



HAL
open science

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

Anne Hertenstein, Theresa Schumacher, Ulrike Litzenburger, Christiane A. Opitz, Christine S. Falk, Tito Serafini, Wolfgang Wick, Michael Platten

► **To cite this version:**

Anne Hertenstein, Theresa Schumacher, Ulrike Litzenburger, Christiane A. Opitz, Christine S. Falk, et al.. Suppression of human CD4⁺ T cell activation by 3,4-dimethoxycinnamonyl-anthranilic acid (tranilast) is mediated by CXCL9 and CXCL10. *Biochemical Pharmacology*, 2011, 82 (6), pp.632. 10.1016/j.bcp.2011.06.013 . hal-00722842

HAL Id: hal-00722842

<https://hal.science/hal-00722842>

Submitted on 5 Aug 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

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

Authors: Anne Hertenstein, Theresa Schumacher, Ulrike Litzenburger, Christiane A. Opitz, Christine S. Falk, Tito Serafini, Wolfgang Wick, Michael Platten

PII: S0006-2952(11)00378-9
DOI: doi:10.1016/j.bcp.2011.06.013
Reference: BCP 10937

To appear in: *BCP*

Received date: 16-4-2011
Revised date: 6-6-2011
Accepted date: 7-6-2011

Please cite this article as: Hertenstein A, Schumacher T, Litzenburger U, Opitz CA, Falk CS, Serafini T, Wick W, Platten M, Suppression of human CD4+ T cell activation by 3,4-dimethoxycinnamonyl-anthranilic acid (tranilast) is mediated by CXCL9 and CXCL10, *Biochemical Pharmacology* (2010), doi:10.1016/j.bcp.2011.06.013

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.



1
2
3
4
5
6
7 **Suppression of human CD4+ T cell activation by 3,4-dimethoxycinnamonyl-anthranilic**
8 **acid (tranilast) is mediated by CXCL9 and CXCL10**
9

10 Anne Hertenstein ^{a,b}, Theresa Schumacher ^{a,b}, Ulrike Litzenburger ^{a,b}, Christiane A. Opitz ^{a,b},
11 Christine S. Falk ^{c,d}, Tito Serafini ^e, Wolfgang Wick ^{a,f}, Michael Platten ^{a,b,*}
12

13
14 ^a Department of Neurooncology, University Hospital Heidelberg, 69120 Heidelberg, Germany
15

16 ^b Experimental Neuroimmunology Unit, German Cancer Research Center (DKFZ), 69120
17 Heidelberg, Germany
18

19 ^c Immune Monitoring Unit, German Cancer Research Center (DKFZ), National Center for
20 Tumor Diseases, Translational Oncology and Institute for Immunology, University
21 Heidelberg, Heidelberg, 69120 Heidelberg, Germany
22

23 ^d Institute for Transplantation Immunology, IFB-Tx, Hannover Medical School, 30625
24 Hannover, Germany
25

26 ^e Nuon Therapeutics Inc., 2755 Campus Drive, Suite 175 San Mateo, CA 94403, U.S.A.
27

28 ^f Clinical Cooperation Unit Neurooncology, German Cancer Research Center (DKFZ), 69120
29 Heidelberg, Germany
30

Abstract

1
2
3
4
5 3,4-dimethoxycinnamonyl-anthranilic acid (tranilast) is an orally available anti-allergic drug
6
7 with structural and functional homologies to immunosuppressive catabolites of the essential
8
9 amino acid tryptophan and broad anti-inflammatory properties. It has recently been shown to
10
11 be effective in animal models of multiple sclerosis and rheumatoid arthritis, two autoimmune
12
13 diseases that are mediated by auto-aggressive Th1-polarized CD4⁺ T lymphocytes. Here we
14
15 demonstrate potent suppressive effects of tranilast on the function of naïve human CD4⁺ T
16
17 cells. Tranilast inhibits activation and proliferation of purified CD4⁺ T cells stimulated
18
19 through the T cell receptor with an EC₅₀ of less than 10 μ M, a concentration that is well
20
21 below plasma levels achieved after oral administration of approved doses of 200 – 600 mg in
22
23 humans. The antiproliferative effects were less potent on naïve CD8⁺ T cells. Suppression of
24
25 CD4⁺ and CD8⁺ T cell proliferation was associated with an inhibition of T cell activation.
26
27 Cytokine analyses of naïve CD4⁺ T cells revealed that tranilast interferes with the production
28
29 of cyto- and chemokines driven by signal transducer and activator of transcription 1 (STAT1),
30
31 notably chemokine (C-X-C motif) ligands (CXCL) 9 and 10. Tranilast limited STAT1
32
33 phosphorylation in activated T cells and supplementation of CXCL9 or CXCL10 reversed the
34
35 anti-proliferative effects of tranilast. These data imply CXCL9 and CXCL10 as novel
36
37 therapeutic targets of tranilast in Th1-mediated autoimmune diseases and identify phospho-
38
39 STAT1 and its target chemokines CXCL9 and CXCL10 as potential markers for monitoring
40
41 the bioactivity of tranilast in humans.
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Introduction

1
2
3
4
5 Many autoimmune diseases, such as multiple sclerosis (MS) and rheumatoid arthritis (RA)
6
7 are characterized by an antigen-specific immune response driven by auto-aggressive CD4+ T
8
9 cells polarized to a T helper (Th) 1 phenotype [1]. More recently Th cells producing
10
11 interleukin 17 (IL-17) - Th17 cells - have been shown to be pathogenic in a variety of
12
13 autoimmune disease models [2]. While broad T cell suppressive anti-inflammatory
14
15 compounds are effective in autoimmune diseases they are often associated with severe side
16
17 effects such as opportunistic infections. In addition, therapies using anti-CD4-antibodies have
18
19 failed in multiple sclerosis [3], probably because they also eliminate CD4+ T cells with a
20
21 suppressive or regulatory phenotype [4]. Thus, current therapeutic approaches aim at
22
23 specifically targeting auto-aggressive T cell subsets either by skewing the balance of antigen-
24
25 specific T cell responses to a net regulatory or suppressive mode or by interfering with the
26
27 molecular program that drives the differentiation and activation of pathogenic CD4+ T cells
28
29 [5]. *In vitro* studies and transgenic mouse models have not only identified specific cytokine
30
31 profiles for regulatory and pathogenic Th cells but also helped characterizing the cytokines
32
33 and their downstream signalling pathways necessary to induce the differentiation and
34
35 activation of these Th cell subsets [6]. Gene expression analyses have guided these analyses
36
37 and revealed that transcription factors such as forkhead box (Fox)P3, retinoic acid-related
38
39 orphan nuclear receptor (ROR) γ c, T-bet or GATA binding transcription factor (GATA)3
40
41 signify *Treg*, Th17, Th1 or Th2 polarized Th cells, respectively [7]. Once differentiated,
42
43 specific activation of signal transducer and activator of transcription (STAT) pathway
44
45 signatures reflect the state of T cell activation in autoimmune and primary immunodeficiency
46
47 diseases [8]. This is clinically relevant as for instance phosphorylation of the interferon (IFN)-
48
49 γ target STAT1 in CD4+ T cells correlates with disease activity in multiple sclerosis [9] and
50
51 other autoimmune diseases [10, 11]. While STAT1 during T cell development and
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 differentiation appears to be important to maintain self-tolerance [12, 13], deletion of IFN- γ
2 and STAT1 in T cells provides a favourable milieu for the generation of *Treg* [14].
3

4 Collectively, suppression of Th1 responses by disrupting IFN- γ -induced STAT1 pathway
5 activation may represents a promising target in Th1-mediated autoimmune diseases.
6

7
8
9
10 Tranilast (3,4-dimethoxycinnamonyl-anthranilic acid) is an anti-inflammatory agent with a
11 favourable pharmacokinetic profile that is approved for the treatment of allergic disorders due
12 to its capacity to inhibit mast cell degranulation [15]. Recent studies in animal models of Th1-
13 mediated autoimmune diseases such as RA and MS have shown that tranilast suppresses
14 pathogenic Th1 cell responses [16-19]. The molecular downstream targets of tranilast-
15 mediated suppression of STAT1 phosphorylation, however, remain enigmatic. In addition, a
16 biomarker for the anti-inflammatory activity of tranilast, which is currently in phase II clinical
17 trials of RA, is lacking. Here we analyze the effects of tranilast on human CD4⁺ T cell
18 function and characterize the molecular pathways influenced by this promising drug for Th1-
19 mediated autoimmune diseases.
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Materials and methods

