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Suppression of human CD4+ T cell activation by 3,4-dimethoxycinnamonyl-anthranilic acid (tranilast) is mediated by CXCL9 and CXCL10

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ABSTRACT

3,4-dimethoxycinnamonyl-anthranilic acid (tranilast) is an orally available anti-allergic drug with structural and functional homologies to immunosuppressive catabolites of the essential amino acid tryptophan and broad anti-inflammatory properties. It has recently been shown to be effective in animal models of multiple sclerosis and rheumatoid arthritis, two autoimmune diseases that are mediated by auto-aggressive Th1-polarized CD4+ T lymphocytes. Here we demonstrate potent suppressive effects of tranilast on the function of naïve human CD4+ T cells. Tranilast inhibited inhibits activation and proliferation of purified CD4+ T cells stimulated through the T cell receptor with an EC50 of less than 10 μM, a concentration that is well below plasma levels achieved after oral administration of approved doses of 200–600 mg in humans. The antiproliferative effects were less potent on naïve CD8+ T cells. Suppression of CD4+ and CD8+ T cell proliferation was associated with an inhibition of T cell activation. Cytokine analyses of naïve CD4+ T cells revealed that tranilast interferes with the production of cyto- and chemokines driven by signal transducer and activator of transcription 1 (STAT1), notably chemokine (C-X-C motif) ligands (CXCL) 9 and 10. Tranilast limited STAT1 phosphorylation in activated T cells and supplementation of CXCL9 or CXCL10 reversed the anti-proliferative effects of tranilast. These data imply CXCL9 and CXCL10 as novel therapeutic targets of tranilast in Th1-mediated autoimmune diseases and identify phospho-STAT1 and its target chemokines CXCL9 and CXCL10 as potential markers for monitoring the bioactivity of tranilast in humans.

1. Introduction

Many autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA) are characterized by an antigen-specific immune response driven by auto-aggressive CD4+ T cells polarized to a T helper (Th) 1 phenotype [1]. More recently Th cells producing interleukin 17 (IL-17) – Th17 cells – have been shown to be pathogenic in a variety of autoimmune disease models [2]. While broad T cell suppressive anti-inflammatory compounds are effective in autoimmune diseases they are often associated with severe side effects such as opportunistic infections. In addition, therapies using anti-CD4-antibodies have failed in MS multiple sclerosis [3], probably because they also eliminate CD4+ T cells with a suppressive or regulatory phenotype [4]. Thus, current therapeutic approaches aim at specifically targeting auto-aggressive T cell subsets either by skewing the balance of antigen-specific T cell responses to a net regulatory or suppressive mode or by interfering with the molecular program that drives the differentiation and activation of pathogenic CD4+ T cells [5]. *In vitro* studies and transgenic mouse models have not only identified specific cytokine profiles for regulatory and pathogenic Th cells but also helped characterizing the cytokines and their downstream signalling pathways necessary to induce the differentiation and activation of these Th cell subsets [6]. Gene expression analyses have guided these analyses and revealed that transcription factors such as forkhead box (Fox)P3, retinoic acid-related orphan nuclear receptor (ROR)γc, T-bet or GATA binding transcription factor (GATA)3 signify T*reg*, Th17, Th1 or Th2 polarized

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Th cells, respectively [7]. Once differentiated, specific activation of signal transducer and activator of transcription (STAT) pathway signatures reflect the state of T cell activation in autoimmune and primary immunodeficiency diseases [8]. This is clinically relevant as for instance phosphorylation of the interferon (IFN)-γ target STAT1 in CD4+ T cells correlates with disease activity in MS multiple sclerosis [9] and other autoimmune diseases [10,11]. While STAT1 during T cell development and differentiation appears to be important to maintain self-tolerance [12,13], deletion of IFN-γ and STAT1 in T cells provides a favourable milieu for the generation of T*reg* [14]. Collectively, suppression of Th1 responses by disrupting IFN-γ-induced STAT1 pathway activation may represent a promising target in Th1-mediated autoimmune diseases.

Tranilast (3,4-dimethoxycinnamonyl-anthranilic acid) is an anti-inflammatory agent with a favourable pharmacokinetic profile that is approved for the treatment of allergic disorders due to its capacity to inhibit mast cell degranulation [15]. Recent studies in animal models of Th1-mediated autoimmune diseases such as RA and MS have shown that tranilast suppresses pathogenic Th1 cell responses [16–19]. The molecular downstream targets of tranilast-mediated suppression of STAT1 phosphorylation, however, remain enigmatic. In addition, a biomarker for the anti-inflammatory activity of tranilast, which is currently in phase II clinical trials of RA, is lacking. Here we analyze the effects of tranilast on human CD4+ T cell function and characterize the molecular pathways influenced by this promising drug for Th1-mediated autoimmune diseases.

2. Materials and methods

2.1. Cell culture and reagents

Jurkat T cells (clone E6.1) (ATCC, Rockville, MD, USA), Peripheral blood mononuclear cells PBMC, and freshly isolated T cells were maintained in RPMI 1640 medium (PAA Laboratories GmbH, Pasching, Austria) containing 10% FBS (Thermo Fisher Scientific Inc., Schwerte, Germany), 100 U/ml penicillin and 100 μg/ml streptomycin (PAA Laboratories GmbH). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere. Tranilast (Nuon Therapeutics, San Francisco, USA) was diluted in DMSO (Roth GmbH, Karlsruhe, Germany) at a 100 mM stock concentration. Human recombinant interferon-γ (IFN-γ) and interleukin-2 (IL-2) were purchased from Immunotools (Friesoythe, Germany), human recombinant CXCL9 and CXCL 10 and anti-human CXCR3 blocking antibody were from R&D Systems (Minneapolis, USA). PBMC were isolated from healthy blood-donors by density gradient centrifugation using lymphocyte separation medium 1077 (PAA Laboratories GmbH, Pasching, Austria). Prior to T cell isolation, monocytes were removed by plastic adherence depletion for 45 min. CD4+ and CD8+ T cells and naive CD4+ and CD8+ T cells were purified by negative or positive selection with magnetic-associated cell sorting (MACS) according to the manufacturer's protocol (human CD4+ T cell Isolation Kit II, human CD8+ T cell Isolation Kit, human naive CD4+ T cell Isolation Kit II, and human naive CD8+ T cell Isolation Kit Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Purity was validated by flow cytometry using anti-CD4 or anti-CD8 antibodies, respectively. In all experiments, purity was above 95%. T cells were stimulated with 1.5 μg/ml anti-CD3, 2 μg/ml anti-CD28 (eBiosciences, San Diego, CA, USA) and 200 U/ml IL-2, immediately treated with different concentrations of tranilast or DMSO (vehicle control) and cultured in U-shaped 96-well plates at 100,000 cells per well for four days (proliferation) or six days (flow cytometry analysis). In indicated proliferation and mRNA expression experiments recombinant human CXCL9 or CXCL10 at 25 ng/ml or anti-CXCR3 at 10 μg/ml were added to the stimulated and treated cells, immediately.

