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Tissue Non-specific Alkaline Phosphatase Expression is Needed for the Full Stimulation of T Cells and T Cell-Dependent Colitis

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Abstract

Background and Aims: Two alkaline phosphatase isoforms, intestinal [IAP] and tissue non-specific alkaline phosphatase [TNAP], are coexpressed in mouse colon, with the latter predominating in colitis. We aimed to examine the role of TNAP in T lymphocytes, using heterozygous TNAP$^{+/-}$ mice [as TNAP$^{-/-}$ mice are non-viable].
Methods: *In vitro* primary cultures and *in vivo* T cell models using TNAP$^{+/-}$ mice were used.
Results: Stimulated splenocytes [lipopolysaccharide and concanavalin A] and T lymphocytes [concanavalin A and a-CD3/a-CD28] showed a decreased cytokine production and expression when compared with wild-type [WT] cells. Decreased T cell activation was reproduced by the TNAP inhibitors levamisole, theophylline, and phenylalanine in WT cells. Intraperitoneal administration of anti-CD3 *in vivo* resulted in reduced plasma cytokine levels, and decreased activation of splenocytes and T cells *ex vivo* in TNAP$^{+/-}$ mice. We further tested the hypothesis that TNAP expressed in T lymphocytes is involved in T cell activation and inflammation, using the lymphocyte transfer model of colitis. Rag1$^{-/-}$ mice were transferred with T naïve cells [CD4^{+} CD62L^{+}] from TNAP$^{+/-}$ or WT mice and developed colitis, which was attenuated in the group receiving TNAP$^{+/-}$ cells. Compared with WT, T cells from TNAP$^{+/-}$ mice showed a decreased capacity for proliferation, with no change in differentiation.
Conclusions: Our results offer clear evidence that TNAP modulates T lymphocyte function and specifically T cell-dependent colitis. This was associated with distinct changes in the type of TNAP expressed, probably because of changes in glycosylation.

Key Words: Alkaline phosphatase; T cells; Rag1$^{-/-}$; anti-CD3; colitis

Abbreviations: AP, alkaline phosphatase; CXCL9, chemokine [C-X-C motif] ligand 9; ConA, concanavalin A; IAP, intestinal alkaline phosphatase; KLF4, Kruppel-like factor 4; LPS, Lipopolysaccharide; MLNC, mesenteric lymph node cells; MPO, myeloperoxidase; noF, not fractionated; OPN, osteopontin; TNAP, tissue non-specific alkaline phosphatase.

1. Introduction

Alkaline phosphatases are a large family of enzymes distributed from bacteria to man, which cleave phosphate moieties with release of inorganic phosphate at alkaline pH. There are four main alkaline phosphatase [AP] isoforms: the intestinal [IAP], the placental, and the germ cell isoforms, which are tissue-specific, plus the tissue non-specific alkaline phosphatase [TNAP] which is widely expressed. The TNAP gene in turn gives rise to three different enzyme variants that differ only in the glycosidic fraction, namely the liver, bone, and kidney isoforms, which are predominantly found in these organs. The tissue-specific AP forms are also glycosylated.

IAP has a pivotal role in intestinal homeostasis and health, and its expression and activity are modulated by the diet. IAP regulates lipid absorption across the apical membrane of enterocytes,[1] takes part in the regulation of bicarbonate secretion and of duodenal surface pH,[2,3] limits bacterial translocation across the mucosal barrier, and detoxifies lipopolysaccharides [LPS] from Gram-negative bacteria, thereby attenuating LPS-mediated toxicity and inflammation.[4–6] IAP also preserves the normal homeostasis of intestinal microbiota preventing dysbiosis and infections of pathogenic bacteria.[7] Moreover, healthy people with low stool levels of IAP [less than 65.0 U/g stool] might have increased susceptibility to developing metabolic syndrome. In fact, it has been estimated that there is a 35% increased risk of diabetes with each 25 U/g decrease in stool IAP.[8] Furthermore, endogenous and orally supplemented IAP was found to prevent and reverse metabolic syndrome by inhibiting the absorption of endotoxin [LPS] in mice fed with a high fat diet.[9]

TNAP has been widely studied due to its involvement in various liver, bone, and colonic disorders. In the liver, TNAP has an important role in the inhibition of bile secretion and the detoxification of bacterial LPS, and it is increased along with elevated LPS in cholestasis.[10] Additionally, TNAP is known to be essential for the development of bone and teeth. Hypophosphatasia is a condition which results from TNAP gene mutations that lead to accumulation of PP_i in the extracellular matrix, which inhibits mineralisation, thereby causing an inheritable form of rickets in children or osteomalacia in adults.[11,12] Furthermore, TNAP has been shown to be essential in axonal growth[13] and neuron proliferation and differentiation.[14] These effects are related to regulation of purinergic signalling by way of ATP hydrolysis, which in turn is linked to the establishment of neuronal circuits.[15] The production of adenosine by TNAP acting as a 5'-ectonucleotidase in the dorsal spinal cord is essential in maintaining a good purinergic tone in nociceptive circuits.[16] On the other hand, new evidence points to a neurotoxic effect of TNAP, as it dephosphorylates hyperphosphorylated tau protein, a key player in Alzheimer's and other neurodegenerative diseases.[17]

In addition to the above, the Alpl gene encoding TNAP is highly expressed late in adipogenesis during adipose terminal differentiation in mice, being involved in lipid and energy metabolism of fat cells, and TNAP might regulate glucose metabolism and insulin sensitivity through adipokine synthesis and secretion.[18] The influence of TNAP in the immune responses goes beyond the effect on adipokine production. Thus TNAP is expressed in leukocytes, and it has a role in B cell differentiation into antibody-secreting cells.[19,20] However, little is known about the role of TNAP in T cells.[21] We have reported that the colonic expression of TNAP is augmented in models of inflammatory bowel disease, due both to the influx of leukocytes into the inflamed colonic tissue and to enhanced expression in epithelial cells.[22–24] Colonic epithelial cells express the liver type of TNAP in quiescent conditions, but when under stress the pattern of glycosylation is altered, resulting in increased activity due to the shift to the bone or kidney isoforms.[24] The function that TNAP exerts in this context remains unclear, but a protective role has been advocated.[22]

In the present study, we aimed to characterise the function of TNAP in T lymphocytes, particularly in relation to colitis.

2. Material and Methods

2.1. Reagents

Except where indicated, all reagents and primers were obtained from Sigma [Barcelona, Spain]. For immediate stabilisation of RNA in tissue, RNAlater was used [Qiagen, Madrid, Spain]. Total RNA was isolated with the RNeasy Mini Kit [Qiagen]. Reverse transcription was achieved with the iScript™ cDNA Synthesis Kit [Biorad, Alcobendas, Madrid, Spain] and GoTaq® qPCR Supermix for amplification [Promega, Charbonnières-les-Bains, France]. All the mouse cell isolation kits used in the magnetic separation [Pan T Cell and $CD4^+$ $CD62L^+$ T Cell] and MACS columns were provided by Miltenyi Biotec [Cologne, Germany]. Mouse ELISA kits [IL-6, TNF-α, IFN-γ, and IL-17A] were obtained from eBioscience [San Diego, CA, USA] except for IL-10 [R&D Systems, Minneapolis, MN, USA]. Multiplex assay [Procarta plex Mix&Match mouse 17-plex] was provided by eBioscience [San Diego, CA, USA]. Anti-mouse CD3ε antibodies [clone 145-2C11] and hamster IgG_1 κ Isotype for *in vivo* experiments were purchased from BD Pharmingen [San Agustín, Spain]. *In vitro* experiments were made with anti-mouse CD3ε [clone 145-2C11] and anti-mouse CD28 [clone 37.51] and Armenian hamster IgG iso control [clone Ebio288Arm] of Functional Grade Purified from eBioscience, and LPS from *Escherichia coli* 055:B5. Cytokines and antibodies for mouse naïve T cell differentiation were purchased in BioLegend [San Diego, CA, USA: IL-4, IL-6, anti-IFN-γ, anti-IL-4, anti-IL-12] and R&D systems [Abingdon, UK: IL-2, IL-12 and TGF-β].

