and environmental ligands, provides a molecular mechanisms to adjust the mucosal production of IL-10 and IL-22. Moreover, it provides a potential target for therapeutic intervention.

Methods

Mice

Eight weeks old C57BL/6 and double RAG2- IL-2RG-deficient female mice were purchased from the Jackson Laboratories. AhR-d, IL21R- and RORγt-deficient mice were breed at Harvard Medical School, from breeders provided by the Jackson Laboratories. Mice were kept in a conventional, pathogen-free facility at the Harvard Institutes of Medicine. All mouse handling and *in vivo* experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

T-cell differentiation in vitro

Naïve CD4+ T cells were stimulated were stimulated with plate bound antibodies against CD3 (145-2C11, 2μg/ml) and CD28 (PV-1, 2μg/ml), in the presence of IL-21 (30ng/ml) or IL-21 (30ng/ml) and IL-23 (30ng/ml) to generate Th22 cells; and IL-6 (30ng/ml), TGFβ1 (3ng/ml). Mouse IL-6, IL-21, IL-23 and TGFβ1 were purchased from R&D Systems. In

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

some experiments we used TGFβ1-blocking antibodies at a final concentration of 10 ug/ml from R&D (Cat. #AF-101-NA). FICZ was obtained from Enzo Life Sciences (PA, USA) and CH-223191 from EMD Chemicals (NJ, USA). We used an RPMI-based medium to minimize the activation of the cells by tryptophan-derived AhR ligands present in other tissue culture mediums[22]. RPMI was supplemented with 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml), 10% heat-inactivated FBS (BioWhittaker, Inc.) and 2-mercaptoethanol. To evaluate the stability of IL-22 producing T cells induced with IL-21, in some experiments after an initial phase of activation in the presence of IL-21, T cells were re-stimulated with plate bound antibodies to CD3 and CD28 for 48h in the presence of medium, IL-21, TGFβ1 or TGFβ1and IL-6.

Cytokine Analysis

Secreted cytokines were measured in supernatants of differentiated CD4+ T cells *in vitro* and in supernatants of colon samples *ex vivo* after 48h by ELISA. For intracellular cytokine staining, cells were stimulated for 4h at 37C in culture medium containing PMA (50 ng/ml; Sigma, USA), ionomycin (1 μg/ml; Sigma) and GolgiStop (1 μl/ml; BD Biosciences, USA). After staining for the surface markers CD3 and CD4, cells were fixed and permeabilized using Cytofix/Cytoperm and Perm/Wash according to the manufacturer's instruction (BD Biosciences, USA; eBioscience, USA). All antibodies were purchased from Biolegend and eBioscience. Data were acquired on a FACSCalibur (BD Biosciences) and analyzed using the FlowJo software (Tree Star).

qPCR

RNA was extracted with RNAeasy columns (Qiagen, USA). cDNA was prepared using High Capacity cDNA Reverse Transcriptase kit following the manufacturer's instructions (Applied Biosystems, USA) and used as template for real-time PCR. Probes were purchased from Applied Biosystems, and were used on the ViiA™ 7 Real-Time PCR System (Applied Biosystems). Expression was normalized to the expression of *gapdh*. The following probe mixtures were used: il1r (Mm01226962_m1), *il6r* (Mm00439653_m1), *il21r* (Mm00600319_m1), *il23r* (Mm00519942_m1), *il10* (Mm0043614_m1), *il17* (Mm00439619_m1), *il21* (Mm00517640_m1), *il22* (Mm00444241_m1), ahr (Mm01291777_m1), maf (Mm02581355_s1), rora (Mm01173766_m1), rorc (Mm00441144_g1), stat3 (Mm01219775_m1), foxp3 (Mm00475156_m1), socs3, mt1 (Mm00496660_g1), mt2 (Mm00809556_s1), gzma (Mm01304452_m1), gzmb (Mm00442834_m1), tbx21 (Mm00450960_m1), gapdh (Mm99999915_g1).

Gene expression analysis

Transcriptome analysis was done using Affymetrix microarray MoGene_1_0_st on CD4+ T cells stimulated for 48 h in the presence of medium, IL-21 or IL-6 and TGFβ1. Microarray data was analyzed using GeneSpring version12.5 software package (Agilent, Santa Clara, CA). Samples were normalized using Quantile Normalization Algorithm. Fold change difference between IL-21-treated and untreated naïve CD4+ T cells was calculated to observe the differential expression profile of genes. Hierarchical clustering was performed

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

on genes with fold change >=1.5 using Euclidean distance metric to generate heat-maps. We also performed Principle Component Analysis (PCA) to observe the separation between samples using GeneSpring version 7.3 software package (Agilent, Santa Clara, CA). GEO: microarray data GSE55509.

Western blot analysis

Cells were lysed with RIPA buffer supplemented with protease inhibitor cocktail (Sigma, USA). Total T cell lysates (20 μg) were resolved on 4–12% Bis-Tris Nupage gels (Invitrogen, USA) and transferred onto PVDF membranes (Millipore). The following primary antibodies were used: Anti-AhR from Enzo Life Sciences (BML-SA210; 1/2000 dilution); anti-RORγt from Abcam (Cat. #78007; 1/1000 dilution) and anti-GAPDH from Cell Signaling Technology (Cat. #2111; 1/1000 dilution). Western blot analysis was performed as described[60] and developed using SuperSignal West Femto Maximum Sensitivity Substrate, as instructed by the manufacturer (Pierce).

Chromatin immunoprecipitation (ChIP)

Cells were cross-linked with 1% paraformaldehyde and lysed with 0.35 ml of lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1) containing 1X protease inhibitor cocktail (Roche Molecular Biochemicals, USA). Chromatin was sheared by sonication and supernatants were collected after centrifugation and diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl, pH 8.1). Five μg of antibody was prebound for a minimum of 4 h to protein A and protein G Dynal magnetic beads (Invitrogen, USA) and washed three times with ice-cold PBS plus 5% BSA, and then added to the diluted chromatin and immunoprecipitated overnight. The magnetic bead-chromatin complexes were then washed 3 times in RIPA buffer (50 mM HEPES [pH 7.6], 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5 M LiCl) followed by 2 times with TE buffer. Immunoprecipitated chromatin was then extracted with 1% SDS, 0.1 M $NaHCO_3$ and heated at 65°C for at least 6 h to reverse the paraformaldehyde cross-linking. DNA fragments were purified with a QIAquick DNA purification Kit (Qiagen, USA) and analyzed using SYBR green real time PCR (Takara Bio Inc., USA). We used the following antibodies for ChIP: anti-pSTAT3 (Cat. #9132, Cell Signaling Technology, Inc., USA), anti-AhR (Cat. #9172, Cell Signaling Technology, Inc., USA), anti-cmaf (Cat. #9172, Cell Signaling Technology, Inc., USA), anti-Acetyl-H3 (Cat. #06-599, Millipore, MA, USA), Acetyl-H4 (Cat. #06-866, Millipore, MA, USA), anti-H3K4me3 (Cat. #ab8580, Abcam, MA, USA), anti-H3K9me3 (Cat.#ab8898, Abcam, MA, USA) and anti-H3K27me3 (Cat. #6002, Abcam, MA, USA). The primer pairs used in these experiments are listed in Supplementary Table 1.