Cell culture and reagents

Jurkat T cells (clone E6.1) (ATCC, Rockville, MD, USA), PBMCs, 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 % CO₂ 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 from R&D Systems (Minneapolis, USA). Peripheral blood mononuclear cells (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 were purified from PBMC of healthy volunteers by negative selection with magnetic-associated cell sorting (MACS) (human CD4⁺ T cell Isolation Kit II, human CD8⁺ T cell Isolation Kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Naïve CD4⁺ and CD8⁺ T cells were purified from PBMC of healthy volunteers by negative (naive CD4⁺ T cells) or positive (naive CD8⁺ T cells) selection with magnetic-associated cell sorting (MACS) (human naïve CD4⁺ T cell Isolation Kit II, human naïve CD8⁺ T cell Isolation Kit, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Isolation of T cells was performed according to the manufacturer's protocol, and 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 (Immunotools,

1 Friesoythe, Germany), immediately treated with different concentrations of tranilast or
2 DMSO (vehicle control) and cultured in RPMI 1640 containing 10% FBS, 100 U/ml
3 penicillin and 100 µg/ml streptomycin in U-shaped 96-well plates at 100,000 cells per well
4 for four days (proliferation) or six days (flow cytometry analysis). In indicated proliferation
5 and qPCR experiments recombinant human CXCL9 or CXCL10 (R&D Systems,
6 Minneapolis, USA) at 25 ng/ml [or anti-CXCR3 \(R&D Systems, Minneapolis, USA\) at](#)
7 [10 µg/ml](#) was supplemented to the stimulated and treated cells, immediately.
8
9
10
11
12
13
14
15
16
17
18

19 Proliferation assay

20 T cells or Jurkat T cells were seeded in U-shaped 96-well plates at 100,000 cells per well.
21 Cells were stimulated and treated with tranilast (Nuon Therapeutics, San Francisco, USA) and
22 DMSO (Roth GmbH, Karlsruhe, Germany) as described above. Cultures were pulsed with ³H-
23 methylthymidine (Amersham Radiochemical Centre, Buckinghamshire, U.K.) for [18 h after](#)
24 24 h (Jurkat T cells) or 96 h (primary human T cells) stimulation. Finally, cells were
25 harvested and radionuclide uptake was measured by scintillation counting.
26
27
28
29
30
31
32
33
34
35
36
37
38

39 Analysis of apoptosis

40 Cells were labelled with Annexin V-FITC (Bio Vision, California, USA) and DAPI
41 (Invitrogen, Carlsbad, USA) in Annexin binding buffer (Biolegend, San Diego, USA) for 15
42 min, washed with PBS containing 3 % FBS, 0,01 % NaN₃ and 2 mM EDTA and analyzed by
43 flow cytometry using the BD FACS CANTO II cytometer (BD Biosciences, Heidelberg,
44 Germany).
45
46
47
48
49
50
51
52
53
54
55

56 Flow Cytometry

57 Flow cytometry was performed six days after stimulation. T cells were harvested, FC-blocked
58 with human serum for 15 min and washed in PBS, followed by incubation with the specific
59
60
61
62
63
64
65

1 antibodies against human CD4-PE (eBioscience, San Diego, CA, USA), CD69-APC, CD25-
2 APC (Biolegend, San Diego, CA, USA), CD45RO-PE, CD8-PE (eBioscience, San Diego,
3 CA, USA) for 30 min in PBS containing 3 % FBS, 0,01 % NaN₃ and 2 mM EDTA on ice.
4
5 Cells were washed and analyzed by flow cytometry using a BD FACS CANTO II cytometer
6
7 (BD Biosciences, Heidelberg Germany).
8
9

10 Protein multiplex analysis of cytokines and chemokines

11
12
13
14 The supernatants of the isolated and stimulated T cell subsets were analyzed for different
15
16 cytokines and chemokines after six days by using the Luminex-based multiplex technology
17
18 (BioRad, Hercules, USA). The supernatants (50µl) were incubated with color-coded beads
19
20 coated with the capture antibodies for the respective cytokine / chemokine for 45 min at RT.
21
22 After three washing steps, the beads were incubated with the biotinylated secondary detection
23
24 antibodies for each cytokine / chemokine for 30 min at RT, followed by three washing steps
25
26 and the final incubation with Steptavidin-PE (SA-PE) for 10 min at RT and three washings
27
28 steps. The first laser is responsible for the bead identification while the second laser is used
29
30 for the quantification of the SA-PE intensity. According to the standard curves, the
31
32 concentration of the respective cytokine / chemokine is calculated and given as pg/ml.
33
34
35
36
37
38
39
40
41
42

43 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

44
45 Naïve CD4⁺ T cells were stimulated as described above for 24 h or six days. Total RNA was
46
47 isolated using the Qiagen RNeasy RNA isolation kit (Qiagen, Hilden, Germany) and cDNA
48
49 was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied
50
51 Biosystems, Carlsbad, USA). QRT-PCR was performed according to standard protocols in an
52
53 ABI 7000 thermal cycler using SYBR Green PCR Mastermix (Applied Biosystems, Carlsbad,
54
55 USA). 40S ribosomal protein S13 (RPS13) served as housekeeping gene. PCR reactions were
56
57 checked by omission of templates and by both melting curve and gel analysis. The size of the
58
59
60
61
62
63
64
65

1 amplicons was analyzed by loading the samples and a 100 bp ladder (Invitrogen, Carlsbad,
2 USA) on a 2 % agarose gel, which was then stained with ethidium bromide and analyzed
3
4 under UV light. Standard curves were generated for each gene and the amplification was 90 –
5
6 100 % efficient. Relative quantification of gene expression was determined by comparison of
7
8 threshold values. All results were normalized to RPS13. Primer sequences were (5'-3'
9
10 forward, reverse):
11
12

13 RPS 13 fwd.: AAGTACGTTTTGTGACAGGCA, rev.: GGTGAATCCGGCTCTCTATTAG;
14
15

16 CXCL9: fwd.: AGCACCAACCAAGGGACTAT, rev.:
17
18

19 GGGTTTAGACATGTTTGAAGTCC; CXCL10: fwd.: TGTCCACGTGTTGAGATCAT,
20
21

22 rev.: TTTCAGTAAATTCTTGATGGCC; (all from Sigma, St. Louis, USA);
23
24

25 STAT1 fwd.: AGGAAAAGCAAGCGTAATCTTCA, rev.:
26
27

28 TATCCCCGACTGAGCCTGAT (Invitrogen, Carlsbad, USA)
29
30

31 Mixed leucocyte reaction

32
33

34 Peripheral blood mononuclear cells (PBMCs) were isolated from different healthy blood-
35
36 donors as described before. 2×10^5 irradiated (30 Gy) PBMC as stimulators and $2 \times$
37
38 10^5 PBMC from an unrelated donor as responders were seeded in flat-bottom 96-well plates
39
40 and cultured in 200 μ l medium per well. Cells were treated with different concentrations of
41
42 tranilast or DMSO. Five days after treatment cells were pulsed with ^3H -methylthymidine for
43
44 18 h. Then cells were harvested and the radionuclide uptake was measured by scintillation
45
46 counting.
47
48
49
50
51
52

53 Western Blot Analysis

54
55

56 Freshly isolated PBMC were pretreated with 50 μ M tranilast and DMSO control for 1 h and
57
58 then stimulated with 1 μ g anti-CD3 and 1 μ g anti-CD28 per 1×10^6 PBMCs for another hour.
59
60
61
62
63
64
65

1 Whole cell lysates were prepared in ice cold tris(hydroxymethyl)aminomethane hydrochloride
2 (TRIS-HCl, 50 mM, pH 8,0; Carl Roth, Karlsruhe, Germany) containing 50 mM NaCl (J.T.
3 Baker, Deventer, Holland), 1% NP-40 (AppliChem, Darmstadt, Germany), 10 mM EDTA
4 (Gerbu Biotechnik, Gaiberg, Germany), 1 % Glycerol, 200 mM dithiothreitol (Carl Roth,
5 Karlsruhe, Germany), 100 μ M phenylmethylsulphonyl fluoride (PMSF), complete protease
6 inhibitor (Roche, Grenzach, Germany) and Phosphatase inhibitor cocktail 2 and 3 (Sigma
7 Aldrich, St. Louis, USA).

8
9 The protein concentrations were determined by using the Bio-Rad protein assay (Bio-Rad,
10 Hercules, CA, USA) at 595 nm and the desired amount of protein (25 μ g) was separated by 8
11 % SDS-PAGE. Proteins were electrophoretically transferred onto a 2 μ m nitrocellulose
12 membrane (Whatman, Dassel, Germany). After 1 h of blocking in PBS supplemented with 0.2
13 % Tween 20 (Sigma-Aldrich, St. Louis, USA) and 5 % bovine albumin fraction V powder
14 (Roth GmbH, Karlsruhe, Germany), the membrane was incubated with rabbit anti-pSTAT1 or
15 rabbit anti-STAT1 as loading control, (both 1:1000 dilution, both Cell Signalling Technology,
16 Beverly, USA) overnight at 4°C. After 1 h incubation at room temperature with horseradish
17 peroxidase-conjugated secondary antibodies (donkey anti-rabbit, 1:2000 dilution, GE-
18 Healthcare, Buckinghamshire, UK) proteins were visualized by using ECL Plus reagent (GE
19 Healthcare).