2.2. Animals

All animal procedures in this study were carried out in accordance with existing guidelines and were approved by the Animal Welfare Committee of the University of Granada [registry number: CEEA 2011–354].

We used C57BL/6 [B6.129S7-Akp2^{tm1Sor}/J] heterozygous mice for Alpl [referred to as $TNAP^{+/-}$], with wild-type [WT] littermates used as controls. Mice were obtained from the Jackson Laboratory. Homozygous Alpl KO mice are not viable.[25,26] Genotyping was performed by polymerase chain reaction [PCR] [REDExtract-N-Amp™ PCR ReadyMix™ #R4775, Sigma Aldrich] on ear genomic DNA. The Neomycin cassette was used for genotyping heterozygous mice and IL-2 as internal control. Primers were: Neo sense 5'-GGG TGG AGA GGC TAT TCG GCT ATG A-3', antisense 3'-CCC ATT CGC CGC CAA GCT CTT CAG C-5'; and IL-2 sense 5'-CTA GGC CAC AGA ATT GAA AGA TCT-3', antisense 3'-GTA GGT GGA AAT TCT AGC ATC ATC C-5'. Mice were maintained at the Unit of Animal Research [Biomedical Research Center, University of Granada, Granada, Spain] in specific pathogen-free conditions with free access to autoclaved tap water and food [Harlan-Teklad 2014, Harlan Ibérica, Barcelona, Spain]. Female $Rag1^{-/-}$mice [T cell receptors in colitis transfer model] were obtained from Jackson Laboratory [Sacramento, CA, USA].

2.3. Induction of transfer colitis and experimental design

C57BL/6J [WT] and $TNAP^{+/-}$ mice [16 weeks] were used as donors. Spleen cells were suspended in Dulbecco's Modified

Eagle's medium supplemented with fetal bovine serum [10%], 2 mM L-glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 mg/ml amphotericin B, and passed through a 70-µm filter to obtain a mononuclear suspension. Mononuclear cells were washed and resuspended in MACS buffer (0.5% bovine serum albumin and 2 mM EDTA in phosphate buffered saline [PBS], pH 7.2). $CD4^+$ $CD62L^+$ T cells were obtained from the spleen by negative selection of $CD4^+$ T cells followed by positive selection of CD62L+ cells, and administered intraperitoneally in 100 µl of sterile PBS to C57BL/6J $Rag1^{-/-}$ mice [1×10^6 $CD4^+$ $CD62L^+$ T cells, $n = 10$ for both groups, naïve WT and naïve $TNAP^{+/-}$]. The non-colitic control groups [$Rag1^{-/-}$ background] [$n = 5$, noF WT and noF $TNAP^{+/-}$] were injected with unfractionated splenocytes from WT and $TNAP^{+/-}$ and showed little or no sign of inflammation after up to 6 weeks.

The status of the animals was monitored by general examination and specifically by controlling body weight evolution, faecal blood and diarrhoea. According to these parameters, only mice with established disease were used. Animals were sacrificed by cervical dislocation under isoflurane anaesthesia. Whole-blood samples were obtained for blood count [Mythic 22CT, Orpheé, Geneva, Switzerland]. Plasma samples were collected and kept at −80°C until assayed for cytokine levels [enzyme-linked immunosorbent assay; ELISA]. The colon was cleaned, weighed, and its length measured under a constant load [2 g]. The colon was scored for visible damage by a blinded observer on a 0 to 6 scale according to hyperaemia [0–3] and fibrosis [rigidity, 0–3]. Subsequently several pieces were obtained for RNA isolation [stored at −20°C in RNA later] and biochemical determinations [immediately frozen in liquid nitrogen and kept at −80°C until used]. Another fragment was taken for histology. To measure cytokines from colonic tissue, a piece was homogenised in 0.5 ml Hank's balanced salt solution with 1% Triton X-100 and a protease inhibitor cocktail [Sigma].

Mesenteric lymph node [MLN] cells and splenocytes were cultured and stimulated with concanavalin A [ConA] or LPS from *E. coli*, or directly analysed by flow cytometry or RTqPCR. In addition, a spleen fragment was also frozen in liquid nitrogen and kept at −80°C for RNA isolation. The liver was extracted in pyrogen-free conditions, weighed, and homogenised [1:3 w/v] in sterile PBS [GIBCO®, Waltham, MA, USA] for LPS measurement using a kit based on Lymulus Amebocyte Lysate Assay [Lonza®, Barcelona, Spain].

2.4. Histological assessment of colon damage

Distal colon tissue fragments were fixed in 4% paraformaldehyde [w/v]. After being deparaffinised, sections were rehydrated in serial dilutions of ethanol and water, stained with haematoxylin and eosin [H&E], and scored for damage as follows: infiltrate [0:3], goblet cell loss [0:3], crypt loss [0:3], hyperplasia [0:3], muscle thickening [0:3], and submucosal infiltrate [0:3].

2.5. *In vivo* T cell stimulation with anti-CD3 antibody

Wild-type and $TNAP^{+/-}$ mice were injected intraperitoneally with anti-CD3ε antibody [50 µg/mouse] or isotype IgG control [50 µg/mouse]; 6 hours later, all mice were sacrificed. Whole-blood samples were obtained for blood count. Plasma samples were collected for cytokine measurement. Spleen cells were obtained as above and cultured in 24-well plates. The supernatant was used for cytokine measurement. T lymphocytes were isolated by negative magnetic separation [Pan T Cell Isolation Kit, mouse, Miltenyi® Biotec, Cologne, Germany] and used for RNA extraction.

2.6. Stimulation of primary splenocytes and T lymphocytes *ex vivo*

Primary splenocytes and T lymphocytes isolated by negative magnetic separation were obtained from WT and $TNAP^{+/-}$ C57BL/6J mice as described above. Separation and purification protocols were set up and validated by flow cytometry, using FACS Calibur™ [BD Biosciences]. T cell purity was typically > 95%. Splenocytes were cultured at 1×10^6 cells/ml and were stimulated *in vitro* with ConA [5 µg/ml] or LPS [1 µg/ml], while T lymphocytes were stimulated with ConA [5 µg/mL] or plate-bound anti-CD3 [pretreatment of plates with 2.5 µg/ml overnight] and soluble anti-CD28 [2 µg/ml], or vehicle. In some experiments the cells were treated with the uncompetitive TNAP inhibitors levamisole [1 mM], phenylalanine [5 mM], and theophylline [1 mM] for 2 h at 37°C under shaking before being seeded. After 24 h, cells and supernatants were collected for RNA extraction and cytokine detection, respectively.

2.7. *In vitro* differentiation of mouse Th1, Th2, and Th17 from naïve CD4 T cells

Naïve $CD4^+$ $CD62L^+$ T cells were seeded at 1×10^5 cells/ml in differentiating culture medium for Th0 [1 µg/ml anti-CD28 and 20 ng/ml IL-2], Th1 [1 µg/ml anti-CD28 and 10 µg/ml anti-IL-4 antibodies, 20 ng/ml IL-2, and 20 ng/ml IL-12], Th2 [1 µg/ml anti-CD28, 10 µg/ml anti-IFN-γ, and 10 µg/ml anti-IL-12 antibodies, 20 ng/ml IL-2, and 100 ng/ml IL-4], and Th17 [1 µg/ml anti-CD28, 10 ng/ml anti-IFN-γ, and 10 ng/ml anti-IL-4 antibodies, 100 ng/ml IL-6, and 1 ng/ml TGF-β1]. In all cases, cells were stimulated with anti-CD3ε [5 µg/ml] as above. Percentages of intracellular IFN-γ, IL-4, and IL-17A were determined by fluorescence activated cell sorting [FACS].