Methyl-DNA immunoprecipitation

The T cells were harvested after washing with PBS and genomic DNA was isolated using a DNeasy Blood and Tissue kit (Qiagen) and sheared by sonication to obtain DNA fragments of approximately 500 bp. Ten microgram of sheared DNA was heated at 95°C for 10 min and snap-chilled before mixing with 10 ul of freshly prepared protein A and protein G Dynal magnetic beads (Invitrogen) that were pre-incubated with 5 ug of either anti-5-methyl-cytidine antibody (Cat. #ab10805) or mouse IgG control antibody (Cat. #ab18413), both from Abcam, for a minimum of 4 hr and washed 3 times with ice-cold PBS plus 5% BSA.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

The mixture was then incubated overnight in buffer (20 mM Tris-HCl [pH 8.1], 150 mM NaCl, 2 mM EDTA, 1% Triton X-100). The bead-DNA complexes were then washed three times in RIPA buffer (50 mM HEPES [pH 7.6], 1 mM EDTA, 0.7% Na deoxycholate, 1% NP-40, 0.5 M LiCl) followed by three times with TE buffer (10 mM Tris HCl [pH 8.0], 1 mM EDTA). Immunoprecipitated DNA was then extracted by heating at 65°C in 1% SDS, 0.1 M $NaHCO_3$ for at least 6 hr. DNA fragments were purified with a QIAquick DNA purification Kit (Qiagen) and analyzed using SYBR -green real time PCR. The primer pairs used in these experiments are listed in Supplementary Table 2.

Transfection and luciferase assays

HEK293 cells were grown in DMEM supplemented with 10% fetal bovine serum, and transfected using the FuGENE HD (Roche, USA) transfection reagent according the manufacturer's instructions. *il22* promoter reporter construction was purchased from Dr. Renauld (Ludwig Institute for Cancer Research and Experimental Medicine Unit, Universite Catholique de Louvain, Brussels, Belgium). Vectors coding for AhR and c-Maf were purchased from Gene CopoeiaInc (MD, USA), the vector coding for constitutively activated STAT3 was provided by Dr. David Frank (Dana-Farber Cancer Institute, Boston, MA, USA). Firefly and renilla luciferase activity was analyzed 48 h after transfection using a dual luciferase assay kit (Promega, USA).

T-cell Transfer and Colitis induction

Sorted splenic CD4+ T cells from C57BL/6, AhR-d and IL21R knock out mice were transferred i.p.(10×10^6 cells/mouse) into double RAG2- IL-2RG-deficient mice. One month after T cell transfer, the recipient mice were checked for reconstitution of CD4+ T cells and colitis was induced with DSS. 3% DSS (MW:36.000-50.000. MP Biomedicals, LLC) was dissolved in drinking water and administered for 7 days, and weight was monitored daily. Eight days after the initiation of the treatment with DSS the mice were euthanized, the colons were dissected and their length measured from the ileocecal junction to the anal verge.

Histological evaluation of colitis

Colonic tissue was removed and used for histologic evaluation by 2 independent investigators in a blinded fashion. The total histologic score represents the sum of nonulcerative regions of epithelium and an infiltration score ranging from 0 to 6 as follows: epithelium: 0, normal morphology; 1, loss of goblet cells in small areas; 2, loss of goblet cells in large areas; infiltration: 0, no infiltration; 1, infiltration around crypt basis; 2, infiltration reaching the lamina muscularis mucosae; 3, extensive infiltration reaching the lamina muscularis mucosae and thickening of the mucosa with abundant edema; 4, infiltration of the lamina submucosa.

Ex vivo colon culture and ELISA

Sections of 1cm of the colon were excised, longitudinally halved and washed three times with sterile HBSS+Hepes medium (2% HEPES buffer, 1% penicillin/streptomycin and 0,05% gentamicin). The colon sections were placed into culture in complete Bruff's media

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

and cultured for 72h at 37°C with 5% CO_2. The supernatants were harvested and the concentrations of IL-22 (Antigenix America), IL-6 and IL-23 (eBioscience) were determined by ELISA according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the Prism software (Graph Pad software, La Jolla, CA, USA). $P<0.05$ were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants AI075285, and AI093903 from the National Institutes of Health, by a grant from the Harvard Institute of Translational Immunology/Helmsley Trust and by a pilot grant from the Harvard Digestive Diseases Center to FJQ.

References

1. Pestka S, Krause CD, Sarkar D, Walter MR, Shi Y, Fisher PB. Interleukin-10 and related cytokines and receptors. Annu Rev Immunol. 2004; 22:929–979. [PubMed: 15032600]
2. Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol. 2011; 29:71–109. [PubMed: 21166540]
3. Sonnenberg GF, Fouser LA, Artis D. Border patrol: regulation of immunity, inflammation and tissue homeostasis at barrier surfaces by IL-22. Nat Immunol. 2011; 12:383–390. [PubMed: 21502992]
4. Aujla SJ, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. Nat Med. 2008; 14:275–281. [PubMed: 18264110]
5. Dudakov JA, et al. Interleukin-22 drives endogenous thymic regeneration in mice. Science. 2012; 336:91–95. [PubMed: 22383805]
6. Hanash AM, et al. Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. Immunity. 2012; 37:339–350. [PubMed: 22921121]
7. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Karow M, Flavell RA. Interleukin-22 but not interleukin-17 provides protection to hepatocytes during acute liver inflammation. Immunity. 2007; 27:647–659. [PubMed: 17919941]
8. Zenewicz LA, Yancopoulos GD, Valenzuela DM, Murphy AJ, Stevens S, Flavell RA. Innate and adaptive interleukin-22 protects mice from inflammatory bowel disease. Immunity. 2008; 29:947–957. [PubMed: 19100701]
9. Zheng Y, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. Nat Med. 2008; 14:282–289. [PubMed: 18264109]
10. Huber S, et al. IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. Nature. 2012; 491:259–263. [PubMed: 23075849]
11. Kirchberger S, et al. Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. J Exp Med. 2013
12. Sherlock JP, et al. IL-23 induces spondyloarthropathy by acting on ROR-gammat+ CD3+CD4-CD8- entheseal resident T cells. Nat Med. 2012; 18:1069–1076. [PubMed: 22772566]
13. Zheng Y, et al. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. Nature. 2007; 445:648–651. [PubMed: 17187052]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