20 Statistical analysis

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

Results

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 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 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.

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⁺

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

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).

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-1 β , 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-2R α , 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 μ M) 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)

Tranilast suppresses CD4+ T cell proliferation via STAT1 and CXCL9

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

1 freshly isolated T cells. STAT1-phosphorylation induced by IFN- γ or by TCR stimulation was
2 suppressed by tranilast in PBMC (Fig. 4a). Long-term cultures of activated PBMC and CD4+
3 T cells also resulted in a suppression of STAT1 gene expression (Fig. 4b). As CXCL9 has
4 previously been shown to stimulate CD4+ T cell proliferation [20] we assessed whether
5 tranilast-mediated suppression of CD4+ T cell proliferation was blocked by CXCL9. Indeed,
6 human recombinant CXCL9 restored STAT1 gene expression (Fig. 4b) and proliferation in
7 tranilast-treated activated human CD4+ T cells (Fig. 4c). Of note, human recombinant
8 CXCL10 was equally effective in restoring T cell proliferation suppressed by tranilast (Fig.
9 4c). Moreover, while the addition of CXCL9 and CXCL10 only slightly increased the
10 activation of human naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2, both
11 chemokines were capable of partly restoring the suppressive effects of tranilast on T cell
12 activation (Fig. 4d). Finally blocking signalling through CXCR3, the receptor for CXCL9 and
13 CXCL10 in activated T cells, mimicked the suppressive effects of tranilast on T cell
14 proliferation (Fig. 4e) and activation (Fig. 4f). Collectively, these data suggest, that tranilast
15 suppresses CD4+ T cell proliferation *via* inhibition of STAT1-phosphorylation and CXCL9 /
16 CXCL10 release (Fig. 5).
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Discussion

1
2
3
4 While tranilast has initially been developed - and has long been approved – for allergic
5 diseases, it has now become clear that its cellular activity goes far beyond inhibition of mast
6 cell degranulation. Examples of these pleiotropic effects include inhibition of vascular smooth
7 muscle cell proliferation [21, 22], suppression of fibroblast and endothelial function [23, 24],
8 targeting transforming growth factor (TGF)- β production [25, 26] and inhibition of tumour
9 cell proliferation and motility [26, 27]. These effects that play a key role in vascular diseases,
10 fibrosis and metastatic cancer have been translated into several clinical trials addressing the
11 biological activity in these conditions [28-30]. More recently, we have shown that tranilast
12 displays structural and functional similarities to immunosuppressive catabolites of the
13 essential amino acid tryptophan and demonstrated anti-inflammatory activity in an animal
14 model of MS, where tranilast suppressed the antigen-specific Th1 cell response and induced
15 the generation of antigen-specific *Treg* [18]. Anti-inflammatory activity has subsequently
16 been shown in RA, another Th1-mediated autoimmune diseases [16, 17]. Thus, a clinical
17 phase II study has been done testing the activity of tranilast in patients with RA
18 (ClinicalTrials.gov Identifier NCT00882024). While the effects on antigen-specific CD4+ T
19 cells in mice had been thoroughly characterized [16, 18], the effects on human CD4+ T cells
20 had not been addressed to date. We show that tranilast suppresses activation and proliferation
21 of freshly isolated human naïve CD4+ T cells with an EC50 of less than 10 μ M (Fig. 1 b,c).
22 This effect is clinically relevant as steady-state plasma levels of 30 - 100 μ M are achieved
23 using clinically approved doses of 200 to 600 mg [31]. It is interesting that tranilast was less
24 active in suppressing the proliferation of total CD4+ T cells and of total and naïve CD8+ T
25 cells (Fig. 1c, e, f). Interestingly, this differential activity on CD4+ lymphocyte subsets has also
26 been demonstrated for tryptophan catabolites [32, 33], supporting the notion that tranilast not
27 only structurally but also functionally shares tryptophan catabolite activity [34]. We set out to
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 analyze the molecular target of tranilast in human CD4⁺ T cells mediating the anti-
2 proliferative activity. Early *in vivo* studies have indicated that tranilast is capable of
3
4 suppressing human main histocompatibility (MHC) expression [35]. MHC are typically
5
6 induced on virtually any cell type (class I MHC) and antigen presenting cells (APC),
7
8 particularly (class II MHC), in response to viral infections as a result of host type I and type II
9
10 interferons [36]. In APC this response is driven by IFN- γ -mediated phosphorylation of
11
12 STAT1, which subsequently shuttles to the nucleus to induce MHC class II transcription *via*
13
14 the class II transactivator (CIITA) [37]. Indeed, we have shown previously in mouse
15
16 microglial cells that tranilast blocks IFN- γ -mediated phosphorylation of STAT1 and
17
18 expression of CIITA and MHC class II in mouse microglial cells *in vitro* and *in vivo* [18].
19
20 These observations are in accordance with the data presented here showing that tranilast
21
22 blocks at low doses (< 50 μ M) two key STAT1 target chemokines in human CD4⁺ T cells,
23
24 namely CXCL9 - also termed monokine induced by IFN- γ (MIG) - and CXCL10 - also
25
26 termed IFN- γ -induced protein 10 KD (IP-10) (Fig. 3b). At higher doses (> 50 μ M) we
27
28 observed an increase in apoptosis in CD4⁺ T cells (Fig. 1g) as well as unspecific suppression
29
30 of other pro- and anti-inflammatory cytokines such as IFN- γ and IL-10 (Fig. 3b). IFN- γ
31
32 appears to be a preferred target of tranilast while we did not observe relevant changes in IL-
33
34 17, IL-4 or induction of FoxP3 (data not shown), indicating that in our experimental system
35
36 tranilast preferentially suppresses the differentiation of Th1 cells, which is in line with our
37
38 previous observations in murine Th1-mediated autoimmune neuroinflammation [18]. These
39
40 data may also imply that tranilast targets additional signalling pathways at higher
41
42 concentrations. Indeed, others and we have previously shown that tranilast is capable of
43
44 suppressing the activation of nuclear factor (NF)- κ B [31, 38], protein kinase C (PKC) [23, 39]
45
46 and mitogen activated protein kinases (MAPK) [21, 39]. Based on previous studies
47
48 demonstrating that tranilast interferes with tyrosine phosphorylation [21, 23] it is tempting to
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 speculate that tranilast modulates these diverse signalling pathways with differential affinity
2 and particular activity towards STAT1. Our current and previous results render evidence to
3
4 the hypothesis that inhibition of STAT1 is responsible for the preferential CD4⁺ T cell
5
6 suppressive activity of tranilast in mice and humans [18]. More importantly, our study implies
7
8 the STAT1 target chemokines CXCL9 and CXCL10 are the mediators of the anti-proliferative
9
10 activity of tranilast as exogenous CXCL9 or CXCL10 restored this effect (Fig. 4c). CXCL9
11
12 and CXCL10 share a common receptor, CXCR3 [40], which is also suppressed by tranilast in
13
14 human CD4⁺ T cells (data not shown). Targeting CXCR3 by blocking antibodies, peptidergic
15
16 ligands or small molecules ameliorates disease activity in animal models of MS [41-43] and
17
18 rheumatoid arthritis [44, 45], although knock-out mouse models revealed conflicting results
19
20 [46, 47]. While the anti-inflammatory efficacy of this therapeutic approach has been
21
22 attributed to the limitation of CXCR3⁺ auto-aggressive Th1 cells to inflammatory sites [40],
23
24 it has also been shown that CXCL9 is capable of stimulating the proliferation of CD4⁺ and
25
26 CD8⁺ T cells [20, 48]. The direct proinflammatory function of the CXCL9/CXCL10 -
27
28 CXCR3 pathway in human T cells is supported by our data showing that blocking CXCR3
29
30 suppresses the proliferation and activation of T cells through the TCR, thus mimicking the
31
32 effects of tranilast (Fig. 4e,f).
33
34 In addition to their immunomodulatory properties, CXCR3 and its ligands may also serve as
35
36 biomarkers as they correlate with immune and disease activity in a variety of autoimmune
37
38 conditions [49]. CXCL9 and CXCL10 have been shown to correlate with disease activity in
39
40 MS and RA [50-53]. Thus, these molecular targets of tranilast may in addition to
41
42 phosphorylated STAT1 serve as markers to assess bioactivity of tranilast in clinical trials.
43
44 Further clinical trials will determine whether tranilast targets CXCL9 and CXCL10 in patients
45
46 with Th1-mediated autoimmune diseases and whether assaying CXCL9 and 10 in serum and
47
48 other fluids can monitor clinical activity.
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Acknowledgements

This work was supported by a grant from the Helmholtz Association (VH-NG-306) and Nuon Therapeutics to MP as well as the charitable Hertie-Foundation. The sponsors had no influence in study design, in the collection, analysis, and interpretation of data, in the writing of the report and in the decision to submit the paper for publication.