2.2. Proliferation assay

T cells or Jurkat T cells were seeded in U-shaped 96-well plates at 100,000 cells per well.

Cells were stimulated and treated with tranilast (Nuon Therapeutics, San Francisco, USA) and DMSO (Roth GmbH, Karlsruhe, Germany) as described above. Cultures were pulsed with ^{3}H-methylthymidine (Amersham Radiochemical Centre, Buckinghamshire, UK) for 18 h after 24 h (Jurkat T cells) or 96 h (primary human T cells) stimulation. Finally, cells were harvested and radionuclide uptake was measured by scintillation counting.

2.3. Analysis of apoptosis

Cells were labelled with Annexin V-FITC (Bio Vision, California, USA) and DAPI (Invitrogen, Carlsbad, USA) in Annexin binding buffer (Biolegend, San Diego, USA) for 15 min, washed with PBS containing 3% FBS, 0.01% NaN3 and 2 mM EDTA and analyzed by flow cytometry using a BD FACS CANTO II cytometer (BD Biosciences, Heidelberg, Germany).

2.4. Flow cytometry

Flow cytometry was performed six days after stimulation. T cells were harvested, Fc-blocked with human serum for 15 min and washed in PBS, followed by incubation with specific antibodies against human CD4-PE (eBioscience, San Diego, CA, USA), CD69-APC, CD25-APC (Biolegend, San Diego, CA, USA), CD45RO-PE, and CD8-PE (eBioscience, San Diego, CA, USA) for 30 min in PBS containing 3% FBS, 0.01% NaN3 and 2 mM EDTA on ice. Cells were washed and analyzed by flow cytometry using a BD FACS CANTO II cytometer (BD Biosciences, Heidelberg Germany). Statistical analysis was performed using FlowJo software (version 7.6.1, FlowJo, Ashland, OR, USA).

2.5. Protein multiplex analysis of cytokines and chemokines

Supernatants of the isolated and stimulated T cell subsets were analyzed for different cytokines and chemokines after six days by using the Luminex-based multiplex technology (BioRad, Hercules, USA). The supernatants (50 μl) were incubated with color-coded beads coated with the capture antibodies for the respective cytokine/chemokine for 45 min at RT. After three washing steps, the beads were incubated with the biotinylated secondary detection antibodies for each cytokine/chemokine for 30 min at RT, followed by three washing steps and the final incubation with Steptavidin-PE (SA-PE) for 10 min at RT and three washings steps. The first laser is responsible for the bead identification while the second laser is used for the quantification of the SA-PE intensity. According to the standard curves, the concentration of the respective cytokine/chemokine is calculated and given as pg/ml.

2.6. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Naïve CD4+ T cells were stimulated as described above for 24 h or six days. Total RNA was isolated using the Qiagen RNAeasy RNA isolation kit (Qiagen, Hilden, Germany) and cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, USA). QRT-PCR was performed according to standard protocols in an ABI 7000 thermal cycler using SYBR Green PCR Mastermix (Applied Biosystems, Carlsbad, USA). 40S ribosomal protein S13 (RPS13) served as housekeeping gene. PCR reactions were checked by omission of templates and by melting curve and