2.8. Cells labelling for flow cytometry

For FACS analysis, mesenteric lymph node cells [MLNC] from transfer colitis were stained with antibodies to the following markers; IFNγ-PE and CD4-APC from BD and FoxP3-PerP and mouse Fc-Block Anti-CD16/CD32 from eBioscience. Naïve $CD4^+CD62L^+$ T cells differentiated into T help lineage were stained with the following antibodies: Th1 [IFNγ-PE, CD4-PerCP], Th2 [CD4-PerCP, IL4-APC], and Th17 [IL17-PE, CD4-PerCP], and mouse Fc-Block Anti-CD16/CD32. For the intracellular staining of IFN-γ, IL-4, and IL-17, cells were boosted with phorbol-12-myristate-13-acetate [PMA, 50 ng/ml] and ionomycin [250 ng/ml] for 5 h in Iscove's Modified Dulbecco's Medium following the manufacturer's instructions of the Cytofix/Cytoperm Fixation/Permeabilization Solution Kit with BD GolgiPlug [BD]. For the intracellular staining of Foxp3, cells were permeabilised for 2 h with Foxp3 Staining Buffer Set [eBioscience]. Stained cells were analysed with a FACS Calibur [BD] and the data processed with the FlowJo [Treestar, CA, USA] software.

2.9. [^{3}H]-thymidine uptake

T cell proliferation was measured by [^{3}H]-thymidine incorporation. T lymphocytes, isolated as above, were cultured [1×10^6 cells/ml] with [^{3}H]-thymidine [1 µCi/ml, GE Healthcare, Bucks, UK] after being stimulated with ConA or a-CD3/a-CD28 for 48 or 24 h, respectively. Cells were harvested, washed with cold PBS, fixed in ice-cold 10% trichloroacetic acid, and solubilised in 1% sodium dodecyl sulphate [SDS]/1 N NaOH for 30 min at room temperature. Radioactivity was counted with a liquid scintillation counter [Beckman LS-6000, Madrid, Spain]. [^{3}H]-thymidine uptake is expressed as DPM [disintegrations per minute].

2.10. Cell viability and toxicity assay

Cell viability was quantified with the Trypan blue exclusion assay. Cell toxicity was measured in cell culture supernatant using Pierce LDH citotoxicity assay kit [Thermo Fisher Scientific, MA, USA], following the protocol recommended by the manufacturer.

2.11. Cytokine plasma levels

A blood sample was drawn from the heart and spun to obtain plasma, which was snap-frozen at −80°C until assayed for cytokine content by commercial ELISA, following the protocols recommended by the manufacturer. The cytokines determined were IFN-γ and TNF-α. Plates [Nunc™ Immuno plate, Roskilde, Denmark] were read at 450–665 nm using a plate reader [Biorad model 680 XR].

2.12. Myeloperoxidase [MPO] and alkaline phosphatase [AP] activities

Colonic tissue homogenisation was carried out with the protocol for intestinal tissue homogenisation in a Bullet Blender® [Next Advance, Averill Park, NY, USA]. MPO and AP activity was measured spectrophotometrically as described. The sensitivity to the AP inhibitor levamisole was also tested *in vitro*.[22,24]

2.13. RNA isolation and quantitative reverse-transcription polymerase chain reaction analysis

RNA quantity and integrity were assessed by spectrophotometry. Specific DNA sequences were amplified with a Biorad CFX connect real-time polymerase chain reaction [PCR] device [Alcobendas, Madrid, Spain]. Primers used are shown in Table 1. Results are expressed as 2^{-ddCt} using 18S as reference gene.

2.14. Data and statistical analysis

Samples were run at least in triplicate and results are expressed as mean ± standard error of the mean [SEM]. The obtained values are the result of the average values between males and females. Differences among means were tested for statistical significance by two-way analysis of variance [ANOVA] and *a posteriori* Fisher's least significant difference [LSD] tests, or Student's t test for pairwise comparisons. PCR data were log transformed before analysis. All analyses were carried out with the GraphPad Prism 6 [GraphPad Software Inc., La Jolla, CA, USA]. Differences were considered significant at $p < 0.05$.

3. Results

3.1. AP expression in TNAP$^{+/-}$ mice

TNAP$^{+/-}$ mice were used in this study, since homozygous KO mice are not viable. The phenotype of TNAP heterozygous mice was perfectly normal, and they were indistinguishable from WT mice in terms of appearance and general behaviour. TNAP expression (Alpl mRNA by reverse-transcription polymerase chain reaction [RT-qPCR]) and AP activity were measured to characterise TNAP$^{+/-}$ mice. As expected, the presence of a single Alpl allele resulted in diminished mRNA levels, roughly by 50–60%, in all the tissues analysed [Figure 1A]. This correlated with a similar degree of reduction in tissue AP enzymatic activity in the kidney and ileum, but not in other organs, where there was no significant change [Figure 1B]. The isoform-specific inhibitor levamisole was used to further characterise enzyme expression in TNAP$^{+/-}$ mice. Bone and kidney TNAP isoforms are sensitive to levamisole, whereas liver TNAP and intestinal AP are resistant and can even be enhanced by it. As shown in Figure 1C, the AP activity of TNAP$^{+/-}$ in organs was generally less inhibited *in vitro* by levamisole and, in those instances where levamisole had a stimulatory effect [shown as negative values of inhibition, as in the colon and liver], AP stimulation tended to be enhanced. In keeping with these results, AP activity in plasma was not modified significantly [Figure 1D], but it was much less sensitive to inhibition by levamisole *in vitro* [Figure 1E]. These data indicate that the lack of one Alpl allele in TNAP$^{+/-}$ mice results in relatively minor changes at the activity level, whereas the differential sensitivity to levamisole suggests a change in isoform, presumably linked to differences in the glycosylation pattern.[23]

Table 1. Primers used in the RT-qPCR [reverse-transcription quantitative polymerase chain reaction] analysis.

Gene	Sense 5'-3'	Antisense 3'-5'
18s	ACACGGACAGGATTGACAGATTG	GCCAGAGTCTCGTTCGTTATCG
Alpl	ATTCCCACTATGTCTGGAAC	CTCAAAGAGACCTAAGAGGTAG
Cd11b	AAGATCTTTGCAATTGAGGG	CTCTGGTTGTGTTGATGAAG
Cd40	ATAATGAGATCTTACCCCCTG	CTCTCTTTACCATCCTCCTG
Cxcl9	GAGGAACCCTAGTGATAAGG	GTTTGATCTCCGTTCTTCAG
Emr1	TTTCAAATGGATCCAGAAGG	CAGAAGGAAGCATAACCAAG
Foxp3	AATAGTTCCTTCCCAGAGTTC	GGTAGATTTCATTGAGTGTCC
Gata3	TATTAACAGACCCCTGACT	CACCTTTTTGCACTTTTTCG
Ifng	TGAGTATTGCCAAGTTTGAG	CTTATTGGGACAATCTCTTCC
Il10	CAGGACTTTAAGGGTTACTTG	ATTTTCACAGGGGAGAAATC
Il17a	ACGTTTCTCAGCAAACTTAG	CCCCTTTACACCTTCTTTTC
Il22	ATCAGTGCTACCTGATGAAG	CATTCTTCTGGATGTTCTGG
Il4	CTGGATTCATCGATAAGCTG	TTTGCATGATGCTCTTTAGG-
Il5	AGACTTCAGAGTCATGAGAAG	GCTGGTGATTTTTATGAGTAGG
Il6	AAGAAATGATGGATGCTACC	AGTTTCTGTATCTCTCTGAAG
Klf4	CCCCTCTCTCCATTATCAAG	CTCTTGGTATAGGTTTTGCC
S100a8	GCCCTCTACAAGAATGACTTCAAG	ATCACCATCGCAAGGAACTCC
Spib	ACACTTAAGCTGTTTGTACC	TCTGAATCTGGGTAACTGAAG
Spp1	GGATGAATCTGACGAATCTC	GCATCAGGATACTGTTCATC
Tbx21	ACGTCTTTACTTTCCAAGAG	GTACATGGACTCAAAGTTC
Tnf	CGTGGAACTGGCAGAAGAGG	CAGGAATGAGAAGAGGCTGAGAC