14. Spits H, et al. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol. 2013; 13:145–149. [PubMed: 23348417]
15. Kiss EA, et al. Natural aryl hydrocarbon receptor ligands control organogenesis of intestinal lymphoid follicles. Science. 2011; 334:1561–1565. [PubMed: 22033518]
16. Qiu J, et al. The aryl hydrocarbon receptor regulates gut immunity through modulation of innate lymphoid cells. Immunity. 2012; 36:92–104. [PubMed: 22177117]
17. Sonnenberg GF, Artis D. Innate lymphoid cell interactions with microbiota: implications for intestinal health and disease. Immunity. 2012; 37:601–610. [PubMed: 23084357]
18. Kinnebrew MA, et al. Interleukin 23 production by intestinal CD103(+)CD11b(+) dendritic cells in response to bacterial flagellin enhances mucosal innate immune defense. Immunity. 2012; 36:276–287. [PubMed: 22306017]
19. Shaw MH, Kamada N, Kim YG, Nunez G. Microbiota-induced IL-1beta, but not IL-6, is critical for the development of steady-state TH17 cells in the intestine. J Exp Med. 2012; 209:251–258. [PubMed: 22291094]
20. Quintana FJ, et al. Control of T(reg) and T(H)17 cell differentiation by the aryl hydrocarbon receptor. Nature. 2008; 23:23.
21. Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. Proc Natl Acad Sci U S A. 2008; 105:9721–9726. [PubMed: 18607004]
22. Veldhoen M, Hirota K, Christensen J, O'Garra A, Stockinger B. Natural agonists for aryl hydrocarbon receptor in culture medium are essential for optimal differentiation of Th17 T cells. J Exp Med. 2009; 206:43–49. [PubMed: 19114668]
23. Veldhoen M, et al. The aryl hydrocarbon receptor links TH17-cell-mediated autoimmunity to environmental toxins. Nature. 2008; 453:106–109. [PubMed: 18362914]
24. Sugimoto K, et al. IL-22 ameliorates intestinal inflammation in a mouse model of ulcerative colitis. J Clin Invest. 2008; 118:534–544. [PubMed: 18172556]
25. Liang SC, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med. 2006; 203:2271–2279. [PubMed: 16982811]
26. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. Nat Immunol. 2009; 10:857–863. [PubMed: 19578369]
27. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. Nat Immunol. 2009; 10:864–871. [PubMed: 19578368]
28. Rutz S, et al. Transcription factor c-Maf mediates the TGF-beta-dependent suppression of IL-22 production in T(H)17 cells. Nat Immunol. 2011; 12:1238–1245. [PubMed: 22001828]
29. Basu R, et al. Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria. Immunity. 2012; 37:1061–1075. [PubMed: 23200827]
30. Korn T, et al. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007; 448:484–487. [PubMed: 17581588]
31. Nurieva R, et al. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature. 2007; 448:480–483. [PubMed: 17581589]
32. Zhou L, et al. IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol. 2007; 8:967–974. [PubMed: 17581537]
33. Chung Y, et al. Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. Immunity. 2009; 30:576–587. [PubMed: 19362022]
34. Wu C, et al. Metallothioneins negatively regulate IL-27-induced type 1 regulatory T-cell differentiation. Proc Natl Acad Sci U S A. 2013; 110:7802–7807. [PubMed: 23630250]
35. Gandhi R, et al. Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3(+) regulatory T cells. Nat Immunol. 2010; 11:846–853. [PubMed: 20676092]
36. Franke A, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. Nature genetics. 2010; 42:1118–1125. [PubMed: 21102463]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

37. Gao J, et al. MUC1 is a downstream target of STAT3 and regulates lung cancer cell survival and invasion. International journal of oncology. 2009; 35:337–345. [PubMed: 19578748]
38. Nishida A, et al. The membrane-bound mucin Muc1 regulates T helper 17-cell responses and colitis in mice. Gastroenterology. 2012; 142:865–874. e862. [PubMed: 22202458]
39. Ciofani M, et al. A validated regulatory network for Th17 cell specification. Cell. 2012; 151:289–303. [PubMed: 23021777]
40. Okey AB, Vella LM, Harper PA. Detection and characterization of a low affinity form of cytosolic Ah receptor in livers of mice nonresponsive to induction of cytochrome P1-450 by 3-methylcholanthrene. Mol Pharmacol. 1989; 35:823–830. [PubMed: 2543914]
41. Quintana FJ, et al. An endogenous aryl hydrocarbon receptor ligand acts on dendritic cells and T cells to suppress experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A. 2010; 107:20768–20773. [PubMed: 21068375]
42. Quintana FJ, et al. Aiolos promotes T(H)17 differentiation by directly silencing Il2 expression. Nature Immunology. 2012; 13:770–777. [PubMed: 22751139]
43. Apetoh L, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. Nat Immunol. 2010; 11:854–861. [PubMed: 20676095]
44. Zentner GE, Henikoff S. Regulation of nucleosome dynamics by histone modifications. Nat Struct Mol Biol. 2013; 20:259–266. [PubMed: 23463310]
45. Kanno Y, Vahedi G, Hirahara K, Singleton K, O'Shea JJ. Transcriptional and epigenetic control of T helper cell specification: molecular mechanisms underlying commitment and plasticity. Annu Rev Immunol. 2012; 30:707–731. [PubMed: 22224760]
46. O'Shea JJ, Lahesmaa R, Vahedi G, Laurence A, Kanno Y. Genomic views of STAT function in CD4+ T helper cell differentiation. Nat Rev Immunol. 2011; 11:239–250. [PubMed: 21436836]
47. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. Annu Rev Immunol. 2002; 20:495–549. [PubMed: 11861611]
48. Powell N, et al. The transcription factor T-bet regulates intestinal inflammation mediated by interleukin-7 receptor+ innate lymphoid cells. Immunity. 2012; 37:674–684. [PubMed: 23063332]
49. Ota N, et al. IL-22 bridges the lymphotoxin pathway with the maintenance of colonic lymphoid structures during infection with Citrobacter rodentium. Nat Immunol. 2011; 12:941–948. [PubMed: 21874025]
50. Alam MS, et al. Notch signaling drives IL-22 secretion in CD4+ T cells by stimulating the aryl hydrocarbon receptor. Proc Natl Acad Sci U S A. 2010; 107:5943–5948. [PubMed: 20231432]
51. Liu SM, King C. IL-21-producing Th cells in immunity and autoimmunity. Journal of immunology (Baltimore, Md : 1950). 2013; 191:3501–3506.
52. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. Annu Rev Immunol. 2009; 27:485–517. [PubMed: 19132915]
53. Rasheed MA, et al. Interleukin-21 is a critical cytokine for the generation of virus-specific long-lived plasma cells. Journal of virology. 2013; 87:7737–7746. [PubMed: 23637417]
54. Skyberg JA, et al. Apple polyphenols require T cells to ameliorate dextran sulfate sodium-induced colitis and dampen proinflammatory cytokine expression. J Leukoc Biol. 2011; 90:1043–1054. [PubMed: 21693591]
55. Hepworth MR, et al. Innate lymphoid cells regulate CD4+ T-cell responses to intestinal commensal bacteria. Nature. 2013; 498:113–117. [PubMed: 23698371]
56. Araki A, et al. Role of interleukin-21 isoform in dextran sulfate sodium (DSS)-induced colitis. Cytokine. 2013; 62:262–271. [PubMed: 23557800]
57. O'Connor W Jr, et al. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. Nat Immunol. 2009; 10:603–609. [PubMed: 19448631]
58. Wu HY, et al. In Vivo Induction of Tr1 Cells via Mucosal Dendritic Cells and AHR Signaling. PLoS One. 2011; 6:e23618. [PubMed: 21886804]
59. Izcue A, Coombes JL, Powrie F. Regulatory lymphocytes and intestinal inflammation. Annu Rev Immunol. 2009; 27:313–338. [PubMed: 19302043]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