Accepted Manuscript

References

- 1
2
3
4
5 [1] Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2
6 hypothesis of T cell-mediated tissue damage. *Nat Med* 2007;13:139-45.
- 7 [2] Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 Cells. *Annu Rev*
8 *Immunol* 2009;27:485-517.
- 9 [3] van Oosten BW, Lai M, Hodgkinson S, Barkhof F, Miller DH, Moseley IF, et al.
10 Treatment of multiple sclerosis with the monoclonal anti-CD4 antibody cM-T412:
11 results of a randomized, double-blind, placebo-controlled, MR-monitored phase II
12 trial. *Neurology* 1997;49:351-7.
- 13 [4] Rep MH, van Oosten BW, Roos MT, Ader HJ, Polman CH, van Lier RA. Treatment
14 with depleting CD4 monoclonal antibody results in a preferential loss of circulating
15 naive T cells but does not affect IFN-gamma secreting TH1 cells in humans. *The*
16 *Journal of clinical investigation* 1997;99:2225-31.
- 17 [5] O'Brien K, Gran B, Rostami A. T-cell based immunotherapy in experimental
18 autoimmune encephalomyelitis and multiple sclerosis. *Immunotherapy* 2010;2:99-115.
- 19 [6] O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of
20 helper CD4+ T cells. *Science* 2010;327:1098-102.
- 21 [7] Zhu J, Yamane H, Paul WE. Differentiation of effector CD4 T cell populations (*).
22 *Annu Rev Immunol* 2010;28:445-89.
- 23 [8] Adamson AS, Collins K, Laurence A, O'Shea JJ. The Current STATus of lymphocyte
24 signaling: new roles for old players. *Curr Opin Immunol* 2009;21:161-6.
- 25 [9] Frisullo G, Angelucci F, Caggiula M, Nociti V, Iorio R, Patanella AK, et al. pSTAT1,
26 pSTAT3, and T-bet expression in peripheral blood mononuclear cells from relapsing-
27 remitting multiple sclerosis patients correlates with disease activity. *J Neurosci Res*
28 2006;84:1027-36.
- 29 [10] Frisullo G, Nociti V, Iorio R, Patanella AK, Plantone D, Bianco A, et al. T-bet and
30 pSTAT-1 expression in PBMC from coeliac disease patients: new markers of disease
31 activity. *Clin Exp Immunol* 2009;158:106-14.
- 32 [11] Madia F, Frisullo G, Nociti V, Conte A, Luigetti M, Del Grande A, et al. pSTAT1,
33 pSTAT3, and T-bet as markers of disease activity in chronic inflammatory
34 demyelinating polyradiculoneuropathy. *J Peripher Nerv Syst* 2009;14:107-17.
- 35 [12] Nishibori T, Tanabe Y, Su L, David M. Impaired development of CD4+ CD25+
36 regulatory T cells in the absence of STAT1: increased susceptibility to autoimmune
37 disease. *J Exp Med* 2004;199:25-34.
- 38 [13] Bettelli E, Sullivan B, Szabo SJ, Sobel RA, Glimcher LH, Kuchroo VK. Loss of T-
39 bet, but not STAT1, prevents the development of experimental autoimmune
40 encephalomyelitis. *J Exp Med* 2004;200:79-87.
- 41 [14] Caretto D, Katzman SD, Villarino AV, Gallo E, Abbas AK. Cutting edge: the Th1
42 response inhibits the generation of peripheral regulatory T cells. *J Immunol*
43 2010;184:30-4.
- 44 [15] Hara M, Ono K, Hwang MW, Iwasaki A, Okada M, Nakatani K, et al. Evidence for a
45 role of mast cells in the evolution to congestive heart failure. *J Exp Med*
46 2002;195:375-81.
- 47 [16] Inglis JJ, Criado G, Andrews M, Feldmann M, Williams RO, Selley ML. The anti-
48 allergic drug, N-(3',4'-dimethoxycinnamonyl) anthranilic acid, exhibits potent anti-
49 inflammatory and analgesic properties in arthritis. *Rheumatology (Oxford)*
50 2007;46:1428-32.
- 51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [17] Nagate T, Tamura T, Sato F, Kuroda J, Nakayama J, Shibata N. Tranilast suppresses
2 the disease development of the adjuvant- and streptococcal cell wall-induced arthritis
3 in rats. *J Pharmacol Sci* 2007;105:48-56.
- 4 [18] Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM, et al. Treatment of
5 autoimmune neuroinflammation with a synthetic tryptophan metabolite. *Science*
6 2005;310:850-5.
- 7 [19] Shiota N, Kovanen PT, Eklund KK, Shibata N, Shimoura K, Niibayashi T, et al. The
8 anti-allergic compound tranilast attenuates inflammation and inhibits bone destruction
9 in collagen-induced arthritis in mice. *Br J Pharmacol* 2010;159:626-35.
- 10 [20] Whiting D, Hsieh G, Yun JJ, Banerji A, Yao W, Fishbein MC, et al. Chemokine
11 monokine induced by IFN-gamma/CXC chemokine ligand 9 stimulates T lymphocyte
12 proliferation and effector cytokine production. *J Immunol* 2004;172:7417-24.
- 13 [21] Watanabe S, Matsuda A, Suzuki Y, Kondo K, Ikeda Y, Hashimoto H, et al. Inhibitory
14 mechanism of tranilast in human coronary artery smooth muscle cells proliferation,
15 due to blockade of PDGF-BB-receptors. *Br J Pharmacol* 2000;130:307-14.
- 16 [22] Takahashi A, Taniguchi T, Ishikawa Y, Yokoyama M. Tranilast inhibits vascular
17 smooth muscle cell growth and intimal hyperplasia by induction of
18 p21(waf1/cip1/sdi1) and p53. *Circ Res* 1999;84:543-50.
- 19 [23] Koyama S, Takagi H, Otani A, Suzuma K, Nishimura K, Honda Y. Tranilast inhibits
20 protein kinase C-dependent signalling pathway linked to angiogenic activities and
21 gene expression of retinal microcapillary endothelial cells. *Br J Pharmacol*
22 1999;127:537-45.
- 23 [24] Inoue H, Ohshima H, Kono H, Yamanaka M, Kubota T, Aihara M, et al. Suppressive
24 effects of tranilast on the expression of inducible cyclooxygenase (COX2) in
25 interleukin-1beta-stimulated fibroblasts. *Biochem Pharmacol* 1997;53:1941-4.
- 26 [25] Charng MJ, Wu CH. Transcriptional activation of p21 by Tranilast is mediated via
27 transforming growth factor beta signal pathway. *Br J Pharmacol* 2006;147:117-24.
- 28 [26] Platten M, Wild-Bode C, Wick W, Leitlein J, Dichgans J, Weller M. N-[3,4-
29 dimethoxycinnamoyl]-anthranilic acid (tranilast) inhibits transforming growth factor-
30 beta release and reduces migration and invasiveness of human malignant glioma
31 cells. *Int J Cancer* 2001;93:53-61.
- 32 [27] Prud'homme GJ, Glinka Y, Toulina A, Ace O, Subramaniam V, Jothy S. Breast cancer
33 stem-like cells are inhibited by a non-toxic aryl hydrocarbon receptor agonist. *PLoS*
34 *One* 2010;5:e13831.
- 35 [28] Izumi K, Mizokami A, Shima T, Narimoto K, Sugimoto K, Kobori Y, et al.
36 Preliminary results of tranilast treatment for patients with advanced castration-
37 resistant prostate cancer. *Anticancer Res* 2010;30:3077-81.
- 38 [29] Soma J, Sato K, Saito H, Tsuchiya Y. Effect of tranilast in early-stage diabetic
39 nephropathy. *Nephrol Dial Transplant* 2006;21:2795-9.
- 40 [30] Holmes DR, Jr., Savage M, LaBlanche JM, Grip L, Serruys PW, Fitzgerald P, et al.
41 Results of Prevention of REStenosis with Tranilast and its Outcomes (PRESTO) trial.
42 *Circulation* 2002;106:1243-50.
- 43 [31] Spiecker M, Lorenz I, Marx N, Darius H. Tranilast inhibits cytokine-induced nuclear
44 factor kappaB activation in vascular endothelial cells. *Mol Pharmacol* 2002;62:856-
45 63.
- 46 [32] Boasso A, Herbeuval JP, Hardy AW, Winkler C, Shearer GM. Regulation of
47 indoleamine 2,3-dioxygenase and tryptophanyl-tRNA-synthetase by CTLA-4-Fc in
48 human CD4+ T cells. *Blood* 2005;105:1574-81.
- 49 [33] Frumento G, Rotondo R, Tonetti M, Damonte G, Benatti U, Ferrara GB. Tryptophan-
50 derived catabolites are responsible for inhibition of T and natural killer cell
51 proliferation induced by indoleamine 2,3-dioxygenase. *J Exp Med* 2002;196:459-68.
- 52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [34] Opitz CA, Wick W, Steinman L, Platten M. Tryptophan degradation in autoimmune
2 diseases. *Cell Mol Life Sci* 2007;64:2542-63.
- 3 [35] Kawano Y, Noma T. Cell action mechanism of tranilast--effect on the expression of
4 HLA-class II antigen. *Int J Immunopharmacol* 1993;15:487-500.
- 5 [36] Rouse BT, Sehrawat S. Immunity and immunopathology to viruses: what decides the
6 outcome? *Nat Rev Immunol* 2010;10:514-26.
- 7 [37] Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene
8 expression by the class II transactivator. *Nat Rev Immunol* 2005;5:793-806.
- 9 [38] Platten M, Wick W, Wischhusen J, Weller M. N-[3,4-dimethoxycinnamoyl]-
10 anthranilic acid (tranilast) suppresses microglial inducible nitric oxide synthase
11 (iNOS) expression and activity induced by interferon-gamma (IFN-gamma). *Br J*
12 *Pharmacol* 2001;134:1279-84.
- 13 [39] Platten M, Eitel K, Wischhusen J, Dichgans J, Weller M. Involvement of protein
14 kinase Cdelta and extracellular signal-regulated kinase-2 in the suppression of
15 microglial inducible nitric oxide synthase expression by N-[3,4-dimethoxycinnamoyl]-
16 anthranilic acid (tranilast). *Biochem Pharmacol* 2003;66:1263-70.
- 17 [40] Lacotte S, Brun S, Muller S, Dumortier H. CXCR3, inflammation, and autoimmune
18 diseases. *Ann N Y Acad Sci* 2009;1173:310-7.
- 19 [41] Sporici R, Issekutz TB. CXCR3 blockade inhibits T-cell migration into the CNS
20 during EAE and prevents development of adoptively transferred, but not actively
21 induced, disease. *Eur J Immunol* 2010;40:2751-61.
- 22 [42] Ni J, Zhu YN, Zhong XG, Ding Y, Hou LF, Tong XK, et al. The chemokine receptor
23 antagonist, TAK-779, decreased experimental autoimmune encephalomyelitis by
24 reducing inflammatory cell migration into the central nervous system, without
25 affecting T cell function. *Br J Pharmacol* 2009;158:2046-56.
- 26 [43] Kohler RE, Comerford I, Townley S, Haylock-Jacobs S, Clark-Lewis I, McColl SR.
27 Antagonism of the chemokine receptors CXCR3 and CXCR4 reduces the pathology of
28 experimental autoimmune encephalomyelitis. *Brain pathology (Zurich, Switzerland)*
29 2008;18:504-16.
- 30 [44] Mohan K, Issekutz TB. Blockade of chemokine receptor CXCR3 inhibits T cell
31 recruitment to inflamed joints and decreases the severity of adjuvant arthritis. *J*
32 *Immunol* 2007;179:8463-9.
- 33 [45] Xie JH, Nomura N, Lu M, Chen SL, Koch GE, Weng Y, et al. Antibody-mediated
34 blockade of the CXCR3 chemokine receptor results in diminished recruitment of T
35 helper 1 cells into sites of inflammation. *J Leukoc Biol* 2003;73:771-80.
- 36 [46] Muller M, Carter SL, Hofer MJ, Manders P, Getts DR, Getts MT, et al. CXCR3
37 signaling reduces the severity of experimental autoimmune encephalomyelitis by
38 controlling the parenchymal distribution of effector and regulatory T cells in the
39 central nervous system. *J Immunol* 2007;179:2774-86.
- 40 [47] Liu L, Huang D, Matsui M, He TT, Hu T, Demartino J, et al. Severe disease, unaltered
41 leukocyte migration, and reduced IFN-gamma production in CXCR3-/- mice with
42 experimental autoimmune encephalomyelitis. *J Immunol* 2006;176:4399-409.
- 43 [48] Rosenblum JM, Shimoda N, Schenk AD, Zhang H, Kish DD, Keslar K, et al. CXC
44 chemokine ligand (CXCL) 9 and CXCL10 are antagonistic costimulation molecules
45 during the priming of alloreactive T cell effectors. *J Immunol* 2010;184:3450-60.
- 46 [49] Lee EY, Lee ZH, Song YW. CXCL10 and autoimmune diseases. *Autoimmun Rev*
47 2009;8:379-83.
- 48 [50] Kuan WP, Tam LS, Wong CK, Ko FW, Li T, Zhu T, et al. CXCL 9 and CXCL 10 as
49 Sensitive markers of disease activity in patients with rheumatoid arthritis. *J Rheumatol*
50 2010;37:257-64.
- 51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

