
**TNF-α–induced protein 3 (TNFAIP3)/A20 acts as a master switch in TNF-α blockade–driven IL-17A expression.**

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TNFAIP3/A20 acts as master switch in TNFα blockade-driven IL-17A expression

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ABSTRACT

Background: Anti-TNF inhibitors successfully improve life quality of patients suffering from inflammatory disease. Unfortunately, not all patients respond to anti-TNF therapy and some patients show paradoxical immune side-effects, which is poorly understood. Surprisingly, anti-TNF agents were shown to promote IL-17A production, with as yet unknown clinical implications.

Objective: To investigate the molecular mechanism underlying anti-TNF driven IL-17A expression and the clinical implications of this phenomenon.

Methods: FACS, RNA-sequencing, RT-qPCR, western blotting, siRNA interference and kinase-inhibitors, were used to study the molecular mechanism in isolated human CD4+ T cells from healthy donors. The clinical implication was studied in blood samples of inflammatory bowel disease (IBD) patients under anti-TNF therapy.

Results: Here we show that anti-TNF treatment results in inhibition of the anti-inflammatory molecule TNFAIP3/A20 in memory CD4+ T cells. We found an inverse relationship between TNFAIP3/A20 expression levels and IL-17A production. Inhibition of TNFAIP3/A20 promotes kinase activity of p38-MAPK and PKC, which drives IL-17A expression. Regulation of TNFAIP3/A20 expression and cognate IL-17A production in T cells is specifically mediated via TNFR2-signaling. Ex vivo, in IBD patients treated with anti-TNF, we found further evidence for an inverse relationship between TNFAIP3/A20 expression levels and IL-17A producing T cells.

Conclusion: Anti-TNF treatment interferes in the TNFAIP3/A20 mediated anti-inflammatory feedback-loop in CD4+ T cells and promotes kinase activity. This puts TNFAIP3/A20, combined with IL-17A expression, on the map as a potential tool in predicting therapy responsiveness or side effects of anti-TNF therapy. Moreover, it provides novel targets, related to TNFAIP3/A20 activity, for superior therapeutic regimens in IBD patients.
Key Messages:

- Anti-TNF treatment inhibits the anti-inflammatory mediator TNFAIP3/A20 in activated CD4⁺ memory T cells.
- Anti-TNF treatment enhances IL-17A production of activated CD4⁺ memory T cells by neutralization of TNFα mediated TNFR2-signaling.
- Inhibition of TNFAIP3/A20 expression by anti-TNF or siRNA-inhibition of TNFAIP3/A20 activated CD4⁺ enhances IL-17A production.
- P38 MAPK and PKC signaling are required for anti-TNF mediated enhancement of IL-17A expression in activated CD4⁺ T cells.
- Increased numbers of IL-17 expressing CD4⁺ T cells and a concomitant reduction in TNFAIP3 expression was observed in 50% of IBD patients following anti-TNF therapy.
- Combined TNFAIP3/A20 and IL-17A expression profiling might aid in predicting therapy responsiveness (or side effects) of anti-TNF therapy.
- This study provides novel targets, related to TNFAIP3/A20 activity, for improved therapeutic regimens in IBD patients.

Capsule Summary:

Anti-TNF treatment interferes in the TNFAIP3/A20 mediated anti-inflammatory feedback-loop in CD4⁺ T cells, thereby promoting kinase activity and IL17A expression. Current findings support patient risk stratification in anti-TNF therapy IBD patients and provide new therapeutic targets.
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Key words: TNF-alpha, A20, TNFAIP3, anti-TNF, IL-17A, IBD

Abbreviation:

ADL, Adalimumab
ETN: Etanercept
FVD: Fixable Viability Dye
IFX: Infliximab
rhIL-2: recombinant human Interleukin 2
mIgG: mouse IgG
p38i: p38 MAPK inhibitor
PKCi: PKC inhibitor
rhIgG: Recombinant human IgG
RNA-seq: RNA sequencing
Tmem: CD4+ memory T cell
Tnaïve: CD4+ naïve T cell
TNFAIP3: Tumor necrosis factor alpha-induced protein 3
INTRODUCTION

Anti-TNF therapy successfully induces clinical remission, and improvement of quality of life in many patients affected by auto-inflammatory disorders, such as psoriasis, rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) \(^1-^3\). Paradoxically, anti-TNF treatment of IBD as well as of RA patients has been associated with unexpected side effects, such as a psoriasis-like syndrome \(^4,^5\). This suggests that depletion of TNF\(\alpha\) might inadvertently trigger specific forms of immune pathology, and highlights the pleiotropic nature of TNF\(\alpha\). This feature has so far received limited attention. Relevant in this respect is the finding that anti-TNF agents are capable of promoting IL-17 expression in CD4\(^+\) T cell lines from Juvenile Idiopathic Arthritis (JIA) \(^6\) and RA patients \(^7,^8\), and can promote monocyte-driven IL-17 production of T cells \(^7\).

As IL-17 is a potent pro-inflammatory cytokine, responsible for the pathogenesis and progression of psoriasis and many other autoimmune disorders, this may explain some of the above mentioned clinical findings. Importantly, the molecular mechanism behind the anti-TNF mediated induction of IL-17 by T cells is as yet not understood, and the phenomenon has not been clinically evaluated.

TNF\(\alpha\) is present in two forms, membrane-bound uncleaved TNF\(\alpha\) (\(\sim 75\) kDa) or soluble cleaved TNF\(\alpha\) (\(\sim 55\) kDa). TNF\(\alpha\)-signaling through TNF-receptor-1 (TNFR1) or TNFR2 on immune cells primarily activates NF-\(\kappa\)B and promotes transcription of genes related to inflammatory processes, such as proliferation, cytokine production, and cell survival \(^9\). Notably, NF\(\kappa\)B activation also results in the expression of anti-inflammatory regulators, such as the tumor necrosis factor alpha-induced protein 3 (TNFAIP3), that encodes the deubiquitinating enzyme (DUB) A20 \(^10\), a renowned negative regulator of NF\(\kappa\)B activation \(^11,^12\). This NF\(\kappa\)B-induced TNFAIP3/A20 negative feedback loop was mostly studied in innate immune cells \(^13\), but there are indications
that TNFAIP3/A20 also negatively regulates CD4$^+$ T cells$^{14}$. Polymorphisms in the
TNFAIP3 gene or region have been associated with several autoimmune diseases$^{15-18}$ and also anti-TNF responsiveness in IBD and psoriasis$^{19,20}$ that suggests an
undetermined relationship between the lack of TNFα-signaling, deviant TNFAIP3/A20
expression and immune activity.