60. Mascanfroni ID, et al. IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. Nature immunology. 2013; 14:1054–1063. [PubMed: 23995234]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

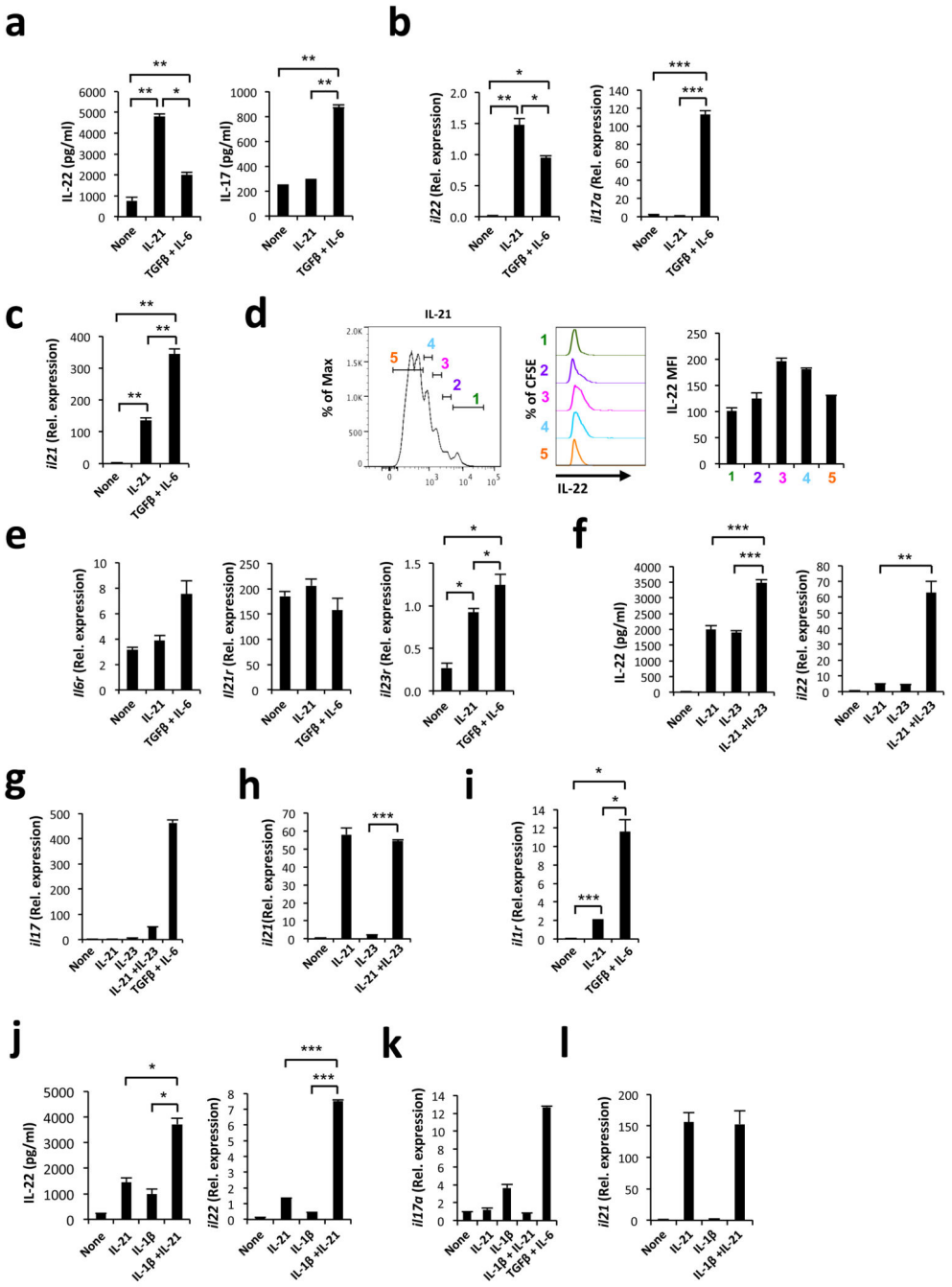

Figure 1. IL-21 promotes the differentiation of CD4+ T cells that produce IL-22 but not IL-17

Naïve WT CD4+ T cells were stimulated *in vitro* with antibodies to CD3 and CD28 in the presence of different cytokines as indicated. (**a-b**) Effect of IL-21 in the production of IL-22 and IL-17 measured by ELISA in culture supernatants (**a**) and qPCR (**b**). (**c**) Effect of IL-21 on the expression of *il21*. (**e**) Effect of IL-21 on the expression of *il6r*, *il21r*, *il23r*. (**d**) Relationship between cell division and IL-22 expression in CD4+ T cells. CFSE-labeled naïve CD4+ T cells were activated in the presence of IL-21 and the production of IL-22 was analyzed by intracellular staining. The data are shown as the Mean fluorescence intensity

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(MFI) for IL-22 for each division cycle. (**e**) Effects of IL-21 on the expression of *il6r*, *il21r* and *il23r*. (**f-h**) Synergistic effects of IL-21 and IL-23 on IL-22 **(f)** and IL-17 **(g)** cytokine and *il21* expression (**h**). **(i)** Effect of IL-21 on *il1r* expression. (**j-l**) Synergistic effects of IL-21 and IL-1β on IL-22 **(j)**, *Il17a* **(k)** and *il21* **(l)** expression. mRNA expression is shown relative to *gapdh*. Results are representative of 3-5 independent experiments.* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA).