3.2. Splenocytes from TNAP$^{+/-}$ mice have an attenuated response to ConA and LPS

In order to assess the role of TNAP in immune cell function, the response of WT vs TNAP$^{+/-}$ splenocytes to LPS *ex vivo* was studied. First, TNAP expression was evaluated as above. Alpl mRNA levels were very low in basal conditions, but increased several fold after stimulation of WT splenocytes with LPS [Figure 2A]. This increase was markedly lower in TNAP$^{+/-}$ cells. In terms of immune response, activation of spleen immune cells with LPS resulted in upregulation of IL-6 and TNF-α, which was 37–70% lower in heterozygous cells [Figure 2B]. Similarly, when ConA was used to stimulate splenocytes, a marked release of IFN-γ and IL-17A was attained as expected, which was attenuated in TNAP$^{+/-}$ cells by 20–50% [Figure 2C]. Basal cytokine production was negligible in all cases [not shown].

3.3. T cells from TNAP$^{+/-}$ mice exhibit a dampened response to *in vitro* stimuli

To evaluate the role of TNAP specifically in T lymphocytes, T cells from WT and TNAP$^{+/-}$ mice were obtained using magnetic cell separation and cultured *in vitro*. First, T cells were stimulated with ConA, and levels of IFN-γ were measured. As with splenocytes, T cells from TNAP$^{+/-}$ displayed a marked reduction in the level of this cytokine [90% approximately, Figure 3A]. A more physiological approach is the stimulation with a-CD3/a-CD28, which partially mimics stimulation by antigen-presenting cells. Antibodies specific for the TCR–CD3 complex provide an initial activation and require the co-stimulatory molecule CD28, critical for T cell activation survival. T cells from TNAP$^{+/-}$ showed again an inhibition of IFN-γ production, albeit less pronounced [~30%, p = 0.14, Figure 3B]. The

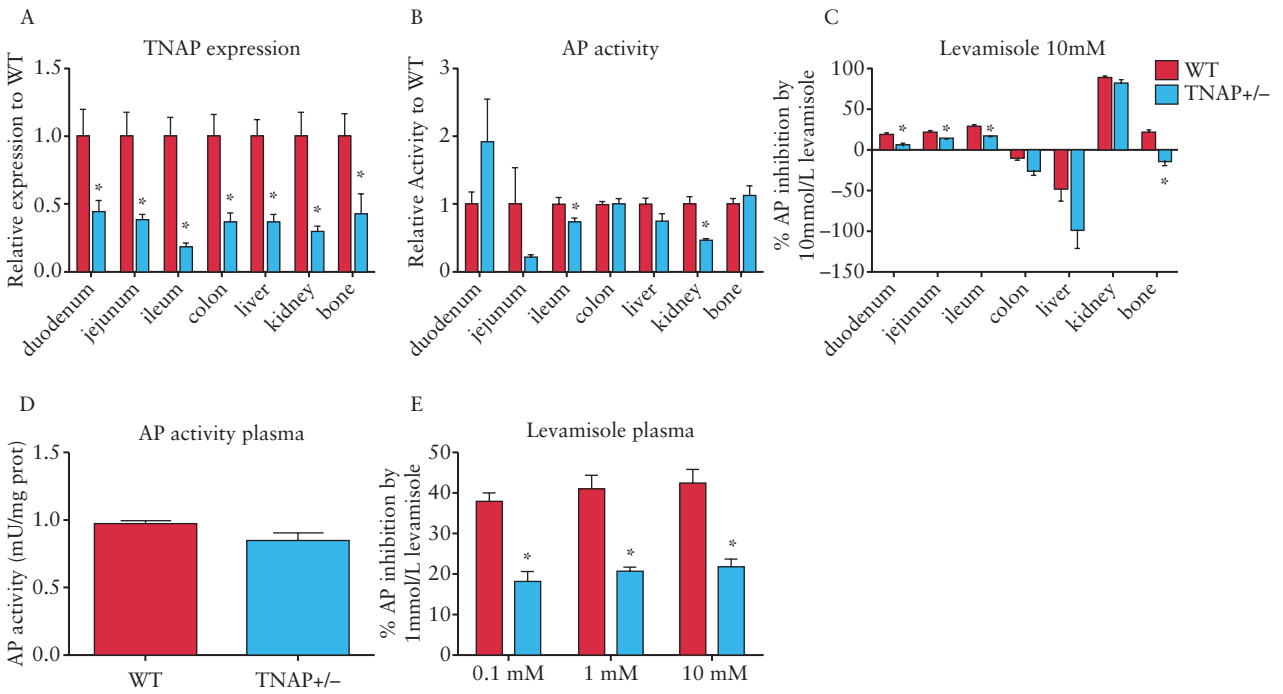

Figure 1. AP activity and expression in TNAP$^{+/-}$ mice. [A] Relative TNAP expression assessed by RT-qPCR and [B] relative AP activities [absolute values are available in Supplementary Table 1, available at *ECCO-JCC* online]. [C] Sensitivity of AP activity to inhibition by levamisole [10 mM] in different tissues. [D] AP activity and [E] AP sensitivity to the specific inhibitor levamisole in the plasma of WT and TNAP$^{+/-}$ mice. Values are means ± SEM; *$p < 0.05$ vs WT mice. AP, alkaline phosphatase; TN, tissue non-specific; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WT, wild type; SEM, standard error of the mean.

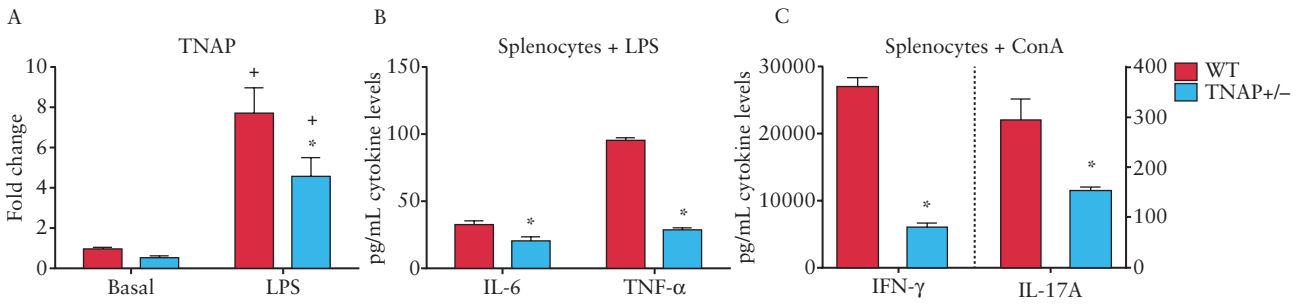

Figure 2. Response of splenocytes from TNAP$^{+/-}$ and WT mice to LPS and ConA. [A] Splenocyte TNAP expression assessed by RT-qPCR. Splenocyte production of [B] IL-6 and TNF-α and **[C]** IFN-γ and IL-17A. Cytokine secretion was negligible under unstimulated conditions [not shown]. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs basal. AP, alkaline phosphatase; TN, tissue non-specific; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WT, wild type;.LPS, lipopolysaccharide; ConA, concanavalin A.