Here, we present evidence that TNFAIP3/A20 acts as master switch in TNFα
blockade-driven IL-17A expression in human CD4$^+$ T cells and showed an inverse
relationship between TNFAIP3/A20 expression levels and IL-17A expressing T cells.
In an ex vivo study of IBD patients treated with the anti-TNF biosimilar Remsima, we
found further evidence for the role of TNFAIP3/A20, by showing that low mRNA levels
of TNFAIP3 were associated with increased IL-17A expression. Reversely, patients
with high TNFAIP3 mRNA levels after Remsima treatment showed low IL-17
expression; most of these patients carried the heterozygous TNFAIP3/A20 region
variant genotype C>G (rs6927172) that has been related to increased TNFAIP3/A20
expression$^{19}$. Our results suggest that combined analysis of, TNFAIP3 mRNA
expression, TNFAIP3 region polymorphisms and IL-17A production by CD4$^+$ T cells
may aid the design of future patient risk stratification protocols in anti-TNF therapy.
Moreover, it provides novel targets for additional, improved therapeutic regimens in
IBD patients.
METHODS

Study approval

The protocols of this study were performed in agreement with the Declaration of Helsinki and approved by the Institutional Review Board of the Radboud University Medical Center in Nijmegen (Radboud UMC), the Netherlands. Registration code: 2015-1838.

Subjects

Voluntary blood donors (Sanquin Bloedvoorziening, Nijmegen, NLD) and healthy volunteers (n= 31) and patients (n=12) gave written informed consent. Inflammatory Bowel Disease patients (ulcerative colitis and Crohn’s disease) were naïve for any biological drug treatment. For more detailed information on the patients see Table E1 in this article’s Online Repository at http://www.jacionline.org. The clinical evaluation of IBD patients was based on disease indexes including the Harvey Bradshaw Index (HBI) for Crohn’s disease and Simple Clinical Colitis Activity Index (SCCAI) for ulcerative colitis.

Peripheral blood mononuclear cell and T cell isolation

Fresh peripheral blood mononuclear cells (PBMC) collected in tubes with acid citrate dextrose (ACD) and derived from healthy blood donors, IBD patients were isolated by Ficoll-paque density gradient centrifugation. Next, CD4\(^+\) T cells were isolated using Rosette Sep CD4 enrichment cocktail (StemCell Technologies) (25-50 \(\mu\)L/ml) according to the instructions of the supplier. To sort CD4\(^+\)CD25\(^-\)CD45RA\(^-\) (Tmem)
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and CD4⁺CD25⁻CD45RA⁺ (T naïve) the purified CD4⁺ cells were washed and stained with BD - anti-CD25-BV510 (M-A251, BD), anti-CD45RA-PE (4KB5, Dako), CD4⁺-PE-Cy5.5 (13B8.2, Beckman-Coulter) and FACS-sorted on a FACSARia™ III machine (BD Biosciences). The purity of the sorted cell populations was >97%.

**Cell culture**

RPMI-1640 Dutch modified (Gibco) culture medium, with sodium bicarbonate and 20 mM HEPES, supplemented with penicillin/streptomycin (100 U/mL), sodium pyruvate (1 mM), glutamine/glutamax and 10% human pooled serum (HPS, Radboud UMC), was used in all experiments. After cell isolation, 2.5 x 10⁴ cells/well were cultured in 96-well U-bottom plates and incubated with Dynabeads Human T-Activator CD3/CD28 (1:5, bead: cell ratio) (Gibco) and recombinant human IL-2 (rhIL-2) (100 U/mL) (Proleukin Prometheus Laboratories). Where indicated, cultures were supplemented with recombinant human (rh) TNFα (rhTNFα, 50 ng/mL, R&D), rhLymphotixin-α (rhLTα 10 ng/mL, R&D) or TNFα inhibitors 5 μg/mL, etanercept (ETN - Enbrel®, Pfizer), infliximab (IFX - Remicade®, Janssen Biotech) or adalimumab (ADL – Humira®, AbbVie), TNFR2 agonist (2.5 μg/mL, MR2-1, Hycult Biotech), anti-human TNFR1 (10 μg/mL; anti-human CD120a, H398, eBioscience), anti-human TNFR2 (10 μg/mL; anti-human CD120b, 3G7A02, Biolegend), recombinant human IgG1 (rhIgG, AbD18705_hIgG1, Bio-Rad), mouse IgG1 (mlG, 11711, R&D), or CysTNFR2 (50 ng/mL) with monoclonal antibodies Mab 80M2 (2 μg/mL)²². To examine the effect of pharmaceutical inhibitors of PKC (AEB071, 10 μM; Sotrastaurin, Novartis) or p38α/β kinase (50 μM, UR13870 previously known as ORG48762-0, Palau Pharma)²³,²⁴ isolated cells were pre-incubated for 30 min, prior to stimulation.
Flow cytometry
Flow cytometry was performed using a 10-colors Navios Flow cytometer (Beckman Coulter). To evaluate cytokine production, we re-stimulated PBMC and CD4\(^+\) T cells subsets for four hours with PMA (12.5 ng/mL), ionomycin (500 ng/mL) and Brefeldin A (5 μg/mL) (Sigma-Aldrich). After the incubation, in some cases the cells were stained with fixable viability dye\(^{-}\)APC-780 (FVD, eBioscience) for 30 min, following by surface, fixation/permeabilization (Intracellular Fixation & Permeabilization Buffer Set, eBioscience) and intracellular staining.