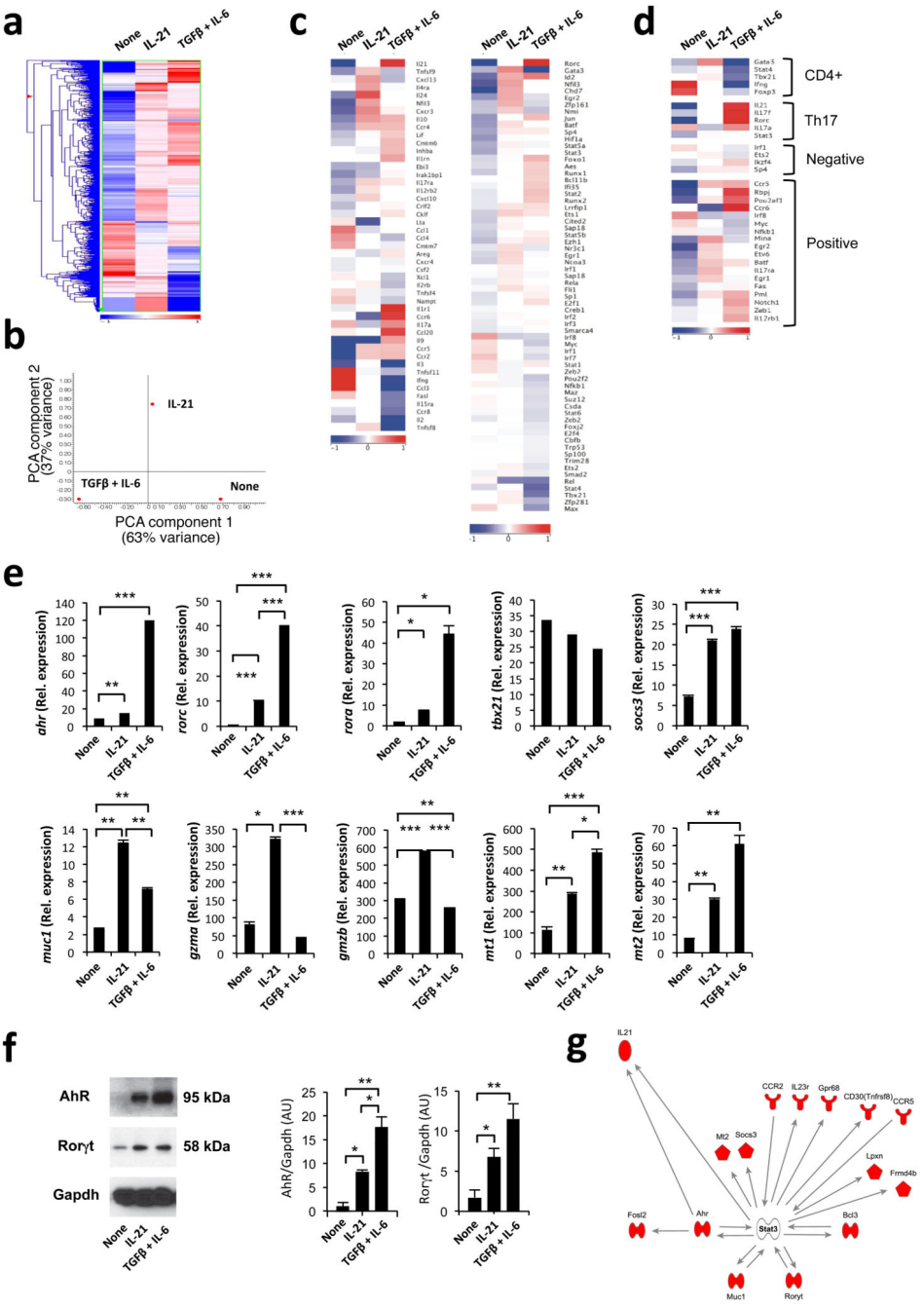

Figure 2. Transcriptional profiling of IL-21-stimulated CD4+ T cells

Naïve WT CD4+ T cells were stimulated *in vitro* with antibodies to CD3 and CD28 in the presence of IL-21, IL-6 and TGFβ1 or without addition of exogenous cytokines. **(a)** Heatmap showing genes with at least a 1.5 fold change in expression relative to Th0 as determined by whole-genome microarrays. **(b)** Principal-component analysis (PCA). **(c-d)** Heatmaps depicting the expression of key cytokines, chemokines, receptors and transcription factors **(c)** and transcriptional modules **(d)** that control the differentiation of Th17 cells as defined in[34]. **(e)** Quantitative RT-PCR analysis of the expression of genes in

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Fig. 2a. mRNA expression is shown relative to *gapdh*. **(f)** Western blot analysis of the expression of AhR (95kDa) and RORγt (58kDa) on T cells activated in the presence of IL-21 or under Th17-polarizing conditions (left panel). Quantification of western blot reactivity (right panel). **(g)** Ingenuity Pathways Analysis of the transcriptional response of naïve CD4+ T cells to IL-21. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