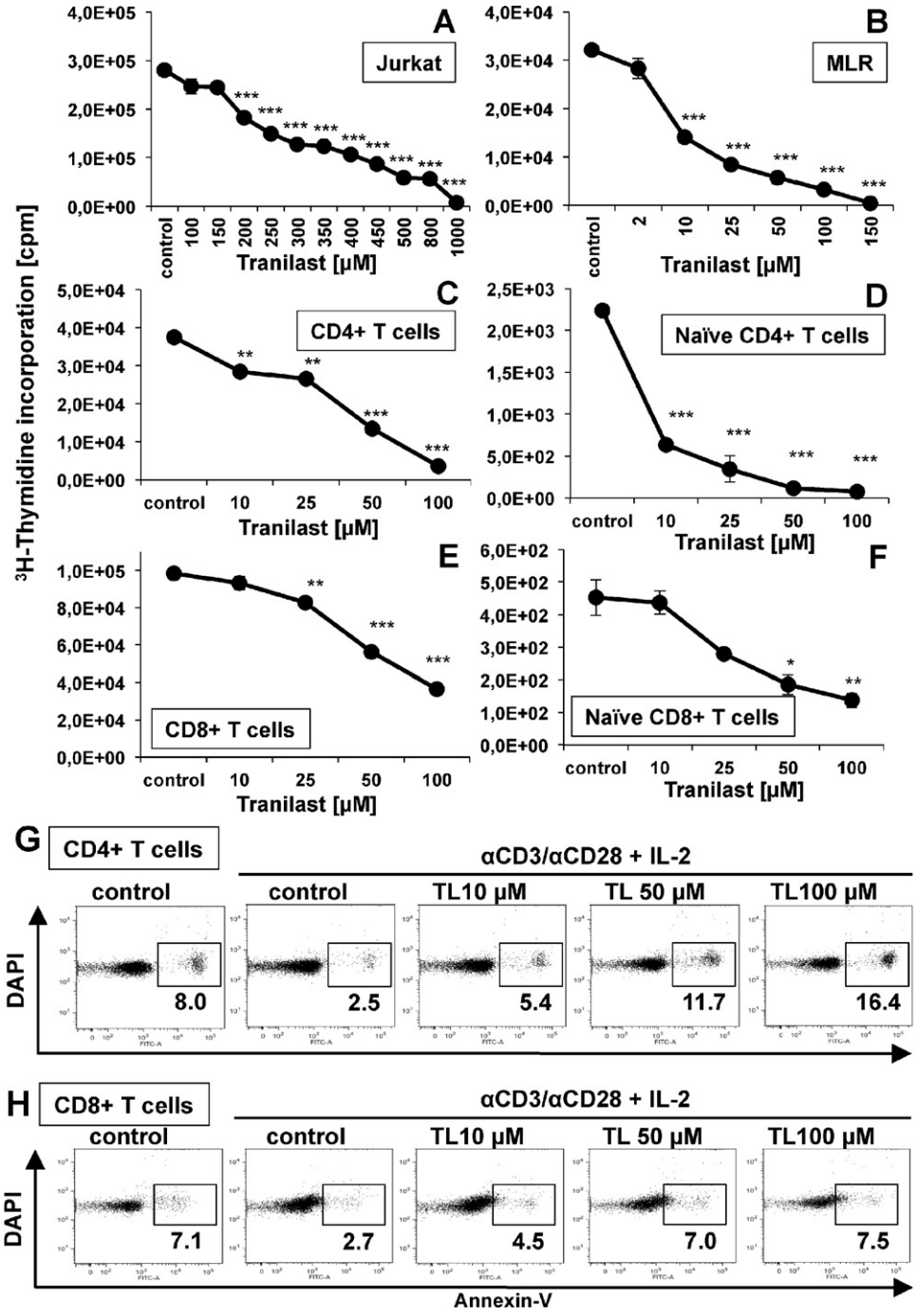

Fig. 1. *Tranilast suppresses proliferation of T cells.* ^{3}[H] methylthymidine incorporation of Jurkat T cells (A, 24 h), PBMC (B, 96 h) and primary T cells (C–F, 96 h) treated with tranilast or DMSO (control). Jurkat T cells were activated with anti-CD3 and anti-CD28, PBMC were activated with allogeneic irradiated PBMC and primary T cells were activated with anti-CD3, anti-CD28 and IL-2. Data are representative of three independent experiments. $n = 3 \pm$ SEM, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. G, H: Apoptosis of primary CD4+ (G) or CD8+ (H) T cells left untreated or after activation with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast or DMSO (control) using flow cytometric analysis of Annexin V and DAPI staining. Data are representative of three independent experiments.

agarose gel electrophoresis. Standard curves were generated for each gene and the amplification was 90–100% efficient. Relative quantification of gene expression was determined by comparison of threshold values. All results were normalized to RPS13. Primer sequences were (5′-3′ forward, reverse):

RPS 13 fwd.: AAGTACGTTTTGTGACAGGCA, rev.: GGTGAATCCGGCTCTCTATTAG; CXCL9: fwd.: AGCACCAACCAAGGGACTAT, rev.: GGGTTTAGACATGTTTGAACTCC; CXCL10: fwd.: TGTCCACGTGTTGAGATCAT, rev.: TTTCAGTAAATTCTTGATGGCC; (all from Sigma, St. Louis, USA);

STAT1 fwd.: AGGAAAAGCAAGCGTAATCTTCA, rev.: TATTCCCCGACTGAGCCTGAT (Invitrogen, Carlsbad, USA).

2.7. Mixed leukocyte reaction

Peripheral blood mononuclear cells (PBMCs) were isolated from different healthy blood-donors as described before. 2×10^5 irradiated (30 Gy) PBMC as stimulators and 2×10^5 PBMC from an unrelated donor as responders were seeded in flat-bottom 96-well plates and cultured in 200 µl medium per well. Cells were

treated with different concentrations of tranilast or DMSO. Five days after treatment cells were pulsed with ^{3}H-methylthymidine for 18 h. Cells were harvested and the radionuclide uptake was measured by scintillation counting.

2.8. Western Blot analysis

Freshly isolated PBMC were pretreated with 50 μM tranilast and DMSO control for 1 h and then stimulated with 1 μg anti-CD3 and 1 μg anti-CD28 per 1×10^{6} PBMCs for another hour.

Whole cell lysates were prepared in ice cold tris(hydroxymethyl)aminomethane hydrochloride (TRIS–HCl, 50 mM, pH 8.0; Carl Roth, Karlsruhe, Germany) containing 50 mM NaCl (J.T. Baker, Deventer, Holland), 1% NP-40 (AppliChem, Darmstadt, Germany), 10 mM EDTA (Gerbu Biotechnik, Gaiberg, Germany), 1% Glycerol, 200 mM dithiothreitol (Carl Roth, Karlsruhe, Germany), 100 μM phenylmethylsulphonyl fluoride (PMSF), complete protease inhibitor (Roche, Grenzach, Germany) and Phosphatase inhibitor cocktail 2 and 3 (Sigma Aldrich, St. Louis, USA).

Protein concentrations were determined by Bio-Rad protein assay (Bio-Rad, Hercules, CA, USA) at 595 nm and 25 μg of protein was separated by 8% SDS-PAGE. Proteins were transferred onto a 2 μm nitrocellulose membrane (Whatman, Dassel, Germany). After 1 h of blocking in PBS supplemented with 0.2% Tween 20 (Sigma–Aldrich, St. Louis, USA) and 5% bovine albumin fraction V powder (Roth GmbH, Karlsruhe, Germany), the membrane was