Measurement of cell culture cytokines
Cell culture supernatant was analyzed for the presence of IL-17A, IFN\(\gamma\) and TNFα using a Bio-Plex Pro Human Th17 Cytokine Assays (Bio-Rad) according to the manufacturer’s instruction. The cytokine concentrations were measured using a Luminex\(^{100}\) machine (Luminex Corp.) and analyzed by the software Bio-Plex 6.1 (Bio-Rad). The lower level detection was 1.9 pg/mL for IL-17A, 11.5 pg/mL for IFN\(\gamma\) and 2.2 pg/mL for TNFα.

Western Blotting
To detect A20 protein in cultured CD4\(^+\) Tmem, the cells were lysed in TBS Urea/Nonidet-P40/Triton X100 buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 8M urea, 0.5% NP-40, 0.5% triton X-100, 10mM DTT and cOmplete\(^{TM}\) EDTA free protease inhibitor cocktail Roche) for 30 min on ice and subsequently prepared for western blot analysis. A20 was detected using a primary mouse anti-human A20
antibody (59A426, Abcam) (2 μg/mL), followed by a secondary polyclonal goat anti-
mouse HRP (P044701-2, Dako). We analyzed expression of the household protein
GAPDH by anti-GAPDH (ab8245, Abcam) to control for protein loading. A
chemiluminescent substrate for HRP (SuperSignal West Femto Maximum Sensitivity
Substrate kit, Thermo Fisher) was used to visualize the proteins using a myECL
Imager system (Bio-Rad).

Quantitative Real-time PCR (RT-qPCR)

Total RNA was extracted by using the RNeasy Plus Micro kit (Qiagen) followed by
cDNA synthesis using the SuperScript III First-Strand Synthesis System and
Oligo(dT)20 primers (Thermo Fisher Scientific). Taqman gene expression assays
were purchased from Thermo Fisher Scientific (see Table E2 in this article’s Online

Single Nucleotide Polymorphism Genotyping

DNA was isolated using the QIAamp DNA Blood Mini Kit according to the
manufacturer's protocol (QIAGEN). SNP genotyping of rs6927172 (for SNP details
see Fig. E1 in this article’s Online Repository at http://www.jacionline.org) was
performed using TaqMan SNP Genotyping Assays on an ABI 7500 system according
to the manufacturer’s instructions using the SNP rs6927172 assay C_1575580_10
(Thermo Fisher Scientific). PCR results were analyzed using 7500 Fast System SDS
software version 1.5 (Thermo Fisher Scientific).

RNA sequencing (RNA-seq)
An initial quality check of RNA quantification of the samples was performed by capillary electrophoresis using the LabChip GX (Perkin Elmer). Non-degraded RNA samples were selected for subsequent sequencing analysis. We carried out Differential expression gene (DEG) analysis using DESeq2, where donor and the batch effect sample preparation were controlled in the model. Principal Component Analysis (PCA) was carried out to visualize the samples given their entire transcriptome and any remaining batch effects (see Fig. E2 in this article’s Online Repository at http://www.jacionline.org).

siRNA Transfection

For siRNA knockdown of TNFAIP3 Accell SMARTpool siRNA (Dharmacon) was used according to the manufacturer's instructions. Briefly, $5 \times 10^4$ CD4+ Tmem cells/well were stimulated with αCD3/CD28 beads (1:5, bead: cell ratio) in Accell delivery media human serum free (Dharmacon) and incubated with Accell siRNA buffer or 1 μM of Cyclophilin B (positive control), non-targeting siRNA or TNFAIP3 siRNA for six days (for siRNA sequences see Table E3 in this article’s Online Repository at http://www.jacionline.org). RT-qPCR and FACS analysis were performed to confirm the knockdown and cytokine expression in CD4+ Tmem.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Windows (GraphPad Software) and R statistical language. For statistical analysis of experiments with more than two groups, a Friedman test followed by Dunn's Multiple comparison was used, while and a Wilcoxon matched-pairs signed rank Test was
used in case of experiments consisting of two groups. For correlation analysis, we applied Spearman's rank correlation coefficient. A method based on negative binomial distribution (DESeq2) was used to test for significant differences in gene expression for RNA-seq analysis.

For detailed description of materials and methods, please see the article’s Online Repository at www.jacionline.org.

RESULTS

Anti-TNF blockade promotes IL-17A expression by CD4⁺ T \textit{in vitro} and \textit{in vivo} in Inflammatory Bowel Disease patients

The impact of TNF blockade on the function of T lymphocytes is barely understood. Therefore we first evaluated the \textit{in vitro} effect of anti-TNF treatment on the pro-inflammatory cytokine producing potential of αCD3/CD28 stimulated PBMC, from healthy blood donors (HD) and inflammatory bowel diseases (IBD) patients, thereby focusing on CD4⁺ T cells. In a first approach, we compared the effects of different clinically used TNFα-antagonists (infliximab - IFX, adalimumab - ADL and etanercept – ETN). Inhibition of TNFα, irrespective of the anti-TNF agent tested, resulted in a significant increased percentage of IL-17A ($p < 0.001$), IFNγ ($p < 0.01$), and IL-17A/IFNγ ($p < 0.01$) expressing CD4⁺ T cells from healthy individuals (Fig. 1\textit{a,b}). As all TNFα antagonists tested induced this same phenomenon, we further focused on ETN in subsequent analyses. Using ETN, we observed that the \textit{in vitro} anti-TNF-induced increase in IL-17A and IFNγ expression was also observed in CD4⁺ T cells from IBD patients prior to the start of therapy (Fig. \textit{1c}). Also anti-TNF induced IL-17 in
To verify whether *in vivo* treatment with anti-TNF agents of patients with inflammatory disorders revealed a similar phenomenon, we analyzed *ex vivo* cytokine expression by PBMC of IBD patients \((n=12)\) that were treated for six weeks with anti-TNF (Remsima®). Although in RA and ankylosing spondylitis a significant increase in IL-17 expressing cells was previously observed \(^7,8,25\), we did not observe a significant increase in the absolute number of IL-17A expressing CD4\(^+\) T cells from IBD patients, but we noted that 50% of the patients revealed a clear increase in the number of IL-17A expressing CD4\(^+\) T cells (Fig. 1d), while the other patients showed a clear reduction in IL-17A expression after anti-TNF treatment (Fig. 1d). Patients that revealed an increase in IL-17A expression did not show an increase in IFN\(\gamma\) expressing cells (see Fig. E4 in this article’s Online Repository at http://www.jacionline.org). Moreover, patients that showed an increase in IL-17A expression following anti-TNF treatment seem to show a better clinical response as compared to patients showing a reduction in IL-17A expression (Fig. 1d, see Table E1 in this article’s Online Repository at http://www.jacionline.org).