- 1 [51] Loos T, Dekeyzer L, Struyf S, Schutyser E, Gijssbers K, Gouwy M, et al. TLR ligands
2 and cytokines induce CXCR3 ligands in endothelial cells: enhanced CXCL9 in
3 autoimmune arthritis. *Lab Invest* 2006;86:902-16.
- 4 [52] Mahad DJ, Howell SJ, Woodroffe MN. Expression of chemokines in the CSF and
5 correlation with clinical disease activity in patients with multiple sclerosis. *J Neurol*
6 *Neurosurg Psychiatry* 2002;72:498-502.
- 7 [53] Sorensen TL, Tani M, Jensen J, Pierce V, Lucchinetti C, Folcik VA, et al. Expression
8 of specific chemokines and chemokine receptors in the central nervous system of
9 multiple sclerosis patients. *The Journal of clinical investigation* 1999;103:807-15.
- 10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure Legends

Figure 1: *Tranilast suppresses proliferation of T cells.* 3 [H] methylthymidine incorporation of Jurkat T cells (A, 24 hours), PBMC (B, 96 hours) and primary T cells (C-F, 96 hours) 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.

Figure 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.

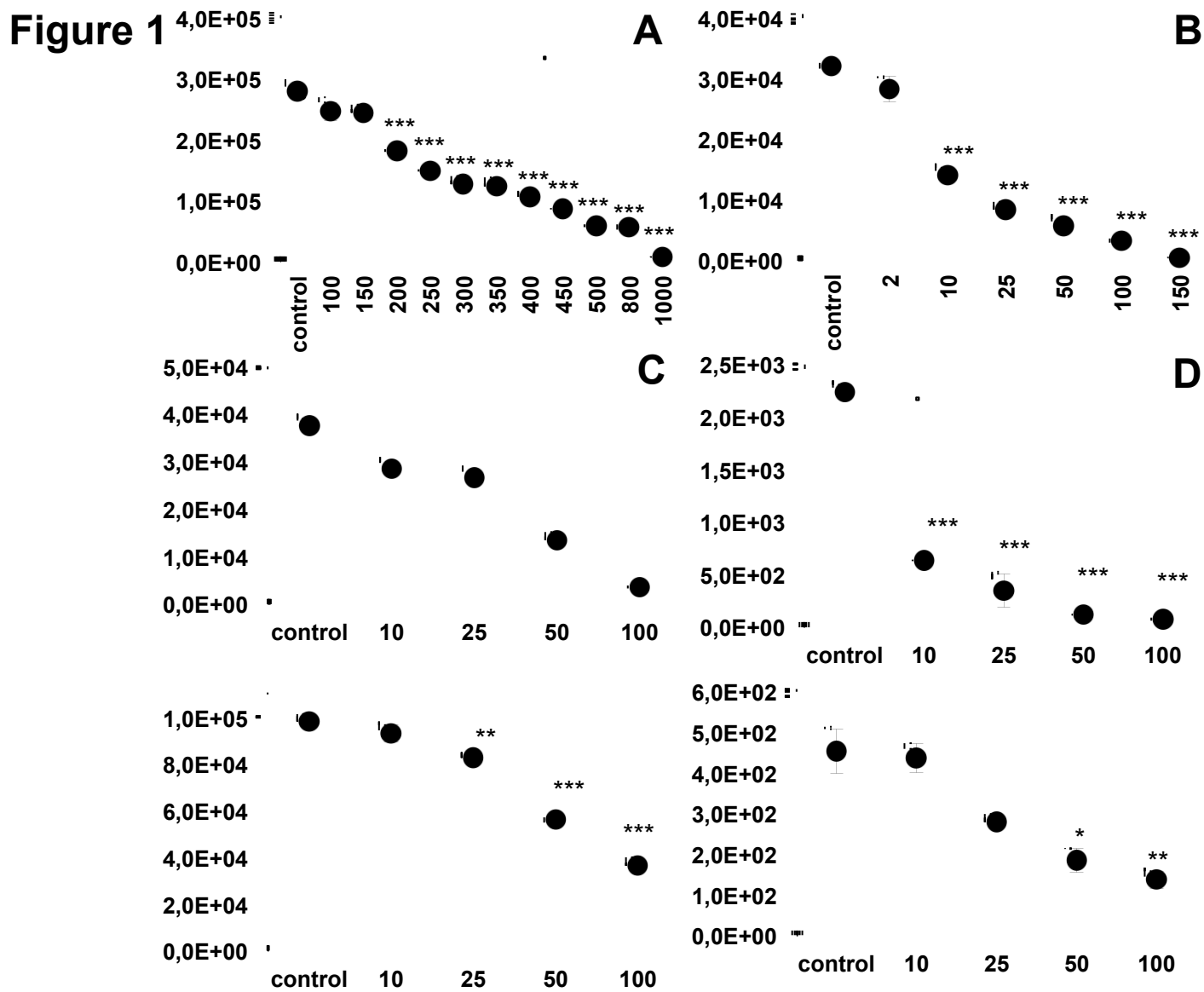
Figure 3: *Modulation of T cell cytokines by tranilast.* A: Multiplex ELISA analysis of cytokine release from naïve CD4+ T cells activated with anti-CD3, anti-CD28 and IL-2 for six days. Data are displayed as fold change over non-activated. The numbers above the bars indicate total release of stimulated 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