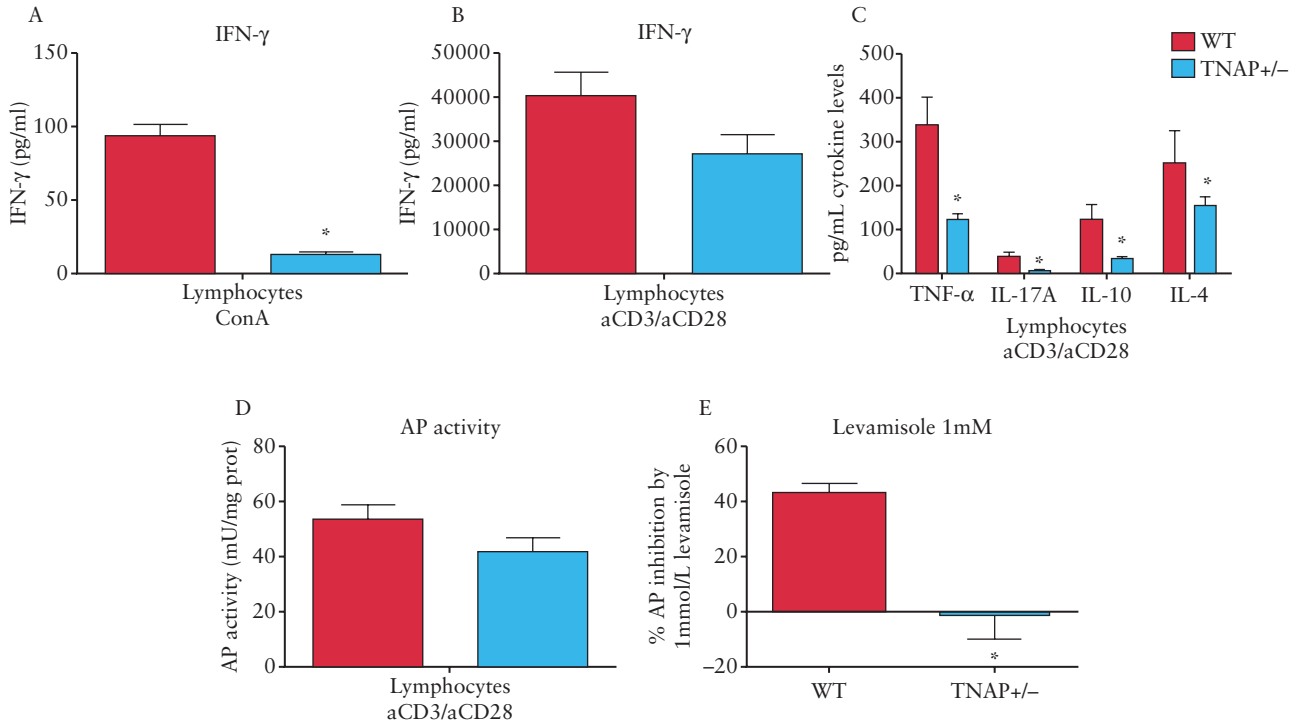

Figure 3. Cytokine profile and AP activity in T lymphocytes from TNAP$^{+/-}$ and WT mice. T lymphocytes were cultured *ex vivo* and IFN-γ was determined by ELISA [A] when stimulated with ConA and [B] when stimulated with a-CD3/a-CD28. [C] TNF-α, IL-17A, IL-10, and IL-4 were measured by Multiplex® when stimulated with a-CD3/a-CD28. Cytokine secretion was negligible under unstimulated conditions [not shown]. [D] AP activity and [E] *in vitro* sensitivity to levamisole [1 mM] in cells stimulated with a-CD3/a-CD28. *$p < 0.05$ vs WT. AP, alkaline phosphatase; TN, tissue non-specific; WT, wild type; ELISA, enzyme-linked immunosorbent assay; ConA, concanavalin A.

reduction was marked in the rest of cytokines studied [corresponding to the Th2, Th17, and Treg lineages] in the Multiplex assay [Figure 3C]. In this experiment, AP activity was not significantly different in WT and TNAP$^{+/-}$ cells, although AP activity in WT lymphocytes was levamisole-sensitive in contrast to TNAP$^{+/-}$ cells [Figure 3D-E].

3.4. *In vitro* inhibition of TNAP mimics the phenotype of TNAP+/-T cells

In order to assess the effect of TNAP inhibition on T cell function, T cells obtained from WT mice were stimulated *in vitro* with a-CD3/a-CD28 as described above in the presence of the uncompetitive TNAP inhibitors levamisole, phenylalanine, and theophylline.[27-29] Compared with other AP isoforms, TNAP [of the bone and kidney subtypes] is sensitive to inhibition with levamisole and theophylline, although it is worth noting that theophylline has well-known additional effects via cAMP. Phenylalanine inhibits all AP isoforms but has partial selectivity for placental and intestinal AP.[30,31] T cells were incubated with the inhibitors for 2 h before stimulation with a-CD3/a-CD28. Multiplex analysis of cell culture supernatants revealed a marked decrease in the levels of all measured cytokines with each of the TNAP inhibitors used, comparable to the down-regulation observed in TNAP$^{+/-}$ vs WT cells [Figure 4A, and 4B]. This marked decrease in cytokine levels was not due to a cytotoxic effect, as the LDH assay revealed no toxic actions of any inhibitor [data not shown]. In parallel experiments, [^{3}H]-thymidine uptake was shown to be lowered by all three inhibitors [with phenylalanine showing the least marked effect] in a-CD3/a-CD28-stimulated T cells, indicating a depressed proliferative response, as that seen in TNAP$^{+/-}$ T cells [Figure 4C].

3.5. T cells from TNAP$^{+/-}$ mice have diminished sensitivity to stimulation *in vivo*

To test whether this difference in cytokine production is reproducible *in vivo*, a model of systemic activation of lymphocytes was used. Intraperitoneal administration of anti-CD3 provoked the characteristic hypothermia of this model in both WT and TNAP$^{+/-}$ mice [Figure 5A] as well as an increase in the plasma levels of TNF-α and IFN-γ [Figure 5B-C]. No differences were seen in body temperature between the treated groups; however, both cytokines were reduced in plasma of anti-CD3-treated TNAP$^{+/-}$ mice [$p = 0.1$ for the latter]. This correlated with reduced IFN-γ production by stimulated splenocytes *ex vivo* [Figure 5D], whereas TNF-α was undetectable [not shown].

To further characterise the phenotype of TNAP$^{+/-}$ T cells, RT-qPCR analysis was applied [Figure 6]. *In vivo* treatment with anti-CD3 produced a lower induction of several genes in T cells isolated from the spleen of TNAP$^{+/-}$ mice in comparison with those of WT mice; specifically, TNAP [Alpl], IFN-γ, CXCL9, IL-4, IL-5, and IL-22 were some of the most affected. Interestingly, TNAP was found to be upregulated in WT activated cells, but not in TNAP$^{+/-}$ cells. This contrasts with the observation that mRNA levels were comparable in basal conditions. These data are similar to those obtained previously in splenocytes [Figure 2A]. TNF-α, Foxp3, and CD40 showed the same trend [non-significant for Foxp3, see Supplementary Figure 1, available at *ECCO-JCC* online]. Spib [Spi-B transcription factor] and Klf4 [Kruppel-like factor 4], involved in proliferation and T function, respectively, were lower in TNAP$^{+/-}$ cells; whereas Spp1, encoding osteopontin, a TNAP-related protein involved in the immune response, displayed a reduced expression in TNAP$^{+/-}$ T cells in basal conditions, with opposite changes after anti-CD3