**Anti-TNF blockade promotes IL-17A production exclusively by ablation of TNF\(\alpha\)-signaling in activated CD4\(^+\) memory T cells**

Next to CD4\(^+\) T cells, PBMC contain additional adaptive and innate immune cells that might also be influenced by anti-TNF agents. To demonstrate that anti-TNF-mediated cytokine production of CD4\(^+\) T cells was a direct consequence of the anti-TNF agent acting on CD4\(^-\) T cells, we FACS-sorted CD4\(^+\) naïve (T naïve) and CD4\(^+\) memory T
cell (Tmem) based on surface expression of CD45RA and CD25, and cultured the cells under αCD3/CD28-stimulation in the presence of recombinant human IL-2 (rhIL-2), with and without anti-TNF treatment and analyzed for IL-17A and IFNγ expression (Fig. 2a). In isolated Tmem, but not T naïve, anti-TNF treatment induced a significant increase in single IL-17A ($p < 0.001$), single IFNγ ($p < 0.002$) and double positive IL-17A/IFNγ producing T cells ($p < 0.0036$) (Fig. 2b) (Analysis strategy see Fig. E5 in this article’s Online Repository at http://www.jacionline.org). Isotype control antibodies did not influence cytokine expression in CD4$^+$ Tmem (see Fig. E6a,b in this article’s Online Repository at http://www.jacionline.org). In contrast to anti-TNFα, supplementation of rhTNFα to CD4$^+$ Tmem led to a significant reduction in the percentage of IL-17A, IFNγ and IL-17A/IFNγ producing T cells compared to the untreated condition (Fig. 2c).

Likewise, supplementation with recombinant human lymphotoxin-alpha (rhLTα), a member of the TNF-superfamily that shares amino acid sequence homology with TNFα and is known to activate the same signaling pathways as TNFα $^{26}$, also inhibited the IL-17A expression of CD4$^+$ Tmem (see Fig. E7 in this article’s Online Repository at http://www.jacionline.org).

Next, to the observed increase in IL-17A intracellular expression upon anti-TNF treatment, both IL17A and IL17F mRNA expression levels (Fig. 2d) as well as IL-17A cytokine secretion in culture supernatants were also increased (see Fig. E8a in this article’s Online Repository at http://www.jacionline.org). This was neither observed for IFNG mRNA expression (Fig. 2d), nor for IFNγ secretion (see Fig. E8b in this article’s Online Repository at http://www.jacionline.org). Th17 and Th1 associated transcription factors RORC (encoding RORγt) and TBX21 (encoding T-bet) were both
up-regulated by anti-TNF treatment (Fig. 2d). These data indicate that anti-TNF treatment promotes a preferential shift towards a Th17 phenotype.

TNFα, either soluble (sTNFα) or membrane-bound TNF (mTNF), signals via the TNF receptors TNFR1 and/or TNFR2, which ultimately results in NF-κB activation \(^{27}\). To confirm that ETN promotes IL-17A expression by inhibition of TNFα-signaling, and not by mTNF-mediated reverse signaling, we evaluated ETN binding to CD4\(^+\) Tmem in our \textit{in vitro} system by flow cytometry and demonstrate that ETN does not bind to Tmem (see Fig. E9 in this article’s Online Repository at http://www.jacionline.org). This indicates that ETN promotes IL-17A production of \textit{in vitro} activated CD4\(^+\) Tmem by the prevention of TNFα-signaling rather than by mTNF-mediated reverse signaling.

\textbf{TNFα-signaling regulates IL-17A production in CD4\(^+\) memory T cells through TNFR2}

Next, we examined which of the TNF receptors is primarily involved in the TNFα-mediated IL-17A regulation of CD4\(^+\) Tmem. First, we evaluated TNFR1 and TNFR2 surface expression on CD4\(^+\) Tmem upon αCD3/CD28 stimulation. Freshly isolated CD4\(^+\) Tmem hardly expressed either TNFR1 or TNFR2, while the majority of the cells showed high expression of TNFR2 after five days of activation; TNFR1 expression at that time point was barely detectable suggesting that TNFR2, but not TNFR1 might play a critical role in CD4\(^+\) Tmem (see Fig. E10a,b in this article’s Online Repository at http://www.jacionline.org). To assess via which receptor TNFα influences the IL-17A producing potential of CD4\(^+\) Tmem, we isolated and stimulated CD4\(^+\) Tmem with αCD3/CD28 and rhIL-2 in the absence or presence of specific TNFR1 or TNFR2 antagonists. Only TNFR2 inhibition led to an increase in IL-17A expression (Fig.}
Reversely, TNFR2 stimulation with a specific TNFR2 agonistic antibody MAb 80M2 (Fig. 3a,b), which ensures full TNFR2 activation, led to a reduction in IL-17A expression (Fig. 3a,b), confirming that TNFR2-signaling plays a crucial role in anti-TNF mediated IL-17 expression.

Induction NF-κB activation is one of the main outcomes of TNFα-mediated TNFR-signaling. Anti-TNFR2 treatment or ETN, in contrast to TNFR2 agonist or rhTNFα, led to strong inhibition of the transcription of NF-κB target genes RELB (Relb), NFKB1 (p50), NFKB2 (p52) and NFKBIA (IκBα) (Fig. 3c,d).

Together, these data demonstrate that TNFR2-signaling plays a significant role in the regulation of IL-17A production in CD4+ Tmem. Moreover, since anti-TNF agents suppress the activation of NF-κB signaling pathways, NF-κB does not seem to directly take part in the enhanced expression of IL-17 by CD4+ Tmem following anti-TNF treatment. At the same time this suggests that NF-κB might be involved in affecting factors or processes that negatively regulate IL-17 expression.