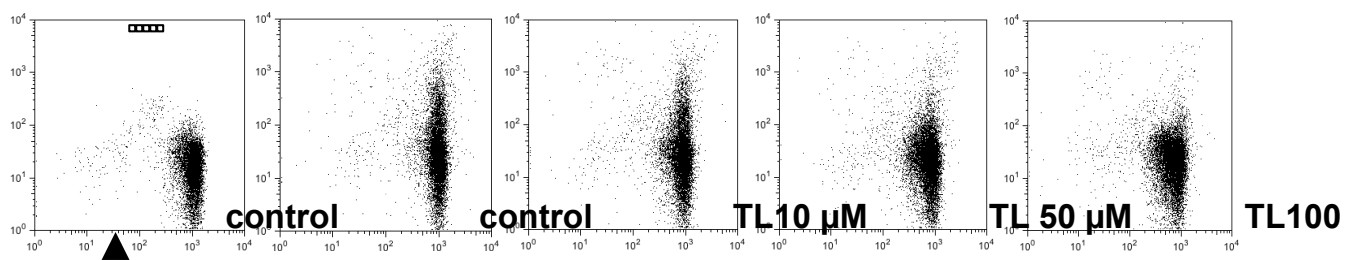
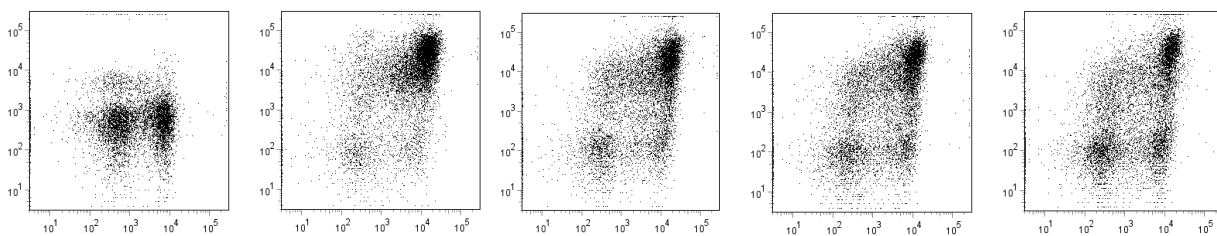
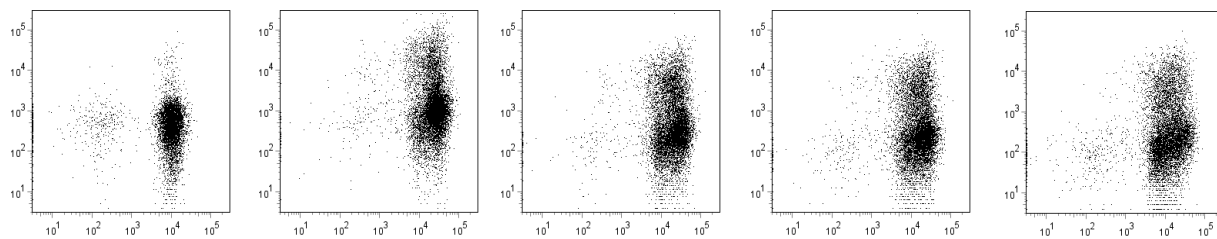
1 experiments. C: RT-PCR analysis of CXCL9 and CXCL10 expression in naïve CD4⁺ T cells
2 activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at 50 μ M or DMSO
3 (control) for six days. Data are representative of two independent experiments. n=3 +/- SEM,
4
5
6
7 ** p<0.01.

11 **Figure 4: *Tranilast suppresses T cell proliferation through STAT1 and CXCL9 / 10.*** A:

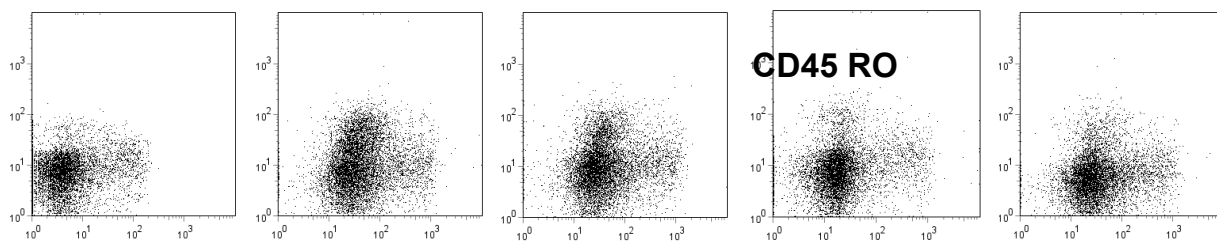
14 Western Blot analysis of STAT1 phosphorylation of human primary PBMC pretreated with
15 50 μ M tranilast or DMSO (control) for one hour and activated with anti-CD3 and anti-CD28
16 for another hour. Blots were reprobed with an antibody against total STAT1 as a loading
17 control. B: RT-PCR analysis of STAT1 expression in naïve CD4⁺ T cells activated with anti-
18 CD3, anti-CD28 and IL-2 in the presence of tranilast at 50 μ M or DMSO (control) and
19 CXCL9 at 25 ng/ml for 24 hours. C: ³[H] methylthymidine incorporation of naïve CD4⁺ T
20 cells activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast at indicated
21 concentrations or DMSO (control) and CXCL9 at 25 ng/ml or CXCL10 at 25 ng/ml for 96
22 hours. ** (p<0.01) represents the difference between the control cells and the cells treated
23 with CXCL9 or CXCL10 D: Flow cytometry of CD4 and CD69 staining in naïve CD4⁺ T
24 cells left untreated or activated with anti-CD3, anti-CD28 and IL-2 in the presence of tranilast
25 at 50 μ M and CXCL9 at 25 ng/ml or CXCL10 at 25 ng/ml for six days. E: ³[H]-
26 methylthymidine incorporation of naïve CD4⁺ T cells activated with anti CD3, anti-CD28
27 and IL-2 in the presence of anti-CXCR3 at 10 μ g/ml for 96 hours. As controls served
28 stimulated untreated cells and stimulated cells treated with Tranilast at 10 μ M. F: Flow
29 cytometry of CD4 and CD69 staining in naïve CD4⁺ T cells activated with anti-CD3, anti-
30 CD28 and IL-2 in the presence of anti-CXCR3 at 10 μ g/ml for six days. As controls served
31 stimulated untreated cells and stimulated cells treated with Tranilast at 10 μ M. Data are
32 representative of two independent experiments. n=3 +/- SEM, * p<0.05, ** p<0.01, ***
33 p<0.001.