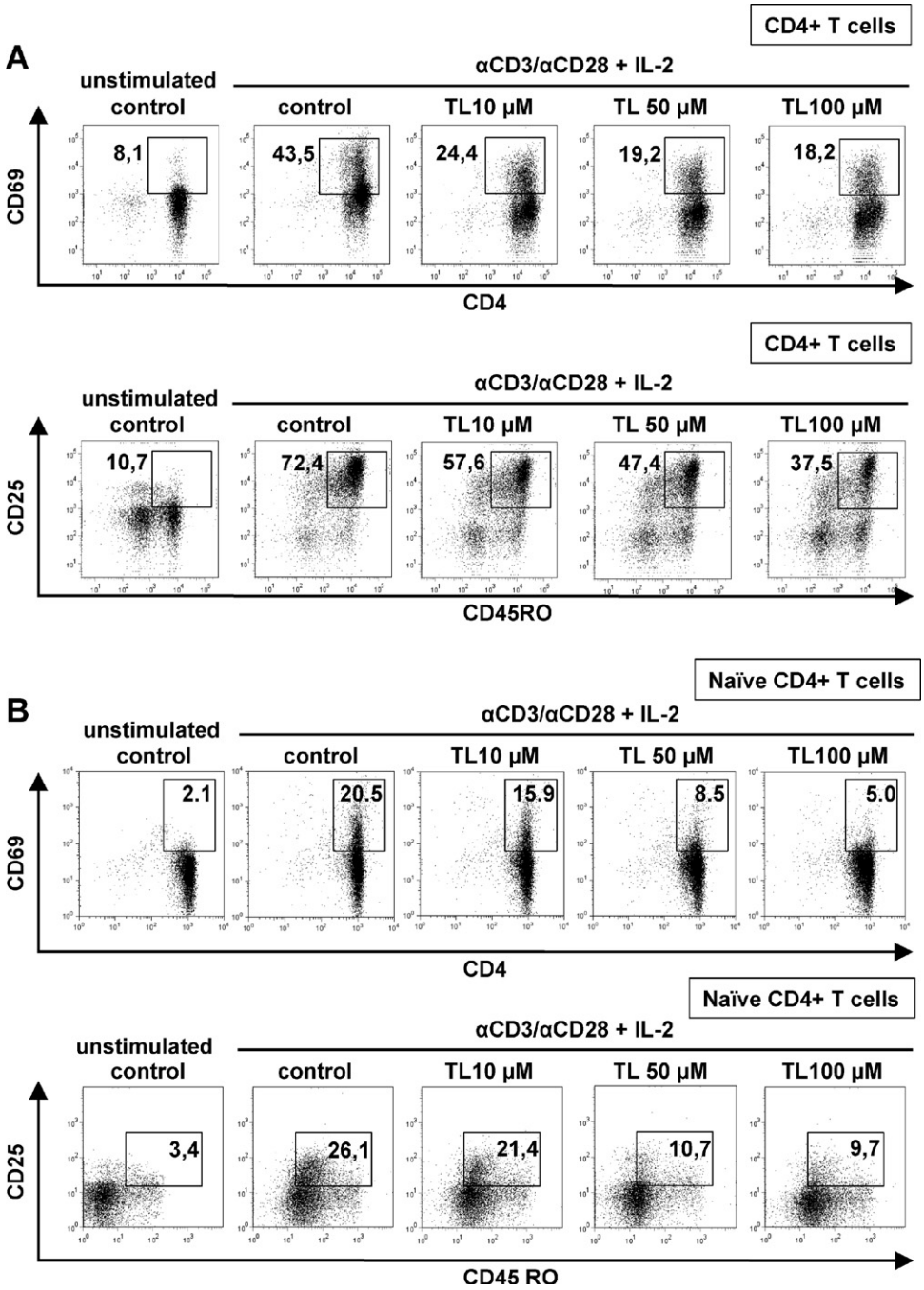

Fig. 2. *Tranilast suppresses activation of T cells.* Flow cytometry of CD4 or CD8 and CD69 staining or CD45 RO and CD25 staining in primary CD4+ T cells (A, B) or CD8+ T cells (C, D), either isolated as total (A, C) or naïve T cells (B, D), left untreated or activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast or DMSO (control) for six days. Data are representative of three independent experiments.