TNF blockade suppresses TNFAIP3/A20 expression, which is associated with enhanced IL-17A expression in activated CD4+ Tmem

For a more in-depth exploration of factors involved in the increased IL-17A expression induced by anti-TNF supplementation of αCD3/CD28 stimulated CD4+ Tmem, whole genome RNA-sequencing was performed. Since supplementation of rhTNFα reduced the IL-17A secreting potential of CD4+ Tmem, this condition was co-analyzed to establish differential gene expression between both conditions. The comparative analysis of CD4+ Tmem exposed to anti-TNF (ETN) versus rhTNFα revealed
opposing transcriptional profiles (Fig. 4a). Of the 751 differentially expressed genes (DEG) (FDR=0.05) between rhTNFα and anti-TNF comparison (For the full DEG list see Table E4 in this article’s Online Repository at http://www.jacionline.org), TNFAIP3 (tumor necrosis factor alpha-induced protein 3, encoding A20 protein), NFKBIA (IkBα), RELB (Relb), BIRC2 (baculoviral IAP repeat containing 2) and LTA (LTα) were suppressed by anti-TNFα (Fig. 4b), while AQP3 (aquaporin 3), SLFN5 (Schlafen family member 5), IL7R (IL-7R), APOL4 (apolipoprotein L4) and ITGA6 (integrin α6) were significantly upregulated (Fig. 4b).

The deubiquitinating enzyme (DUB) TNFAIP3/A20 is a well-known negative regulator of NFκB activation. Activation of NFκB (i.e. by TNFα) enables translocation of NFκB into the nucleus and subsequent target gene activation and protein expression including the anti-inflammatory regulator TNFAIP3/A20. The deubiquitinating activity of TNFAIP3/A20 ultimately avoids the assembly of proximal NF-κB-activating complexes and stops the inflammatory response, triggering an NF-κB auto-regulatory negative feedback loop. Therefore, of the DEG with a fold change >= 2 (red dot plots) from the total of 751 genes identified, we focused on the regulation of TNFAIP3/A20 by anti-TNF since TNFAIP3 is implicated in the pathology of several autoimmune diseases. Moreover, TNFAIP3/A20 has been shown to negatively regulate CD4+ T cell activation.

Using RT-qPCR we confirmed that anti-TNF (ETN) supplementation to cultures of αCD3/CD28 stimulated CD4+ Tmem suppressed TNFAIP3 mRNA, while on the contrary T cell activation in the presence of rhTNFα induced TNFAIP3 transcription (Fig. 4c). Also, we demonstrated that TNFAIP3 mRNA expression inversely correlated with IL17A expression (Spearman test, r= -0.7789, p<0.001) in activated CD4+ Tmem upon anti-TNF and rhTNFα treatment (Fig. 4d). Then, we analyzed if the
observed down-regulation of TNFAIP3 by anti-TNF (ETN) also resulted in reduced protein expression of its cognate protein A20 (~87 kD). To this end, isolated CD4+ Tmem were stimulated with αCD3/CD28 beads and rhIL-2 was added exogenously in the presence or absence of rhTNFα or anti-TNF (ETN) and after five days of culture the cells were harvested and prepared for western blot analysis. Anti-TNF treatment clearly inhibited TNFAIP3/A20 protein expression, while conversely the addition of rhTNFα, led to a significant increase of TNFAIP3/A20 (Fig. 4e).

To reveal a direct relationship between suppression of TNFAIP3/A20 and the increased IL-17A expression, we used a siRNA approach to knockdown TNFAIP3/A20 in isolated CD4+ Tmem. We demonstrate that TNFAIP3-siRNA resulted in significantly enhanced percentages of IL-17A expressing cells (Fig. 4f). Using RT-qPCR we confirmed that TNFAIP3-siRNA inhibition led to a suppression of TNFAIP3/A20 mRNA expression (Fig. 4g). The data implicate TNFAIP3/A20 as an essential regulator of IL-17A in CD4+ Tmem, and reveal the association between anti-TNF-mediated repression of TNFAIP3/A20 and enhancement of IL-17A expression. Moreover, in IBD patients that showed increased IL-17A production after anti-TNF therapy (Fig. 1d), we found a reduced frequency of the heterozygous TNFAIP3/A20 region variant genotype C>G (rs6927172) (Fig. 4h), which has been shown to increase TNFAIP3 expression in IBD, corroborating the reduced TNFAIP3/A20 mRNA expression in CD4+ Tmem (Fig. 4f). These observations further support the clinical relevance of our finding, i.e. deviant TNFAIP3/A20 expression plays an important role in IL-17A expression by CD4+ Tmem via down-modulation of TNFα-mediated signaling.
PKC and p38 MAPK-signaling are required for anti-TNF-mediated enhancement of IL-17A expression in stimulated CD4^+ memory T cells.

In the experiments above, we stimulated T cells by triggering both the T cell receptor (CD3) and co-stimulatory receptor CD28 by αCD3/CD28 beads. PKC-theta (PKC-θ) signaling, which plays an important role in T cell activation by αCD3/CD28 activation has also been implicated in the regulation of the biological activity of A20 in activated T cells and vice versa. Furthermore, PKC signaling has also been shown to contribute to IL-17 production in mouse T cells and human CD4^+ Treg. Interestingly, in our RNA-sequencing analysis (see Table E4 in this article’s Online Repository at http://www.jacionline.org) we observed that anti-TNF treatment induced expression of PRKCQ (encoding PKC-θ) in αCD3/CD28 stimulated CD4^+ Tmem, which we confirmed by RT-qPCR (Fig. 5a). We hypothesized that inhibition of the PKC-signaling of CD4^+ Tmem, which was activated in the presence of ETN/αCD3/CD28 beads, may prevent the anti-TNF mediated increase in IL-17A expression. Supplementation of the pan-PKC inhibitor AEB071 (sotrastaurin, PKCi) to CD4^+ Tmem that were activated by αCD3/CD28 beads in the presence of anti-TNF (ETN), prevented the anti-TNF mediated increase in IL-17A expression (Fig. 5b,c).