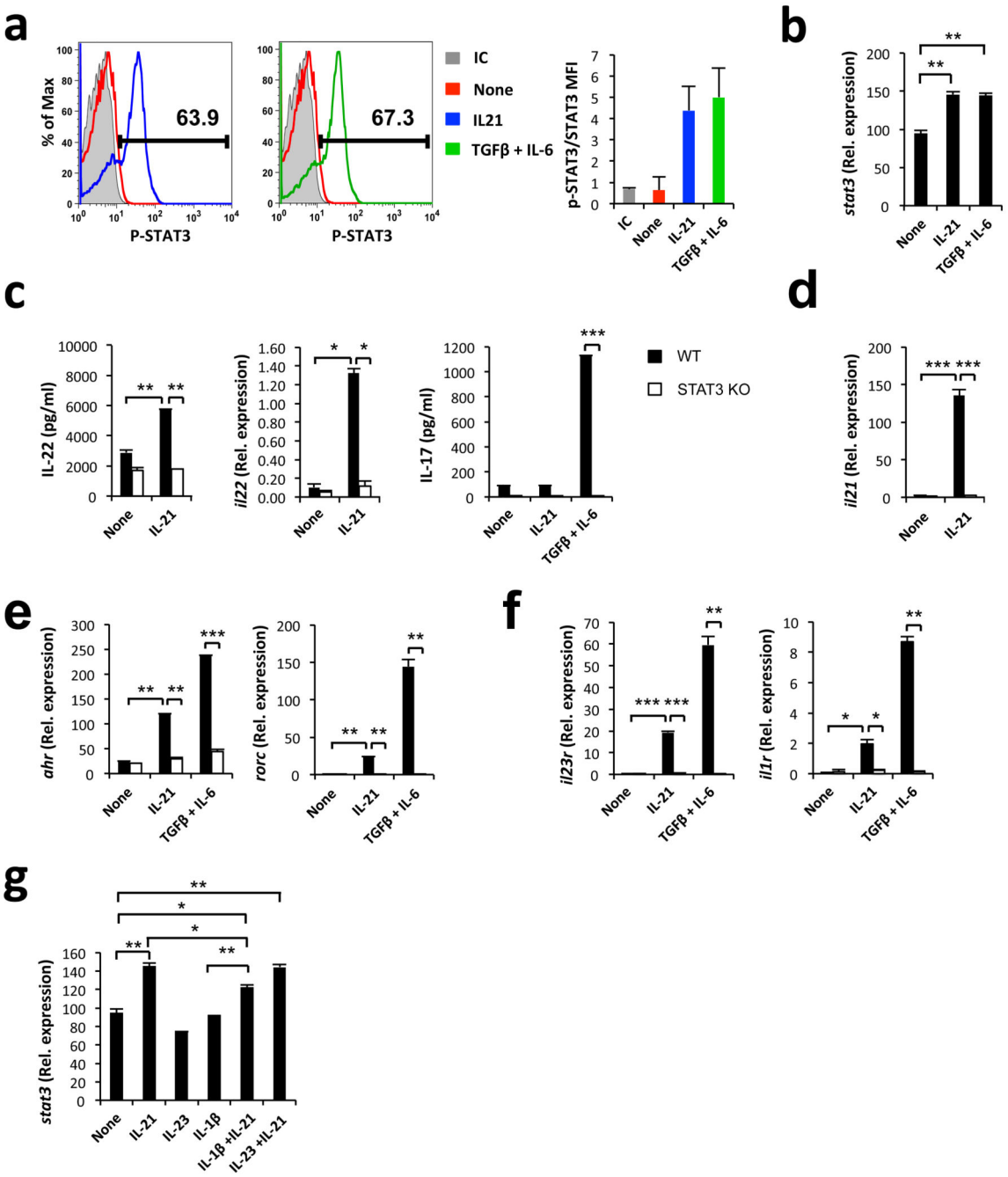

Figure 3. STAT3 controls the production of IL-22 by CD4+ T cells stimulated with IL-21

(**a-b**) NaïveWT CD4+ T cells were activated in the presence of IL-21 or IL-6 and TGFβ1. (**a**) FACS analysis of phosphorylated STAT3. Mean fluorescence intensity (MFI) of phosphorylated STAT3 normalized to total STAT3. (**b**) qPCR analysis of *stat3* expression. (**c-f**) Naïve WT and STAT3-deficient CD4+ T cells were activated in the presence of IL-21 or IL-6 and TGFβ1. (**c**) Effect of STAT3 deficiency on IL-22 and IL-17 production. (**d**) Effect of STAT3 deficiency on *il21* expression. (**e-f**) Effect of STAT3 deficiency on *ahr* and *rorc* expression (**e**) and the receptors *il1r*, *il23r* (**f**). (**g**) Induction of *stat3* by IL-1β, IL-21

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

IL-23, IL-1β and IL-21 or IL-21 and IL-23. mRNA expression is shown relative to *gapdh*. Results are representative of 2-3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA and Student's *t*- test).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