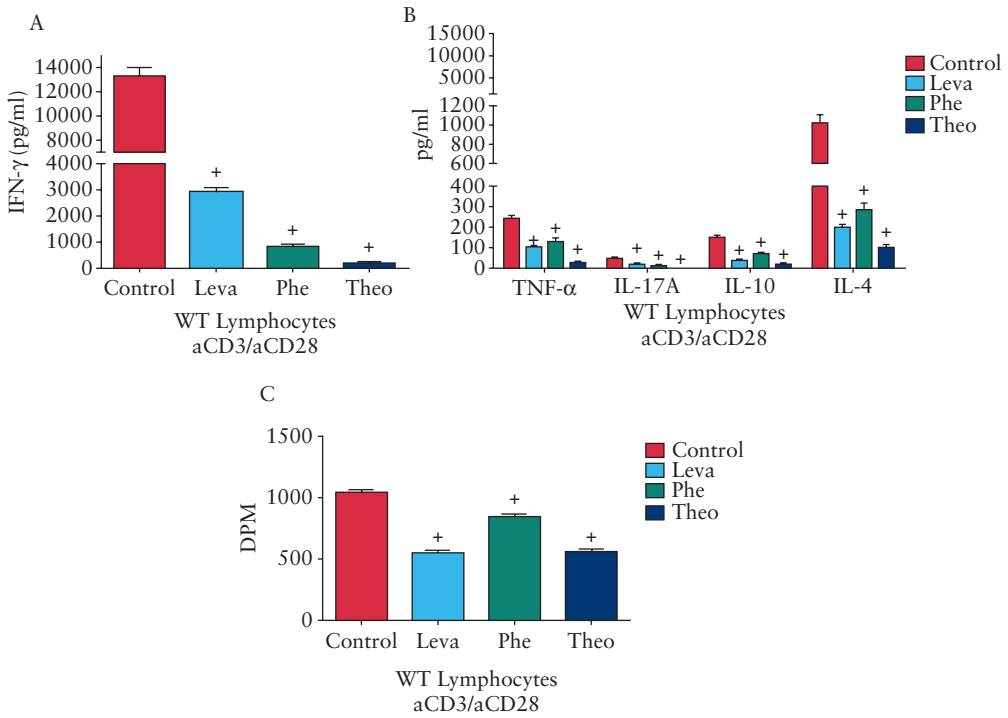

Figure 4. Characterisation of WT T cells exposed to TNAP inhibitors. Lymphocytes were incubated with levamisole [1 mM], phenylalanine [5 mM], or theophylline [1 mM] for 2 h. Then T cells were stimulated with a-CD3/a-CD28 for 24 h. [A] IFN-γ and [B] TNF-α, IL-17A, IL-10, and IL-4 were measured, by ELISA or Multiplex, respectively. **[C]** [^{3}H]-thymidine uptake. $^+p < 0.05$ vs control. AP, alkaline phosphatase; TN, tissue non-specific; WT, wild type; ELISA, enzyme-linked immunosorbent assay.

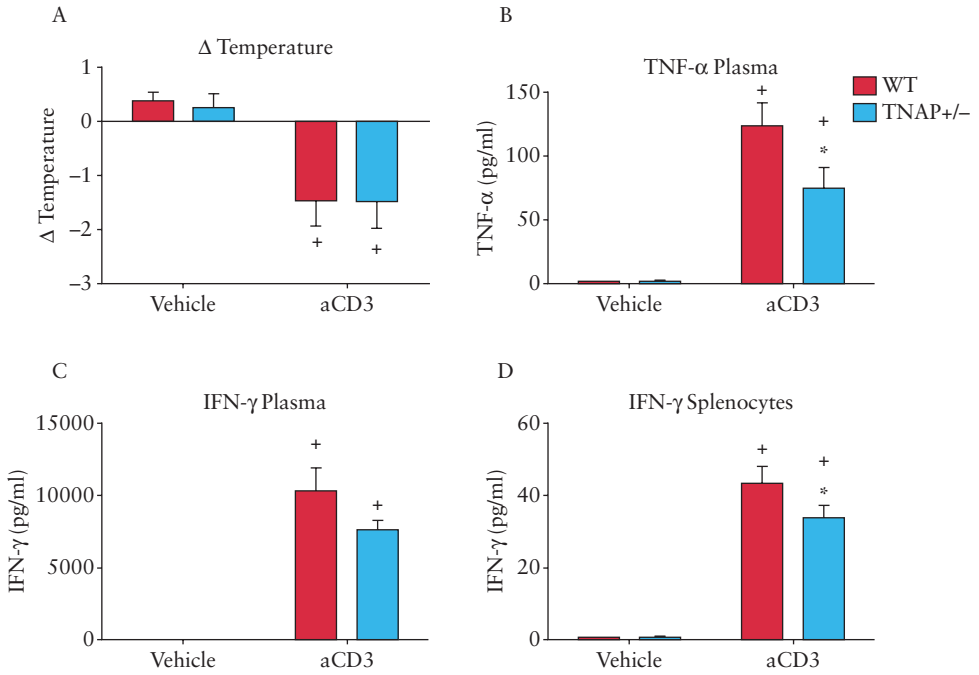

Figure 5. T cell stimulation *in vivo* with anti-CD3. [A] Body temperature changes. Plasma levels of [B] TNF-α and [C] IFN-γ. [D] Splenocyte production of IFN-γ *ex vivo*. $^*p < 0.05$ vs WT; $^+p < 0.05$ vs vehicle. WT, wild type.

administration [$p = 0.059$] [i.e. reduced levels in WT mice and augmented levels in TNAP$^{+/-}$ mice] [see Supplementary Figure 1D–F].

In addition, TNAP$^{+/-}$ mice showed an attenuated lymphopenic response compared with the WT group and monocyte numbers were further reduced [Figure 7A, B].

3.6. T lymphocytes from TNAP$^{+/-}$ induce a milder colitis in the T cell transfer model

We tested the hypothesis that TNAP expressed in T lymphocytes is involved in the inflammatory response *in vivo*. For this purpose, we used the T cell transfer model of colitis. Six weeks after the adoptive