These results suggest that PKC-signaling, most likely as a consequence of TCR and CD28 co-stimulation, plays an important role in the anti-TNF mediated increase in IL-17A expression. However, it is not likely that proximal PKC-signaling is the main driver of anti-TNF mediated IL-17 expression in these activated T cells, rather a more distal signaling pathways will be involved. To further explain the enhanced IL-17A expression by anti-TNF treatment, we focused on the MAPK signaling pathway, as TNFAIP3/A20 has been implicated in the negative regulation of p38 MAPK inhibitor and recently p38 was shown to control IL-17 expression in mouse CD4^+ T cells.
Small molecule p38 MAPK inhibition (p38i) in CD4+ Tmem cells activated in the presence of anti-TNF (ETN), prevented the anti-TNF mediated increase in IL-17 expression (Fig. 5b,d). This indicates that p38-signaling is vital in anti-TNF mediated enhancement of IL-17A expression.

In summary, prevention of TNFα-signaling by anti-TNF in αCD3/CD28 stimulated CD4+ Tmem results in increased IL-17A production by inhibition of the anti-inflammatory mediator TNFAIP3/A20. Under these circumstances, PKC and p38 MAPK are crucial for the production of IL-17A. On the basis of our findings, and as depicted in Fig. 6 we propose a model illustrating the molecular pathways that are involved in anti-TNF treatment mediated enhancement of IL-17A expression in CD4+ T cells.

DISCUSSION

Anti-TNF therapy is successful in many patients with inflammatory disorders, but anti-TNF therapy is also associated with adverse effects such as the unexplained psoriasis-like syndrome. These paradoxical observations following inhibition of TNFα-signaling might be explained by the pleiotropic nature of TNFα that displays both inflammatory and anti-inflammatory bioactivities. So far, more detailed mechanistic insight in how these phenomena are induced is limited, and clinical data supporting such findings are lacking.

Here, we present novel insights, showing that inhibition of TNFα-signaling by anti-TNF agents in activated CD4+ memory T cells prevents expression of the anti-inflammatory mediator TNFAIP3/A20, which consequently promotes IL-17A production. This process requires PKC and p38 MAPK-signaling.
TNFAIP3/A20, encoded by TNFAIP3 is a ubiquitin-editing enzyme consisting of an A20-type zinc finger and an OTU domain, which mediates ubiquitin ligase and deubiquitinase activity, respectively. TNFAIP3/A20 acts as a negative feedback mechanism mainly by preventing NF-κB activation through deubiquitination or ubiquitination of a multitude of proteins such as RIP1. Although it is well established that TNFAIP3/A20 regulates TNFα-signaling, TNFAIP3/A20 has been shown to regulate multiple other immune pathways including TLR-signaling, IL-17R-signaling, Wnt-signaling, TCR-signaling and MAPK-signaling. Not surprisingly, loss of TNFAIP3/A20 expression causes spontaneous inflammatory and autoimmune diseases in mice, and polymorphisms or mutations in TNFAIP3 gene or region that affect A20 expression or function are related to a variety of autoimmune diseases in humans. Interestingly, clinical studies showed that TNFAIP3 region polymorphisms are associated with therapeutic efficacy of anti-TNF treatment in IBD as well as psoriasis patients, indicating an unresolved relationship between the lack of TNFα-signaling, deviant A20 expression and immune activity. Here, using RNA-seq and western blot analyses, we add to the understanding of this relationship by demonstrating that anti-TNF treatment prevents transcription and expression of the anti-inflammatory mediator TNFAIP3/A20 in activated CD4+ Tmem, and we subsequently provide evidence that siRNA-inhibition of TNFAIP3 results in increased IL17A expression.

The impact of TNFAIP3/A20 ablation on the exacerbated expression of IL-17A by CD4+ Tmem following anti-TNF therapy in the clinical setting is still unknown. Here, in a small cohort of IBD patients treated with the anti-TNF biological Remsima, we showed an inverse relationship between numbers of IL-17A-expressing CD4+ Tmem and a reduction of TNFAIP3/A20 mRNA expression, supporting our in vitro findings.
Notably, IBD patients treated with Remsima showing increased numbers of IL-17A expressing CD4+ T cells upon treatment, seemed to have a better clinical response. Also, this group revealed a lower frequency of the heterozygous TNFAIP3 region variant genotype C>G (rs6927172), which previously was shown to increase TNFAIP3/A20 expression, and which was associated with non-responsiveness to anti-TNF therapy in IBD patients. These findings clearly suggest a link between IL-17A expression by CD4+ T cells, TNFAIP3/A20 region polymorphisms, TNFAIP3 expression levels and clinical responsiveness to anti-TNF therapy. To firmly establish this link, further studies are needed.

Until now, a relationship between TNFAIP3/A20 expression and IL-17A production by activated T cells had not been established. Upon T cell stimulation, TNFAIP3/A20 is strictly regulated in order to control the process of T cell activation; TNFAIP3/A20 is highly expressed in resting CD4+ T cells and rapidly depleted after TCR/CD28 stimulation in a process involving both PKC-θ and paracaspase MALT1 activity, which ultimately leads to activation of NF-κB. However, under persistent stimulation, TNFAIP3/A20 is restored and abolishes TCR and NF-κB-signaling. Our data reveal that the inhibition of TNFAIP3 expression, either via blocking of TNFα or via TNFAIP3 knockdown, in activated CD4+ Tmem results in increased IL-17A expression in vitro. To our knowledge, this link between TNFAIP3/A20 and IL-17 has not been described before. However, from mice studies it is known that A20 can prevent MAPK activity, while p38 MAPK activity appeared essential for IL-17 production by CD4+ T cells in an experimental allergic encephalomyelitis (EAE) model. Although we did not evaluate the direct relation between inhibition of A20 and p38 MAPK activity in our experiments, our data show that p38 MAPK inhibition prevented anti-TNF-mediated enhancement of IL-17A expression by activated CD4+ Tmem. Together, this supports the possibility
that ablation of TNFAIP3/A20 by anti-TNF treatment, endorses p38 MAPK activity, thereby promoting in IL-17A expression in CD4+ Tmem.