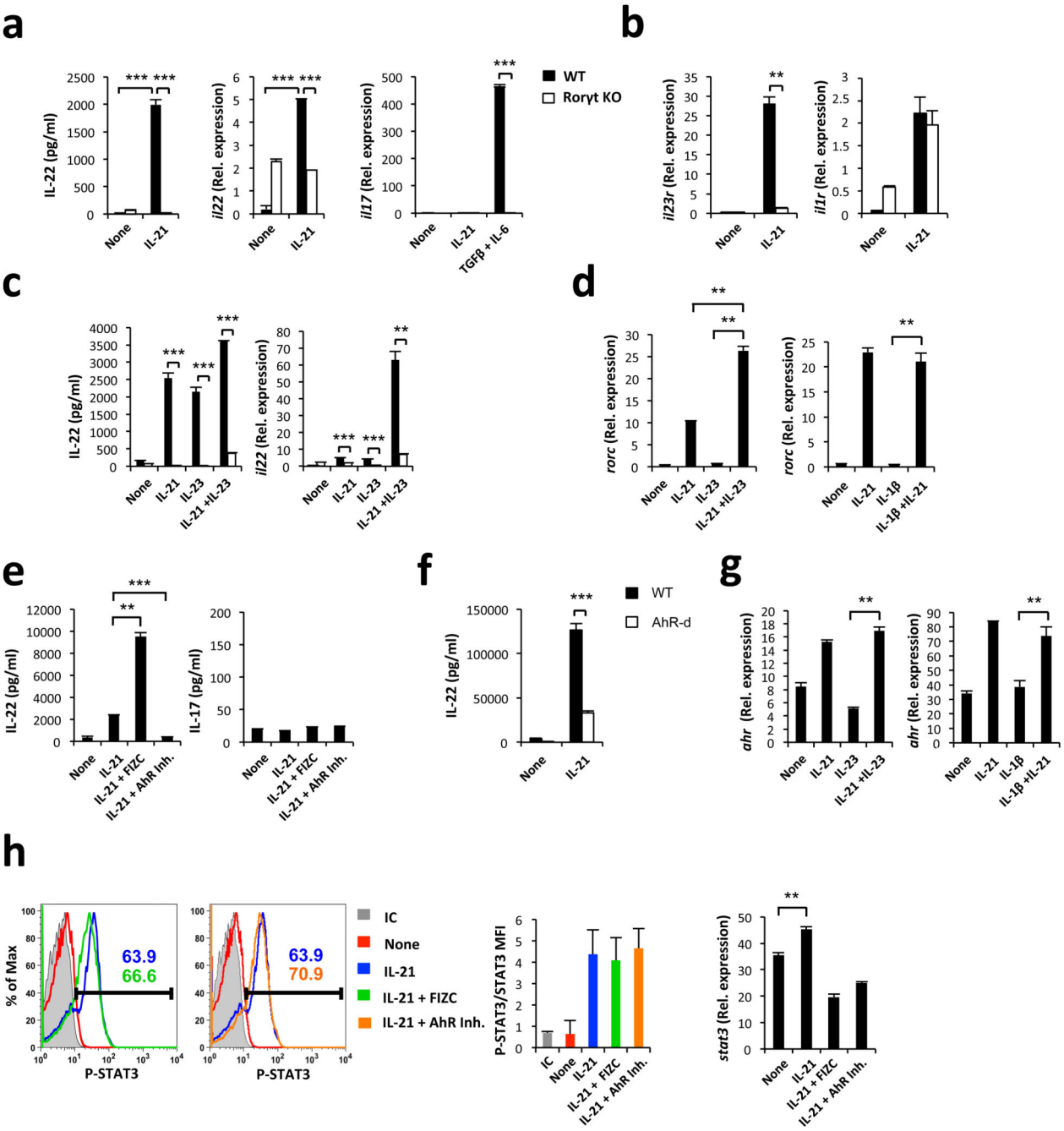

Figure 4. RORγt and AhR control IL-22 production by CD4+ T cells stimulated with IL-21

(a-c) Naïve WT and RORγt-deficient CD4+ T cells were activated in the presence of IL-21 or IL-6 and TGFβ1. (**a**) Effect of RORγt deficiency on IL-22 and IL-17 production. (**b**) Effect of RORγt deficiency on *il23r* and *il1r1*expression. (**c**) Effect of RORγt deficiency on the synergism between IL-21 and IL-23. (**d**) Effect of IL-1β and IL-23 in *rorc* expression. (**e**) Effect of the modulation of AhR activity on IL-22 and IL-17 production. (**f**) IL-22 production by WT and AhR-d CD4+ T cells activated in the presence of IL-21. (**g**) Modulation of *ahr* expression by IL-1β and IL-23. (**h**) Effect of AhR modulation on STAT3

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

phosphorylation and *stat3* expression. MFI of phosphorylated STAT3 normalized to total STAT3. mRNA expression is shown relative to *gapdh*. Representative results of 2-3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA and Student's *t*- test).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

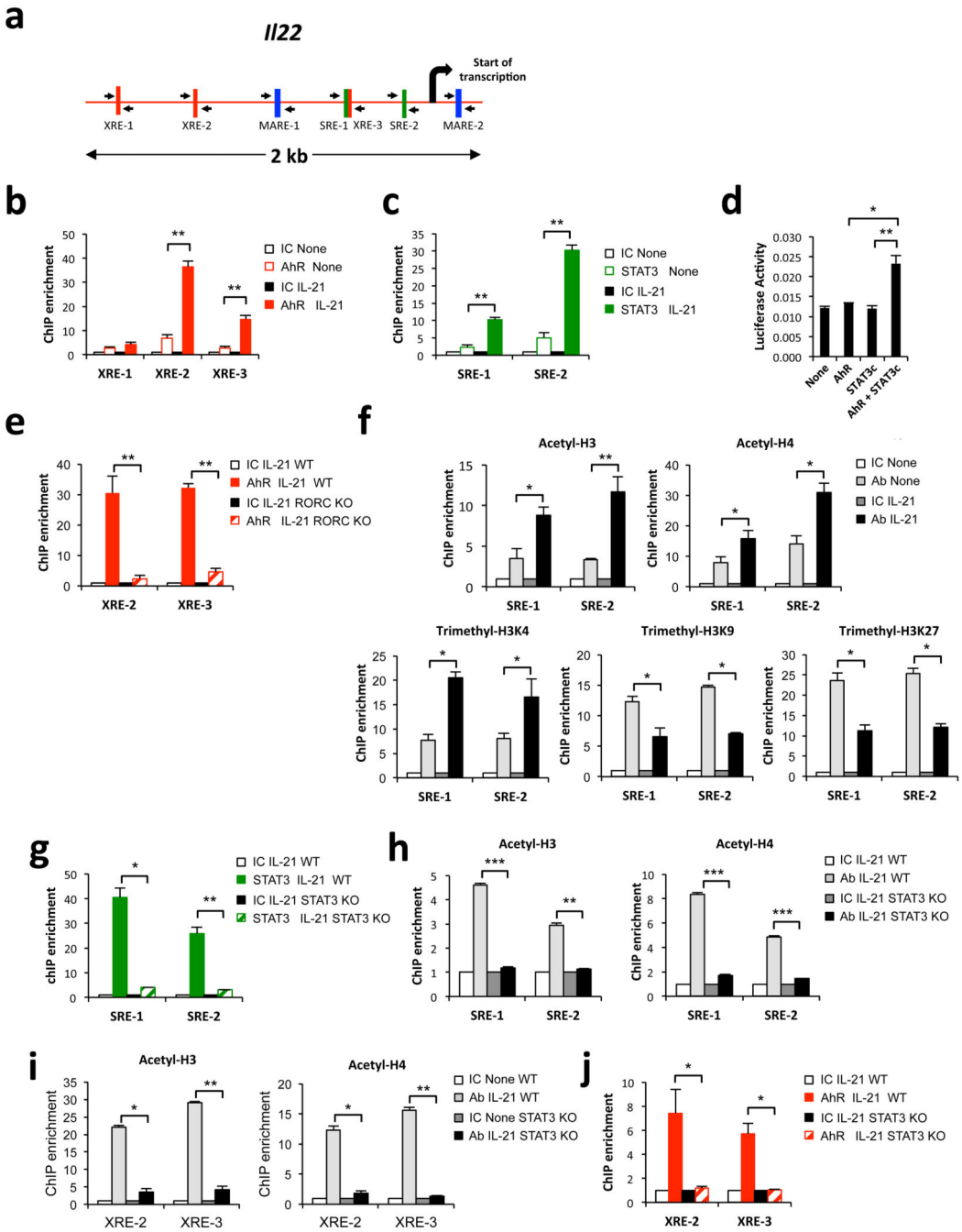

Figure 5. STAT3 controls AhR recruitment to the *il22* promoter in IL-21-stimulated CD4+ T cells