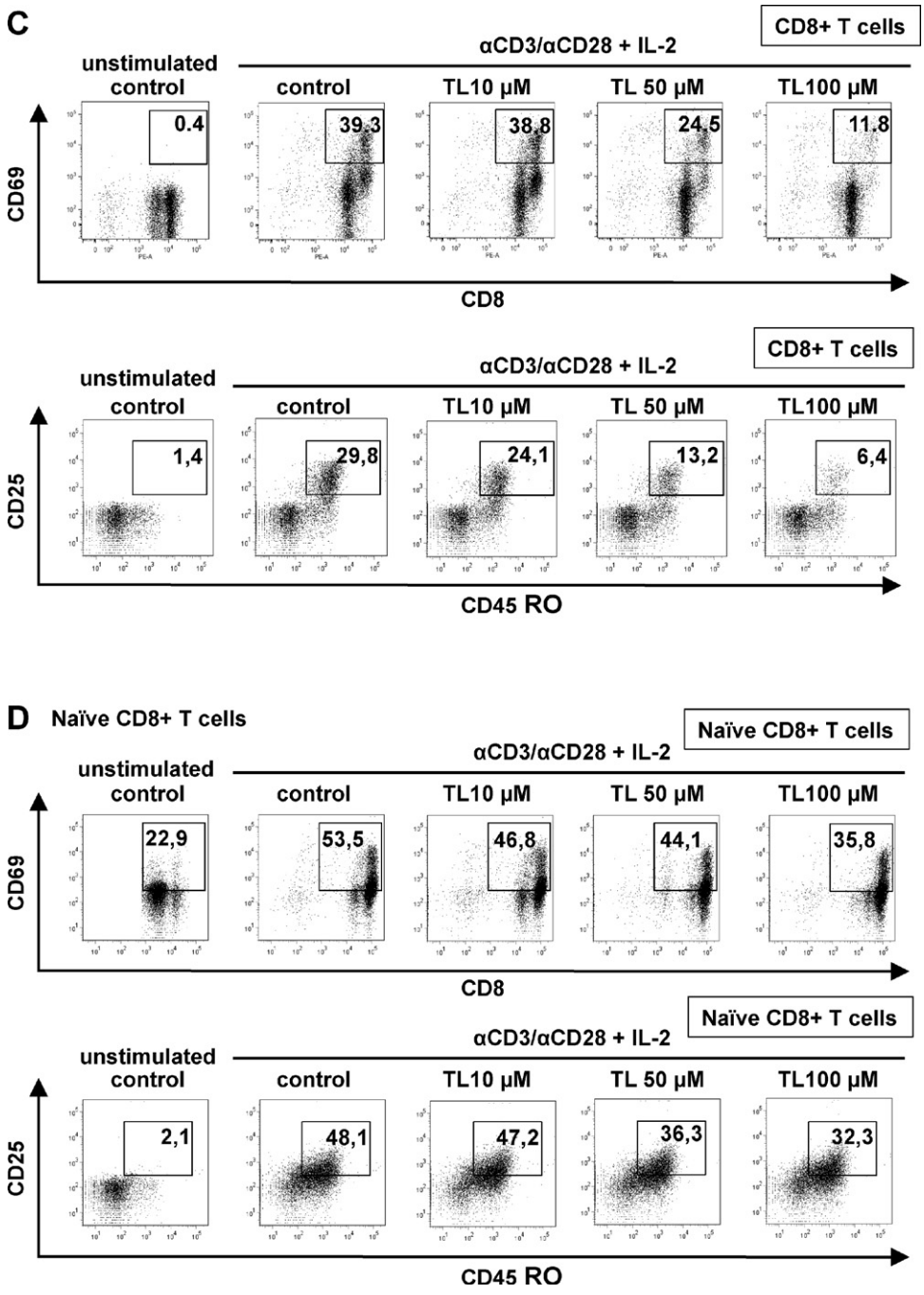

Fig. 2. (*Continued*).

incubated with rabbit anti-pSTAT1 or rabbit anti-STAT1 as loading control (both 1:1000 dilution, both Cell Signalling Technology, Beverly, USA) overnight at 4 °C. After 1 h incubation at room temperature with horseradish peroxidase-conjugated secondary antibody (donkey anti-rabbit, 1:2000 dilution, GE-Healthcare, Buckinghamshire, UK) proteins were visualized by ECL Plus reagent (GE Healthcare).

2.9. Statistical analysis

Data are expressed as mean ± SEM. Analysis of significance was performed using the Student's *t*-test (SigmaPlot, Systat Software Inc., San Jose, CA, USA).

3. Results

3.1. Tranilast suppresses proliferation of human T cells

The impact of tranilast on T cell proliferation was initially studied in Jurkat T cells. While concentrations of up to 150 μM did not result in a reduction of Jurkat T cell proliferation, higher concentrations suppressed proliferation with an EC50 of 300 μM (Fig. 1a). We next assessed the anti-proliferative activity in freshly isolated human peripheral blood monocytes (PBMC) stimulated in a mixed leukocyte reaction (MLR). Strikingly and in contrast to transformed T cells tranilast suppressed allogeneic proliferation of freshly isolated T cells in a concentration-dependent manner with

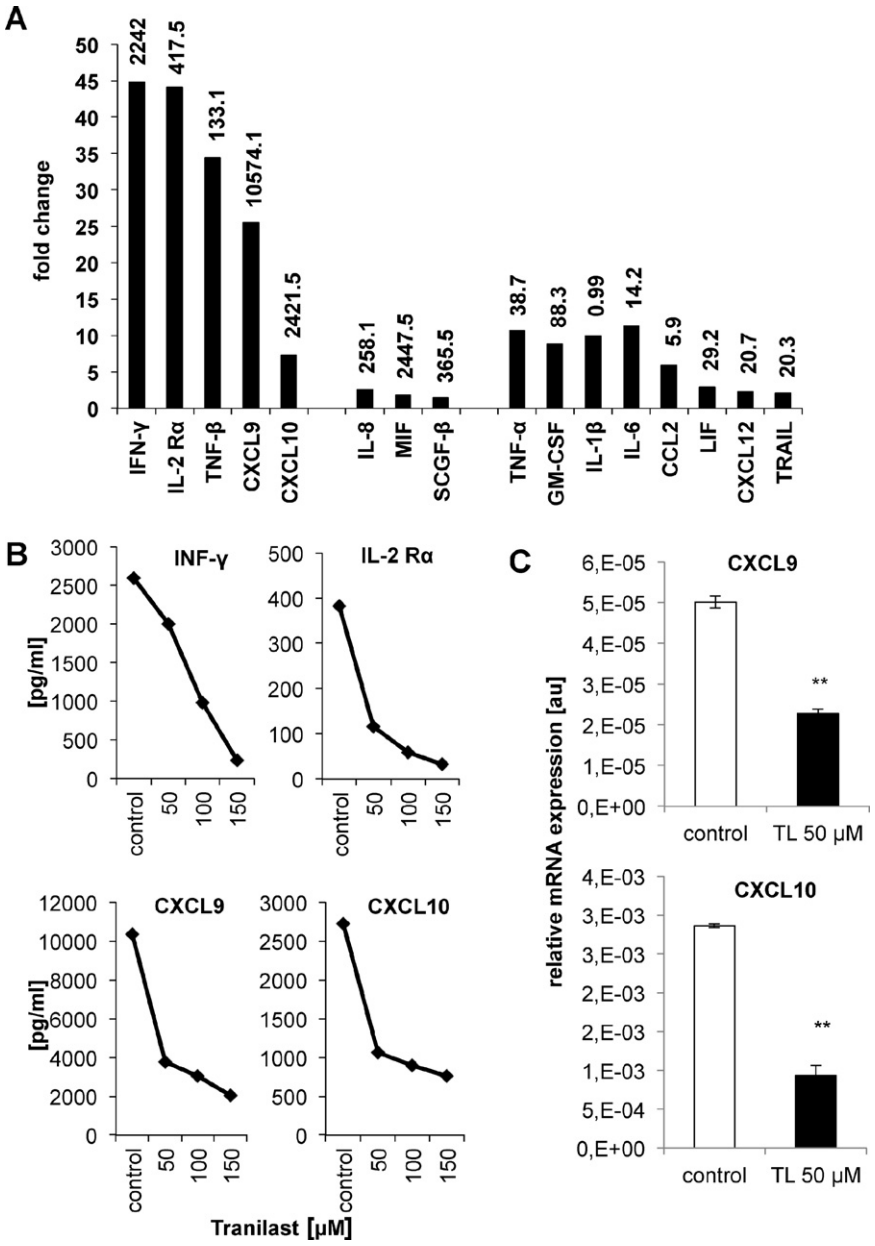

Fig. 3. *Modulation of T cell cytokines by tranilast.* A: Multiplex ELISA analysis of cytokine release from naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 for six days. Supernatants from 100,000 cells per 200μl were analyzed by Luminex-based multiplex technology. Data are displayed as fold change over non-activated. The numbers above the bars indicate total release of stimulated cells in pg/ml. Data are representative of two independent experiments. B: Multiplex ELISA analysis of cytokine release from naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at indicated concentrations or DMSO (control) for six days. Data are representative of two independent experiments. C: QRT-PCR analysis of CXCL9 and CXCL10 expression in naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at 50 μM or DMSO (control) for six days. Data are representative of two independent experiments. $n = 3 \pm$ SEM, $^{**}p < 0.01$.