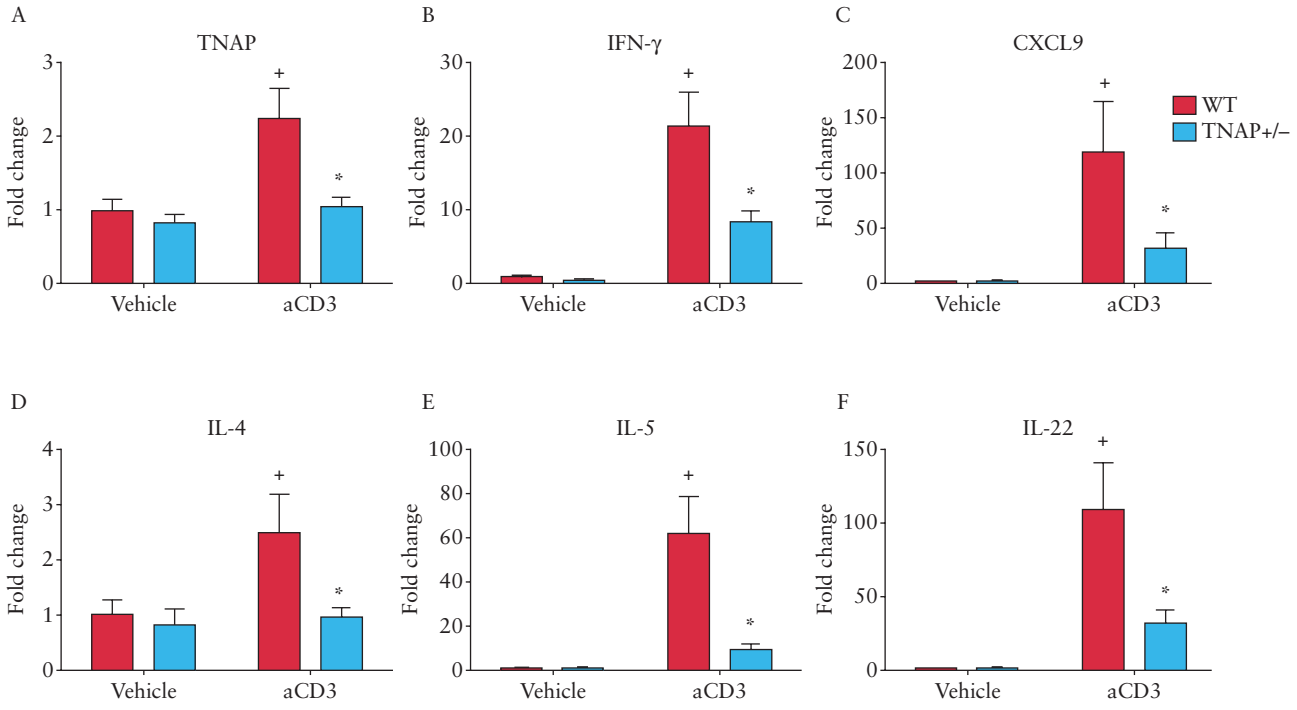

Figure 6. T cell expression of inflammatory markers by RT-qPCR after anti-CD3 administration *in vivo*. T lymphocyte mRNA levels of [A] TNAP, [B] IFN-γ, [C] CXCL9, [D] IL-4, [E] IL-5, and [F] IL-22 are shown. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs vehicle. AP, alkaline phosphatase; TN, tissue non-specific; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WT, wild type.

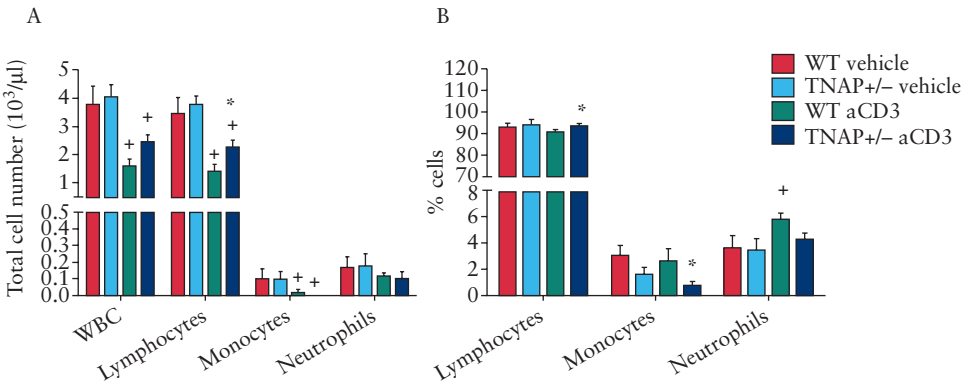

Figure 7. Haematological analysis of anti-CD3 injected mice. [A] Total cell number of white blood cells. [B] Percentage of white blood cells. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs vehicle. WT, wild type.

transfer of naïve T cells obtained from WT or TNAP$^{+/-}$ mice, animals from both groups had signs of colitis and were sacrificed. A haematological study was conducted, showing no differences among the groups, with the notable exception of the noF TNAP$^{+/-}$ group in which a panleukopenic phenotype was detected [Figure 8A]. The colon of transferred mice was thickened, shortened, and hyperaemic, with signs of fibrosis, increased MPO activity [Figure 8], and marked infiltration and tissue injury, resulting in a significantly increased histology score [Figure 9A, B]. Visible colonic damage was evaluated by a macroscopic score which was significantly increased [1.95 vs 0, $p < 0.05$]. Mice receiving TNAP$^{+/-}$ lymphocytes exhibited a lower severity of inflammation, particularly a lower degree of fibrosis, resulting in a significant reduction of the score [1.35, $p < 0.05$]. However, there was no difference in the histological score between the colitic groups [Figure 9B]. Although not significant, it is interesting to note that the colonic weight:length ratio was slightly lower in mice receiving either naïve and unfractionated TNAP$^{+/-}$ T cells than their corresponding WT controls [Figure 8B]. Colonic cytokine expression was assessed with a Multiplex assay, which indicated that IFN-γ, TNF-α, and IL-10 were reduced in the naïve TNAP$^{+/-}$ group, although only IFN-γ was significant. In contrast, levels of IL-17 were increased in this group [Figure 9C]. RT-qPCR results support a lower production of cytokines [significant for IFN-γ and IL-1β] as well as a significantly reduced expression of the Th1 driving transcription factor T-bet [Figure 9E]. No changes were seen in iNOS or S100A8 expression [Figure 9D]. LPS translocation was increased in both colitic groups, more extensively in mice receiving WT cells, but without reaching significance in either case [Figure 8C]. Despite the above, there were no differences in MPO activity [Figure 8D]. There were also no differences in splenomegaly [Supplementary

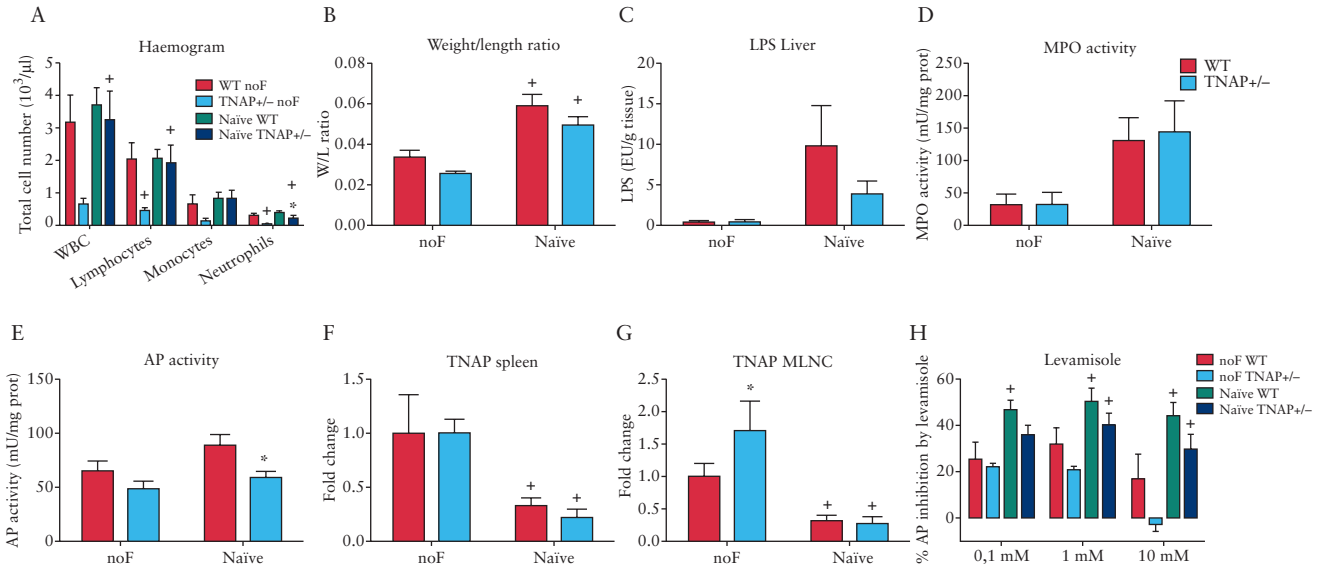

Figure 8. Inflammatory markers in T cell transfer colitis. [A] Total cell number of white blood cells; [B] colonic weight/length ratio; [C] LPS levels in liver; [D] colonic MPO activity; [E] colonic AP activity and [H] AP sensitivity to the specific inhibitor levamisole; [F] spleen and [G] MLNC expression of TNAP. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs noF [control]. AP, alkaline phosphatase; TN, tissue non-specific; WT, wild type; LPS, lipopolysaccharide; MPO, myeloperoxidase; MLNC, mesenteric lymph node cells.