1
2 Figure 5: A molecular model of tranilast-mediated suppression of T cell activation. Upon T
3
4 cell activation STAT1 phosphorylation leads to transcriptional upregulation of CXCL9 and
5
6 CXCL10. These chemokines are secreted and – in an autocrine / paracrine fashion – activate
7
8 CXCR3 on T cells driving proliferation, activation and expression of STAT1. Tranilast
9
10 interferes with STAT1 phosphorylation, thus preventing the upregulation of CXCL9 and
11
12 CXCL10.
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65





CD25



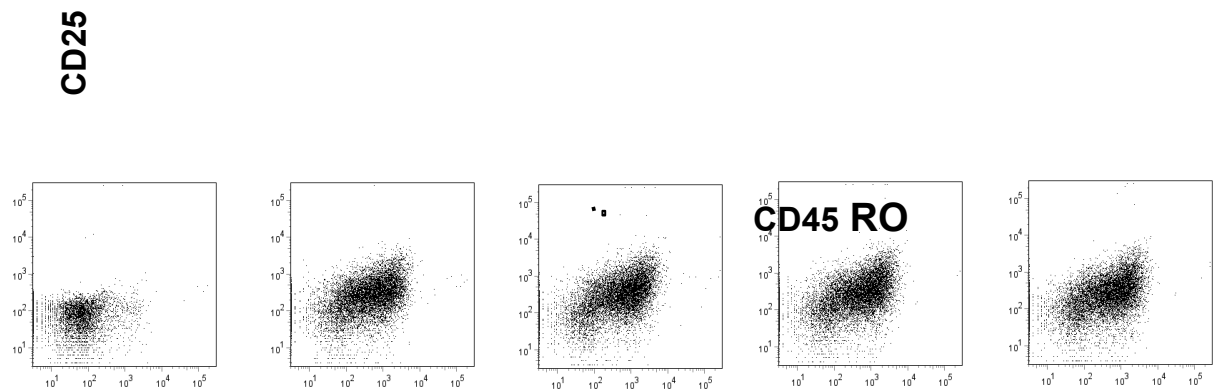
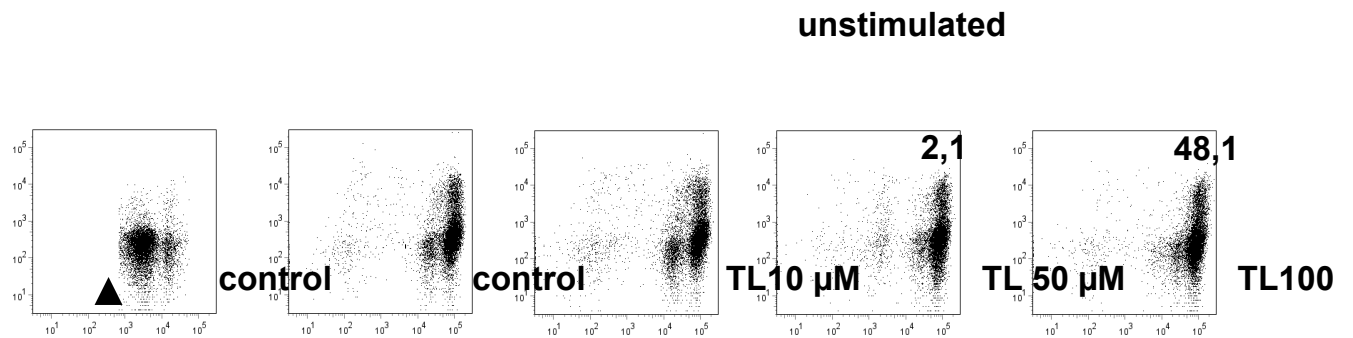
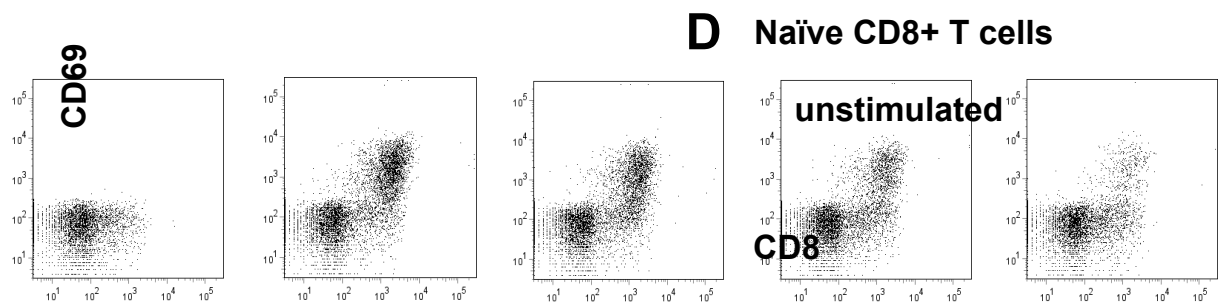
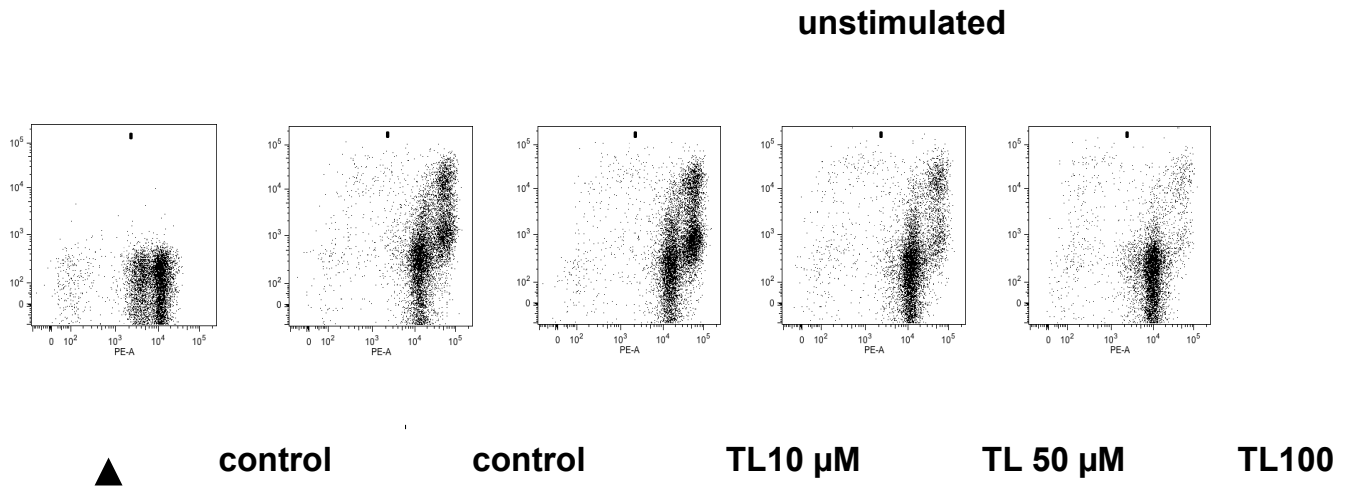
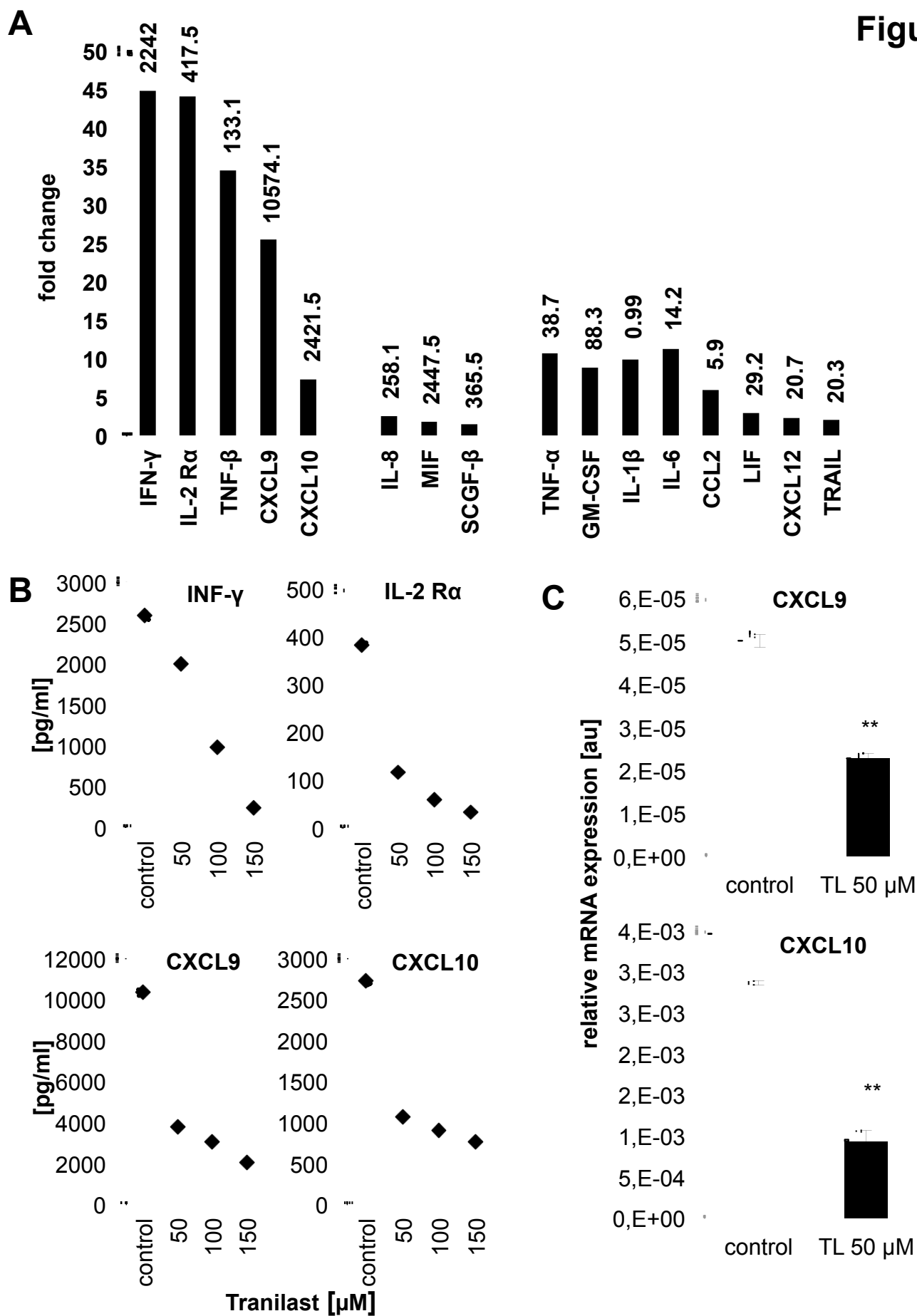
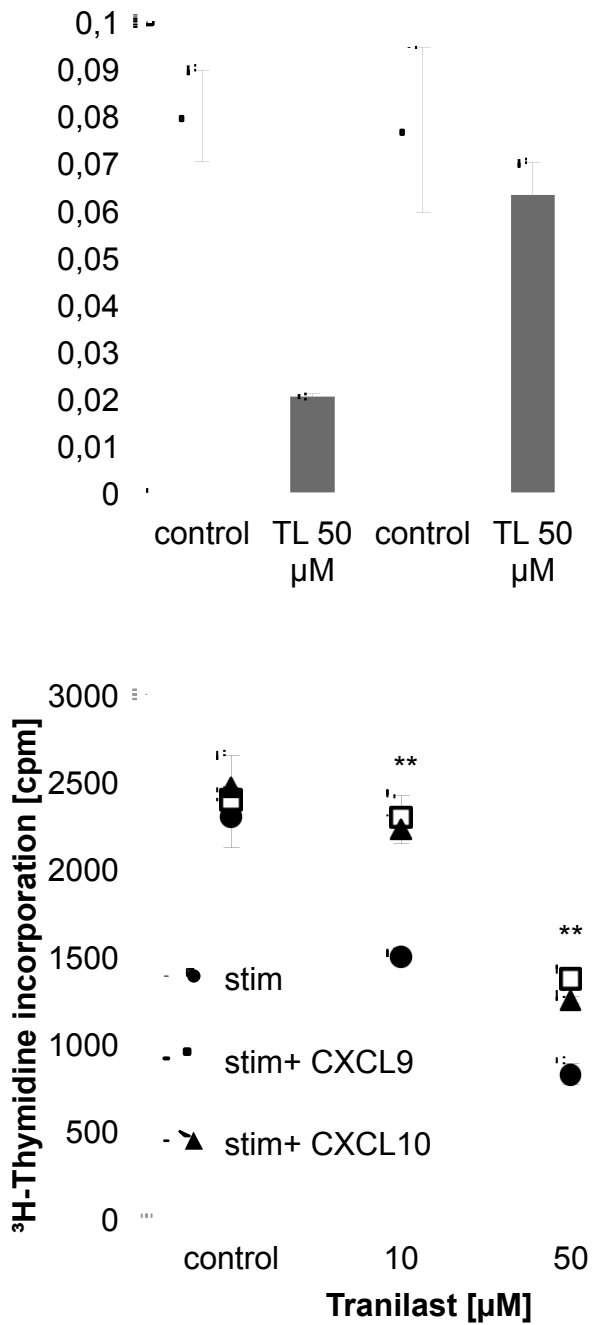
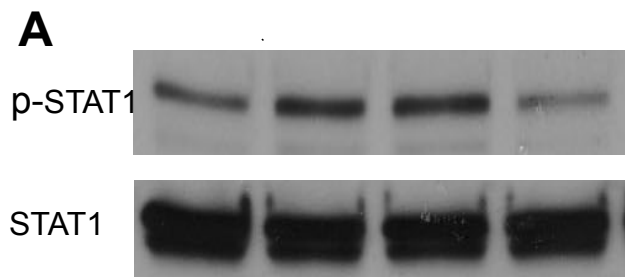
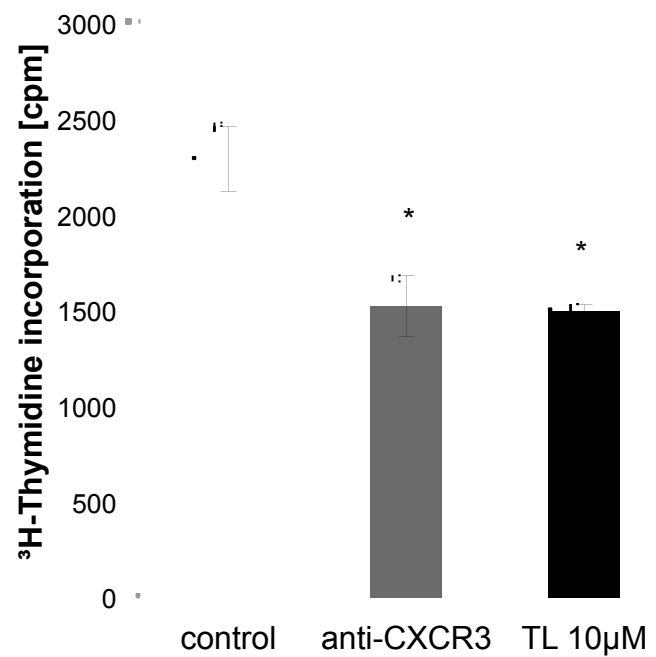
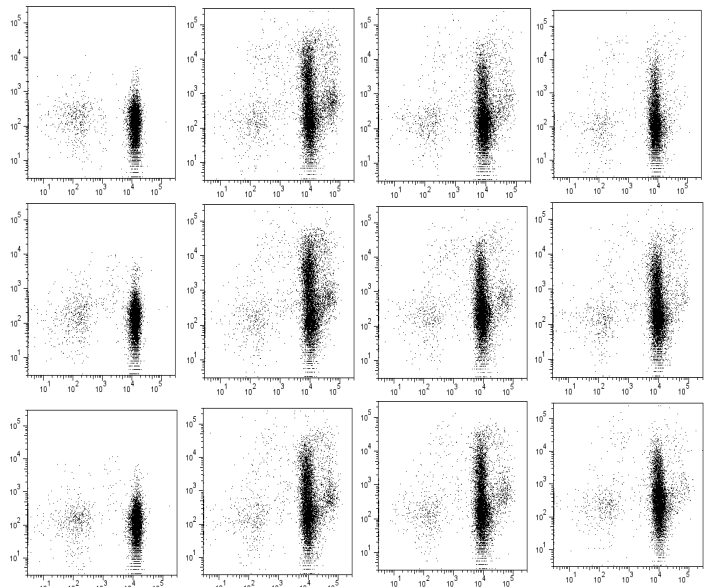
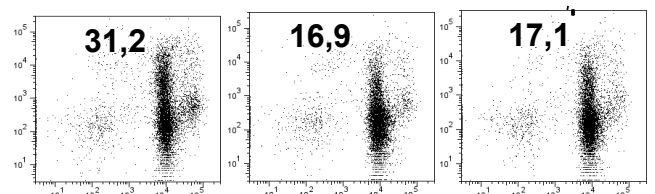
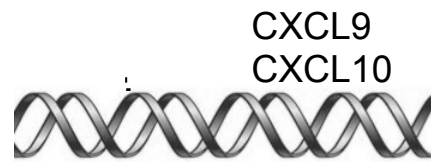


Figure 3



**D** α CD3/ α CD28 + IL-2

.



*Graphical Abstract