Besides its function as a regulator of TCR-signaling in T cell activation, there is strong evidence that TNFAIP3/A20 is an important negative regulator of activation of antigen presenting cells (APCs) in mice. Anti-TNF treatment was shown to result in much stronger production of IL-17 in an in vitro experimental setup where human T cells were stimulated with αCD3 mAb plus monocytes, as compared to T cells that were activated with αCD3/CD28-stimulation beads only. This indicates that the presence of APCs even further boosts IL-17 expression following depletion of TNFα by anti-TNF agents. A possible explanation for this increased IL-17 expression upon monocyte co-culture is that anti-TNF agents might suppress TNFAIP3/A20 expression in these APC resulting in hyper-activated APC, which in turn provoke hyper-stimulation of CD4+ Tmem, thereby triggering high IL-17 expression. In fact, TNFAIP3/A20 deprivation of dendritic cells (DC) in mice led to exacerbated DC activation and these DC strongly promoted overproduction of IL-17 by mouse wild-type T cells in vitro. A limitation of our work is that we only focus on T cells as a source of TNFα, but obviously there are many other cellular sources of TNFα that are influenced by anti-TNF treatment. Next to T cells, also other immune cell types such as basophils and ILC3 were associated with IBD.

In which ways might our findings that TNFAIP3/A20 acts as master switch in TNFα blockade-driven IL-17A expression add to improved clinical regimens? IL-17A producing T cells are strongly associated with various inflammatory diseases. It could well be that the anti-TNF therapy associated reduction in TNFAIP3/A20 and its resultant increase in IL-17A expressing T cells contributes to the immune pathology of paradoxical autoimmune related side effects, such as psoriasis-like syndrome that are
observed in 0.6-5% of the patients treated with anti-TNF medication. Of interest, in pilot experiments we observed that CD4+ memory T cells of IBD patients that developed a psoriasis-like syndrome following anti-TNF treatment in the past, revealed reduced TNFAIP3/A20 mRNA expression levels and an increased potential to express IL-17A as compared to healthy controls or IBD patients that did not develop psoriasis-like syndrome (see Fig. E12 in this article’s Online Repository at http://www.jacionline.org). TNFAIP3/A20 has been shown to prevent p38 MAPK activity in a mouse model, and we here demonstrate that anti-TNF induced inhibition of TNFAIP3/A20 promotes IL-17A expression by facilitating p38 MAPK activity. Although speculative, anti-TNF treatment combined with specific p38 MAPK-inhibition might prevent the anti-TNF associated paradoxical autoimmune side effects. In addition, our data suggest that IL-17A expression and reduced TNFAIP3/A20 mRNA levels by activated CD4+ T cells might contribute to better clinical response in IBD patients treated with anti-TNF.

Although IL-17A is renowned for its pro-inflammatory potential, IL-17 has also been shown to be beneficial in epithelial barrier protection in IBD. Additional support for a protective role of IL-17A in IBD emerged from a placebo-controlled anti-IL-17A monoclonal antibody clinical trial in Crohn’s disease, where it was reported that a substantial number of the patients developed exacerbation of disease. Although anti-IL-17 inhibition was not successful in IBD, small-molecule inhibition of RORyt, a crucial transcription factor for Thelper-17 cell differentiation, might be an alternative approach for the treatment of IBD. Moreover, next to anti-TNF inhibition other biological-based therapeutics, such as adhesion-molecule inhibition are successfully applied in IBD.

Based on our current work we propose that combined analysis of TNFAIP3 mRNA
expression, TNFAIP3 region polymorphisms and IL-17A production by CD4+ T cells may aid the design of future patient risk stratification protocols in anti-TNF therapy. Moreover, it provides novel TNFAIP3/A20-activity related targets for additional improved therapeutic regimens in IBD patients. In conclusion, we add to the understanding of the biological effects of anti-TNF therapy by demonstrating that TNF blockade interferes in an autocrine anti-inflammatory regulatory feedback loop with TNFAIP3/A20 at its heart and resulting in enhanced IL-17A production.

DATA AVAILABILITY

Raw and processed data from RNA-seq have been deposited in the European Bioinformatics Institute (EMBL-EBI) database, under accession number E-MTAB-5622 (at http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-5622).

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Fig. 1. Anti-TNF promotes IL-17 expression in vitro and ex vivo in inflammatory bowel disease patients treated with anti-TNF therapy. Flow cytometry of IL-17A and IFNγ expression of PBMC from healthy controls that were stimulated with αCD3/CD28 beads and IL-2 in the absence or presence of anti-TNF agents. Intracellular IL-17A and IFNγ expression were measured on day five of the cultures after 4 hours restimulation with PMA and ionomycin in the presence of Brefeldin-A. a. Representative dot plots showing IFNγ and IL-17A expression. b. Cumulative data showing the percentage of single and double positive IL-17A and IFNγ-producing CD4+ T cells cultured for 5 days in the presence of TNF antagonists; infliximab (IFX), adalimumab (ADL) or etanercept (ETN) (n=6). c. Percentage of single and double positive IL-17A and IFNγ-producing CD4+ T cells from healthy control donors (HD) (n=6) and IBD patients (n=13) that were cultured as indicated. d. Ex vivo analysis of the absolute number of IL-17A-expressing CD4+ T cells from IBD patients upon anti-TNF treatment (remisa) (n=12). Based on the ex vivo IL-17A expression in PBMC, detected after 4 hours of restimulation with PMA and ionomycin in the presence of Brefeldin-A, the patients were grouped in those who showed reduced (IL-17-) or increased (IL-17+) IL-17A expression after remisa treatment (middle graphs), followed by their clinical response (lower graph). All data are shown as mean ± SEM. For statistical analysis the Friedman test followed by Dunn’s Multiple Comparison test, Wilcoxon matched-pairs signed rank test and Two-way ANOVA were used. P-value < 0.05 (*), < 0.01 (**), ns= no significant.