an EC50 of less than 10 μM (Fig. 1b). Because of its effects on the activation and differentiation of antigen-specific Th1 cells in animal models of autoimmunity [16,18], we then evaluated the proliferation of isolated primary human CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 in response to tranilast. Concentrations of 50 μM tranilast reduced CD4+ T cell proliferation by 60% (Fig. 1c). Strikingly, when primary human naïve CD4+ T cells were similarly activated in the presence of tranilast, concentrations of 10 μM tranilast were sufficient to reduce CD4+ T cell proliferation by 70% (Fig. 1d). In comparison, CD8+ T cells and naïve CD8+ T cells were more resistant to the anti-proliferative effects of tranilast (Fig. 1e, f). While tranilast resulted in a slight increase of apoptosis in CD4+ T cells at concentrations of >50 μM (Fig. 1g), there was no induction of cell death in CD8+ T cells (Fig. 1h). Collectively, these data show that freshly isolated human naïve CD4+ are highly susceptible to anti-proliferative effects of tranilast.

3.2. Tranilast suppresses the activation of primary human T cells

Corresponding to the anti-proliferative effects tranilast concentration-dependently suppressed the expression of the activation markers CD69, CD25 and CD45RO in primary human CD4+ T cells (Fig. 2a) and CD8+ T cells (Fig. 2c). Again, these effects were most pronounced in naïve CD4+ T cells (Fig. 2b) but also observed in naïve CD8+ T cells (Fig. 2d).

3.3. Tranilast differentially regulates the cytokine and chemokine profile of CD4+ T cells

Next, we assessed the impact of tranilast on the release of immunomodulatory soluble factors in freshly isolated human naïve CD4+ T cells stimulated by anti-CD3, anti-CD28 and IL-2. Cytokine analyses revealed that activation of naïve CD4+ T cells induced the release of substantial amounts of interferon (IFN)-γ, IL-2 receptor (R)α, tumour necrosis factor (TNF)-β, and chemokine (C-X-C motif) ligand CXCL9 and 10, while the amounts of TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF), IL-1β, IL-6, chemokine (C-C motif) ligand (CCL) 2, IL-10, IL-17, leukaemia inhibiting factor (LIF), CXCL12 and TNF-related apoptosis inducing ligand (TRAIL) release after T cell activation were negligible (Fig. 3a and data not shown). In contrast, activation of CD4+ T cells resulted only in a small increase of IL-8, macrophage migration inhibitory factor (MIF) and stem cell growth factor (SCGF)-β (Fig. 3a). Of the soluble factors strongly induced by TCR stimulation, tranilast differentially suppressed the release of IFN-γ, IL-2Rα, CXCL9 and CXCL10 (Fig. 3b) while IL-8, SCGF-β, MIF and TNF-β remained unaltered (data not shown). Of note, the STAT1 target cytokines CXCL9 and CXCL10 were particularly suppressed at lower concentrations (i.e. 50 μM) of tranilast (Fig. 3b). The reduction of CXCL9 and CXCL10 by tranilast appeared to be mediated at the level of transcription as tranilast induced a strong reduction in CXCL9 and CXCL10 mRNA expression in activated naïve CD4+ T cells (Fig. 3c).

3.4. Tranilast suppresses CD4+ T cell proliferation via STAT1 and CXCL9/10

Since we have previously shown that STAT1 is a molecular target of tranilast in murine microglial cells [18] we next assessed whether tranilast alters STAT1 phosphorylation in freshly isolated T cells. STAT1-phosphorylation induced by TCR stimulation was suppressed by tranilast in PBMC (Fig. 4a). Long-term cultures of activated PBMC and CD4+ T cells with tranilast also resulted in a suppression of STAT1 gene expression (Fig. 4b). As CXCL9 has previously been shown to stimulate CD4+ T cell proliferation [20] we assessed whether tranilast-mediated suppression of CD4+ T cell proliferation was blocked by CXCL9. Indeed, human recombinant CXCL9 restored STAT1 gene expression (Fig. 4b) and proliferation in tranilast-treated activated human CD4+ T cells (Fig. 4c). Of note, human recombinant CXCL10 was equally effective in restoring T cell proliferation suppressed by tranilast (Fig. 4c). Moreover, while the addition of CXCL9 and CXCL10 only slightly increased the activation of human naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2, both chemokines were capable of partly restoring the suppressive effects of tranilast on T cell activation (Fig. 4d). Finally blocking signalling through CXCR3, the receptor for CXCL9 and CXCL10 in activated T cells, mimicked the suppressive effects of tranilast on T cell proliferation (Fig. 4e) and activation (Fig. 4f). Collectively, these data suggest, that tranilast suppresses CD4+ T cell proliferation *via* inhibition of STAT1-phosphorylation and CXCL9/CXCL10 release (Fig. 5).