(**a**) AhR, c-Maf and STAT3 binding sites in the *il22* promoter. (**b-c**) WT naïve CD4+ T cells were stimulated *in vitro* with antibodies to CD3 and CD28 in the presence or absence of IL-21. ChIP analysis of the interaction of AhR (**b**) and STAT3 (**c**) to the AhRand STAT3 binding sites in the *il22* promoter, respectively. (**d**) Regulation of the transcriptional activity of the *il22* promoter by AhR and STAT3. A reporter construct for the *il22* promoter (il22:Luc) was cotransfected in HEK293 cells with vector coding for AhR and/or

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

constitutively activated STAT3 (STAT3c). Firefly luciferase was determined and normalized to renilla luciferase activity. (**e**) ChIP analysis of the recruitment of AhR to the *il22* promoter in WT and RORγt-deficient CD4+ cells activated in the presence of IL-21. (**f**) WT naïve CD4+ T cells were stimulated *in vitro* with antibodies to CD3 and CD28 in the presence or absence of IL-21, and the epigenetic status of the *il22* promoter was analyzed by ChIP using antibodies to Acetyl-H3, Acetyl-H4 and H3 trimethylated at Lys4, Lys9 or Lys27. (**g**-**j**) ChIP analysis of the epigenetic status of the *il22* promoter in WT and STAT3-deficient CD4+ T cells activated in the presence of IL-21. STAT3 recruitment **(g)**, H3 and H4 acetylation in SREs **(h)** and XREs **(i)** and AhR recruitment **(j)** in the *il22* promoter. Results are representative of 2-3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA).

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

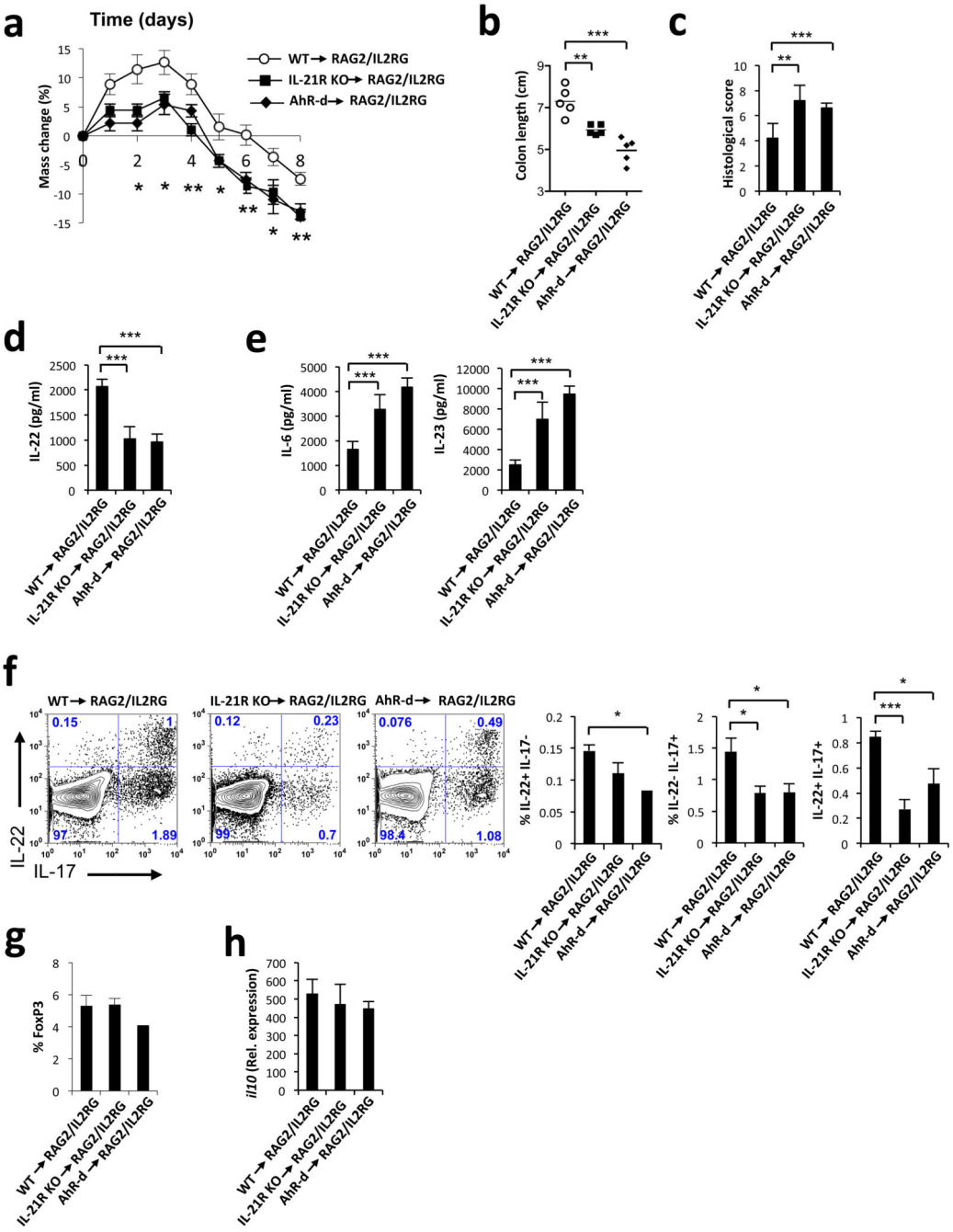

Figure 6. IL-21 and AhR control IL-22 + CD4+ T cells *in vivo* and limit mucosal inflammation

(**a**) RAG2/IL2RG mice transferred with WT, AhR-d or IL-21R deficient CD4+ T cells were given 3 % DSS *ad libitum* in their drinking water for 7 days. Body weight was assessed daily, and the percent mass change from day 0 was calculated. Mean ± s.e.m is shown, n=5 mice per group. Effect of genotype on disease course $P<0.0001$, *$P<0.01$ by two-way ANOVA. (**b**) Colon length at day 7 after treatment with DSS. (**c**) Colonic histological sections were scored as described in Methods. Values are mean ± s.e.m. (**d-e**) Colons were excised on day 7 after the initiation of treatment with DSS, cultured for 3 days and IL-22

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

(**d**), IL-6 and IL-23 (**e**) were quantified in the supernatants by ELISA. (**f-h)** Lymphocytes from the colonic lamina propria (LPL) were isolated. The frequency of IL-22+IL17-, IL-22+IL-17+, IL-22-IL-17+ T cells **(f)** and FoxP3+ Tregs **(g)** was analyzed by FACS and the expression of *il10* was measured by qPCR (**h**). mRNA expression is shown relative to the expression of *gapdh*. Results are representative of 3 independent experiments. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (one-way ANOVA and two-way ANOVA).