Fig. 2. Anti-TNF blockade promotes IL-17A production exclusively by ablation of TNFα-signaling in activated CD4+ memory T cells. a. Representative dot plots showing the FACS-sort strategy of CD25 CD45RA (Tmem), CD25 CD45RA+ (Tnaïve) CD4+ T cells from healthy controls. b. Cumulative data showing the expression of single IL-17A, single IFNγ and IL-17A/IFNγ co-expressing Tnaïve (n=6) or Tmem (n=11). Isotype control analysis is shown in Fig. E6 in the article’s Online Repository at www.jacionline.org. c. Cumulative data plot showing the percentage of single IL-17A, single IFNγ and IL-17A/IFNγ co-expressing CD4+ Tmem that were stimulated as indicated in the presence of recombinant human TNFα (rhTNFα) (n=11). d. Relative mRNA expression levels of IL17A, IL17F, IFNG, RORC and TBX21 in CD4+ Tmem that were cultured as indicated and measured by RT-qPCR (n=8). All data are shown as mean ± SEM. For statistical analysis Friedman test followed by Dunn’s Multiple Comparison test, Wilcoxon matched-pairs signed rank test and Two-way ANOVA were used. P-value < 0.05 (*), < 0.01 (**), < 0.001 (***), ns= no significant.

Fig. 3. TNFR2-signaling, not TNFR1, plays a critical role in regulating IL-17A-expression in CD4+ memory T cells. Flow cytometry of IL-17A expression of FACS-sorted CD4+ Tmem from healthy donors that were stimulated with αCD3/CD28 beads and IL-2 in the absence or presence of TNF-receptor antagonists (anti-TNFR2, anti-TNFR1) (n=7) or TNFR2 agonist (n=9) or cysTNFR2 plus 80M2 (n=6), or rhTNFα or anti-TNF (ETN) (n=8) and cultured for five days. a. Representative FACS dot plots showing CD3+ IL-17A expressing cells and b. cumulative data showing intracellular IL-17A expression in CD4+ Tmem after restimulation with PMA and ionomycin in the presence of Brefeldin-A (n=6-9). c and d. Relative mRNA expression measured by RT-qPCR of NFkB target genes in CD4+ Tmem that were cultured for 4 days in the presence of TNFR2 agonist, or anti-TNF (n=5) or rhTNFα or anti-TNF (ETN). All data are shown as mean ± SEM. Friedman test followed by Dunn’s Multiple Comparison test and Wilcoxon matched-pairs signed rank test were used for statistical analysis. P-value < 0.05 (*), < 0.01 (**), ns= no significance.

Fig. 4. TNFAIP3/A20 plays a central role in anti-TNF-mediated enhancement of IL-17 expression in activated CD4+ memory T cells. RNA-sequencing of healthy blood donor derived FACS-sorted CD4+ Tmem that were activated with αCD3/CD28 beads and IL-2 in the presence of recombinant human TNFα (rhTNFα) or anti-TNF (ETN) for three days. a. Representative heat map from 751 differentially expressed genes (DEG) obtained from DESeq2 analysis (FDR at 5%, adjusted p <= 0.05) is shown (n=4). b. Cumulative volcano plot showing the fold change in DEG when CD4+ Tmem was activated in the presence of ETN or rhTNFα. Red dots refer to genes with a >=2 fold change (n=4). c. Relative mRNA expression of TNFAIP3 in CD4+ Tmem that were activated for four days in the presence of rhTNFα or ETN (n=6). d. Spearman’s rank correlation coefficient of mRNA expression of TNFAIP3 vs IL17A. e. Western blot (WB) analysis showing the cytoplasmic expression of A20 in CD4+ Tmem, household protein expression of GAPDH is included. e. Cumulative data showing the relative protein expression of A20 normalized to GAPDH expression (n=4). The original WB membranes are shown in the Fig. E11. F. FACS analysis of the IL-17A expression in activated CD4+ Tmem where TNFAIP3 was inhibited by siRNA-based approach (n=5). g. TNFAIP3 mRNA inhibition by TNFAIP3 siRNA (n=5). All data are shown as mean ± SEM. h. Single SNP analysis of rs6987172 on IBD patients, those who showed reduced (IL-17-) or increased (IL-17+) IL-17A expression after Remisa treatment (n=12), and their i. relative mRNA expression of TNFAIP3 in FACS-sorted cryopreserved CD4+ Tmem (n=6). DESeq2, Friedman test followed by Dunn’s Multiple Comparison Test, Wilcoxon-Mann-Whitney U Test and Spearman test were used for statistical analysis. P-value < 0.05 (*), < 0.01 (**), ns= no significance.
Fig. 5. Protein kinase C and p38 MAPK are required for anti-TNF-mediated enhancement of IL-17A expression in stimulated CD4+ memory T cells. a. RT-qPCR of PRKCO by CD4+ Tmem that were stimulated with αCD3/CD28 and IL-2 in the absence or presence of rhTNFα or anti-TNF (ETN) for four days (n=8). b. Flow cytometry of the intracellular IL-17A expression of CD4+ Tmem that were activated with or without anti-TNF (ETN) in the absence or presence of PKC or p38 inhibitors (PKCi and p38i) for five days. Representative flow cytometry dot plots are shown. Aggregate flow cytometry data showing the effect of c. PKC inhibition (n=10) and d. p38 inhibition (n=6) on IL-17A expression. Data show mean ± SEM. Statistical significance was analysed by the Wilcoxon matched-pairs signed rank test and Friedman test followed by Dunn’s Multiple Comparison Test. P-value < 0.05 (*) and < 0.001 (**).  

Fig. 6. Model showing the involvement of the anti-inflammatory regulator A20 in anti-TNF mediated expression of IL-17A by activated CD4+ T cells.  
a. Upon T cell receptor activation and CD28 costimulation (1) TNFα is secreted (2) and signals via TNFR2 to promote NFκB expression (3) that leads to high expression of the anti-inflammatory mediator A20 (4) which is known to act as negative feedback loop to suppressing NFκB (5). Our results indicate that A20 may inhibit p38MAPK and PKC activity (6) and prevents IL-17A (7) expression.  
b. Anti-TNF inhibits TNFα-signaling via TNFR2 (2) and as a consequence A20 is not expressed (4). Lack of A20 impair NFκB negative feedback loop (5) and endorses PKC and p38 MAPK-signaling (6), inducing expression of IL-17 (7).  
c. Stratification of anti-TNF responsiveness in IBD patients (blue represent the group of patients with high IL-17 expression after anti-TNF treatment and red represents the group of patients with low IL-17 expression after anti-TNF treatment) based on intracellular IL-17A production by T cells, followed by TNFAIP3 mRNA expression and polymorphisms.