4. Discussion

While tranilast has initially been developed – and has long been approved – for allergic diseases, it has now become clear that its cellular activity goes far beyond inhibition of mast cell degranulation. Examples of these pleiotropic effects include inhibition of vascular smooth muscle cell proliferation [21,22], suppression of fibroblast and endothelial function [23,24], targeting transforming growth factor (TGF)-β production [25,26] and inhibition of tumour cell proliferation and motility [26,27]. These effects that play a key role in vascular diseases, fibrosis and metastatic cancer have been translated into several clinical trials addressing the biological activity in these conditions [28–30]. More recently, we have shown that tranilast displays structural and functional similarities to immunosuppressive catabolites of the essential amino acid tryptophan and demonstrated anti-inflammatory activity in an animal model of MS, where tranilast suppressed the antigen-specific Th1 cell response and induced the generation of antigen-specific T*reg* [18]. Anti-inflammatory activity has subsequently been shown in RA, another Th1-mediated autoimmune disease [16,17]. Thus, a clinical phase II study has been done testing the activity of tranilast in patients with RA (ClinicalTrials.gov Identifier NCT00882024). While the effects on antigen-specific CD4+ T cells in mice had been thoroughly characterized [16,18], the effects on human CD4+ T cells had not been addressed to date. We show that tranilast suppresses activation and proliferation of freshly isolated human naïve CD4+ T cells with an EC50 of less than 10 μM (Fig. 1b, c). This effect is clinically relevant as steady-state plasma levels of 30–100 μM are achieved using clinically approved doses of 200–600 mg [31]. It is interesting that tranilast was less active in suppressing the proliferation of total CD4+ T cells and of total and naïve CD8+ T cells (Fig. 1c, e, f). Interestingly, this differential activity on CD4+ lymphocyte subsets has also been demonstrated for tryptophan catabolites [32,33], supporting the notion that tranilast not only structurally but also functionally shares tryptophan catabolite activity [34]. We set out to analyze the molecular target of tranilast in human CD4+ T cells mediating the anti-proliferative activity. Early *in vivo* studies have indicated that tranilast is capable of suppressing human major histocompatibility complex (MHC) expression [35]. MHC are typically induced on virtually any cell type (class I MHC) and antigen presenting cells (APC), particularly (class II MHC), in response to viral infections as a result of host type I and type II interferons [36]. In APC this response is driven by IFN-γ-mediated phosphorylation of STAT1, which subsequently shuttles to the nucleus to induce MHC class II transcription *via* the class II transactivator (CIITA) [37]. Indeed, we have shown previously in mouse microglial cells that tranilast blocks IFN-γ-mediated phosphorylation of STAT1 and expression of CIITA and MHC class II in mouse microglial cells *in vitro* and *in vivo* [18]. These observations are in accordance with the data presented here showing that tranilast blocks at low doses (<50 μM) two key STAT1 target chemokines in human CD4+ T cells, namely CXCL9 – also termed monokine induced by IFN-γ (MIG) – and CXCL10 – also termed IFN-γ-induced protein 10 KD (IP-10) (Fig. 3b). At higher doses (>50 μM) we observed an increase in apoptosis in CD4+ T cells (Fig. 1g) as well as unspecific suppression of other pro- and anti-inflammatory cytokines such as IFN-γ and IL-10 (Fig. 3b). IFN-γ appears to be a preferred target of tranilast while we did not observe relevant changes in IL-17, IL-4 or induction of FoxP3 (data not shown), indicating that in our experimental system tranilast preferentially suppresses the differentiation of Th1 cells, which is in line with our previous observations in murine Th1-mediated autoimmune neuroinflammation [18]. These data may also imply that tranilast targets additional signalling pathways at higher concentrations. Indeed, others and we have previously shown that tranilast is capable of suppressing the activation of nuclear factor (NF)-κB [31,38], protein kinase C (PKC) [23,39] and mitogen activated protein kinases (MAPK) [21,39]. Based on previous studies demonstrating that tranilast interferes with tyrosine phosphorylation [21,23] it is tempting to speculate that tranilast modulates these diverse signalling pathways with differential affinity and particular activity towards STAT1. Our current and previous results render evidence to the hypothesis that inhibition of STAT1 is responsible for the preferential CD4+ T cell suppressive activity of tranilast in mice and humans [18]. More importantly, our study implies that

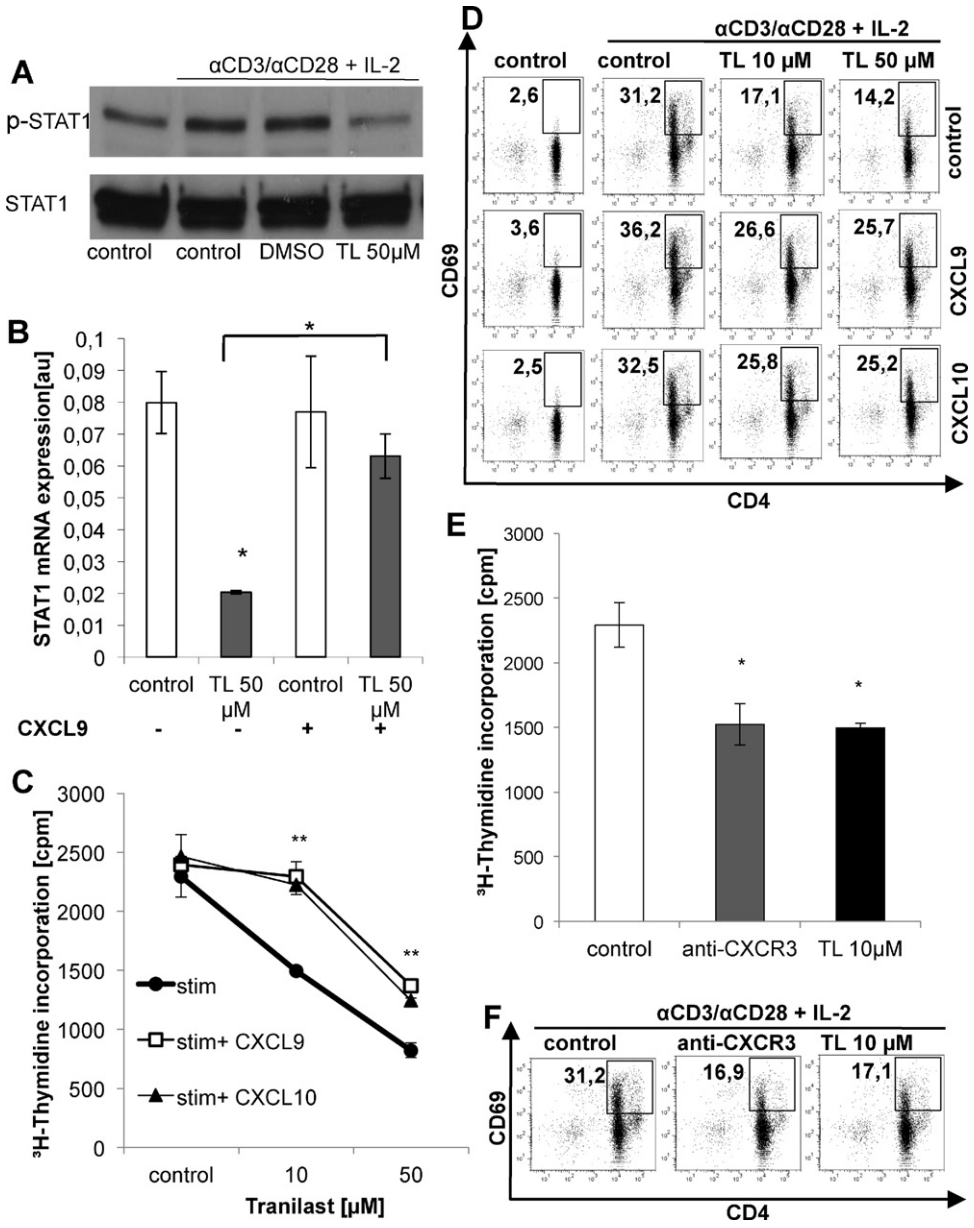

Fig. 4. *Tranilast suppresses T cell proliferation through STAT1 and CXCL9/10.* A: Western Blot analysis of STAT1 phosphorylation of human primary PBMC pretreated with 50 μM tranilast or DMSO (control) for 1 h and activated with anti-CD3 and anti-CD28 for another hour. Blots were reprobed with an antibody against total STAT1 as a loading control. B: QRT-PCR analysis of STAT1 expression in naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at 50 μM or DMSO (control) and CXCL9 at 25 ng/ml for 24 h. C: ^{3}[H] methylthymidine incorporation of naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at indicated concentrations or DMSO (control) and CXCL9 or CXCL10 at 25 ng/ml for 96 h. $^{**}(p < 0.01)$ vs. stimulation without CXCL9 or CXCL10. D: Flow cytometry of CD4 and CD69 staining in naïve CD4+ T cells left untreated or activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at 50 μM and CXCL9 or CXCL10 at 25 ng/ml for six days. E: ^{3}[H]-methylthymidine incorporation of naïve CD4+ T cells activated with anti CD3, anti-CD28 and IL-2 in the presence of anti-CXCR3 at 10 μg/ml for 96 h. As controls served stimulated untreated cells and stimulated cells treated with Tranilast at 10 μM. F: Flow cytometry of CD4 and CD69 staining in naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 in the presence of anti-CXCR3 at 10 μg/ml for six days. As controls served stimulated untreated cells and stimulated cells treated with Tranilast at 10 μM. Data are representative of two independent experiments. $n = 3 \pm$ SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

the STAT1 target chemokines CXCL9 and CXCL10 are the mediators of the anti-proliferative activity of tranilast as exogenous CXCL9 or CXCL10 restored this effect (Fig. 4c). CXCL9 and CXCL10 share a common receptor, CXCR3 [40], which is also suppressed by tranilast in human CD4+ T cells (data not shown). Targeting CXCR3 by blocking antibodies, peptidergic ligands or small molecules ameliorates disease activity in animal models of MS [41–43] and RA [44,45], although knock-out mouse models revealed conflicting results [46,47]. While the anti-inflammatory efficacy of this therapeutic approach has been attributed to the limitation of CXCR3+ auto-aggressive Th1 cells to inflammatory sites [40], it has also been shown that CXCL9 is capable of stimulating the proliferation of CD4+ and CD8+ T cells [20,48]. The direct proinflammatory function of the CXCL9/CXCL10–CXCR3 pathway in human T cells is supported by our data showing that blocking CXCR3 suppresses the proliferation and activation of T cells through the TCR, thus mimicking the effects of tranilast (Fig. 4e, f).

In addition to their immunomodulatory properties, CXCR3 and its ligands may also serve as biomarkers as they correlate with immune and disease activity in a variety of autoimmune conditions [49]. CXCL9 and CXCL10 have been shown to correlate with disease activity in MS and RA [50–53]. Thus, these molecular targets of tranilast may in addition to phosphorylated STAT1 serve as markers to assess bioactivity of tranilast in clinical trials. Further

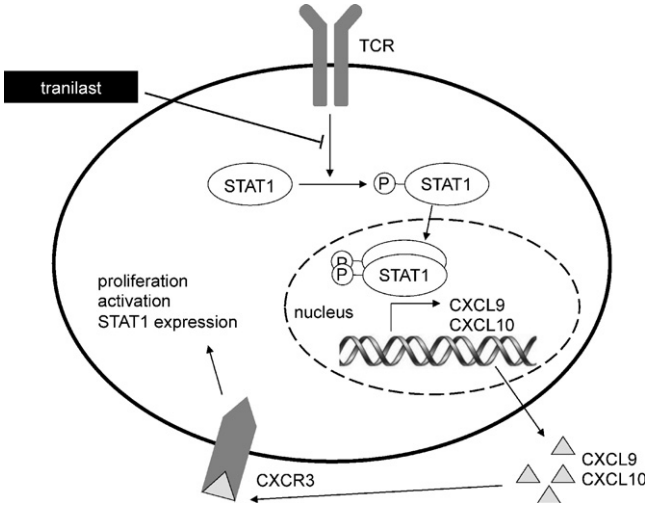

Fig. 5. *A molecular model of tranilast-mediated suppression of T cell activation.* Upon T cell activation STAT1 phosphorylation leads to transcriptional upregulation of CXCL9 and CXCL10. These chemokines are secreted and – in an autocrine/paracrine fashion – activate CXCR3 on T cells driving proliferation, activation and expression of STAT1. Tranilast interferes with STAT1 phosphorylation, thus preventing the upregulation of CXCL9 and CXCL10.

clinical trials will determine whether tranilast targets CXCL9 and CXCL10 in patients with Th1-mediated autoimmune diseases and whether assaying CXCL9 and 10 in serum and other fluids can monitor clinical activity.

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