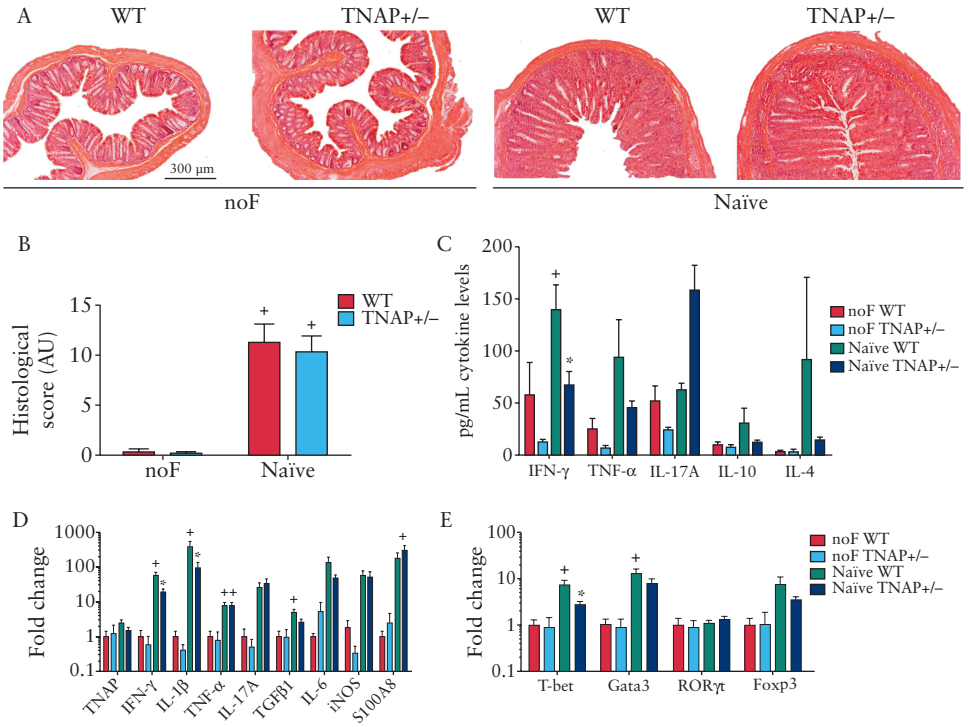

Figure 9. Histological analysis and immune response in T cell transfer colitis. [A] Representative histology sections. [B] Histological score of colon sections stained with H&E, expressed as arbitrary units [AU]. [C] Cytokine levels of IFN-γ, TNF-α, IL-17A, IL-10, and IL-4 detected in colon homogenates determined by Multiplex assay. Colon mRNA levels of immunologically related genes [D] TNAP, IFN-γ, IL-1β, TNF-α, IL-17A, TGFβ1, IL-6, iNOS, S100A8, and T cell lineage markers [E] T-bet [Th1], GATA-3 [Th2], RORγt [Th17], and Foxp3 [Treg]. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs noF [control]. AP, alkaline phosphatase; TN, tissue non-specific; WT, wild type; H&E, haematoxylin and eosin.

Figure 2, available at *ECCO-JCC* online]. As expected, colonic AP activity was higher in the WT transferred mice, although this was of low magnitude and failed to reach significance, presumably due to the relatively mild colitic status of mice in this model [Figure 8E]. Sensitivity of AP activity to levamisole was, however, significantly augmented. Of note, both colonic AP activity and inhibition by levamisole were decreased in the naïve TNAP$^{+/-}$ group [Figure 8H]. Part of the increase in AP activity in the inflamed colon arises from the influx of TNAP-expressing leukocytes[23]; consistent with this, TNAP mRNA was higher in naïve WT than in the noF WT and

noF TNAP$^{+/-}$ mice, but without reaching significance [Supplementary Figure 2]. This trend was not observed in naïve TNAP$^{+/-}$ animals, consistent with a reduced contribution of incoming lymphocytes. There were no differences in TNAP expression in either the spleen or MLNC [Figure 8F-G].

We further analysed plasma to determine the levels of pro-inflammatory cytokines, specifically IFN-γ and TNF-α. ELISA measurement showed an increase in both cytokines in the colitic groups, but naïve TNAP$^{+/-}$ mice exhibited a reduced production of IFN-γ and TNF-α [58% and 17%], with only the former being statistically significant [Figure 10A, B]. A similar result was obtained when culture supernatants from splenocytes stimulated with LPS or ConA were studied, where cytokine production was reduced by 31% and 40% for TNF-α, and 57% and 13% for IFN-γ [non-significant], respectively, in the naïve TNAP$^{+/-}$ group [Figure 10C–F]. We examined different MLN T cell populations, namely CD4$^+$Foxp3$^+$ cells and CD4$^+$IFN-γ$^+$ cells, by flow cytometry. The percentage of CD4$^+$Foxp3$^+$ cells, representative of the Treg population, was increased in the naïve TNAP$^{+/-}$ group in comparison with the naïve WT colitic control [Figure 11A]. RT-qPCR analysis of MLN revealed that both IL-10 and Foxp3 mRNA levels were significantly increased in naïve TNAP$^{+/-}$ mice compared with the naïve WT mice [Figure 11D, E]. This effect was highly specific, since TNF-α and IFN-γ were unaffected [Supplementary Figure 2]. Despite this increase, IL-10 production by splenocytes was inhibited [Figure 11C]. Conversely, the percentage of CD4$^+$IFN-γ$^+$ cells was reduced in the naïve TNAP$^{+/-}$ group [Figure 11B], although there was an increase in both colitic groups compared with the noF noncolitic groups, consistent with plasma IFN-γ levels [Figure 10A]. There was no change in splenocyte ConA-evoked IL-17A and IL-4 production [Supplementary Figure 2].

These observations were generally supported by PCR analysis of gene expression in the spleen. Thus mRNA levels of TNF-α, IFN-γ, IL-10, and IL-17A generally paralleled those of cytokine release, although they failed to reach significance [Supplementary Figure 2]. We also examined the expression of T cell lineage-specific markers, namely T-bet, Gata3, and Foxp3, corresponding to Th1, Th2, and Treg cells, respectively. Of the three, only T-bet expression showed a trend for downregulation, which was non-significant, in the naïve TNAP$^{+/-}$ group compared with naïve WT mice, but a similar profile was noted for Gata3 [Figure 12A-C]. Interestingly, the monocyte/macrophage marker CD11b was also apparently downregulated in the spleen of naïve TNAP$^{+/-}$ mice, but without reaching significance. In order to confirm this observation, another well-established macrophage marker, F4/80, was measured, and its expression found to be significantly reduced. This effect extended to mice which received unfractionated TNAP$^{+/-}$ cells [Figure 12D, E].

3.7. T cells from TNAP$^{+/-}$ mice exhibit a depressed proliferative response but no changes in differentiation

The results described above could be explained by an altered differentiation or proliferation of T cells in response to activation. To contrast this hypothesis, we first carried out T naïve cell polarisation experiments in Th1, Th2, or Th17 effector cells by applying specific stimulatory cytokines and cytokine-blocking antibodies. Flow cytometry analysis revealed no differences between lymphocytes from WT and TNAP$^{+/-}$ mice in any of the polarised populations [Figure 13A and Supplementary Figure 2]. We then focused on possible modifications in T cell proliferation. For this purpose, we stimulated T cells with ConA [Figure 13B], but again no differences were seen between the groups. In turn, when stimulated with a-CD3/a-CD28, T cells from TNAP$^{+/-}$ mice proliferated to a lower extent [almost 50% less] than those from WT mice [Figure 13C]. It is noteworthy that stimulation with anti-CD3, apart from being more physiological, induces a more powerful proliferation than ConA.

4. Discussion

Our results offer clear evidence that TNAP modulates T lymphocyte function and, more specifically, T cell-dependent colitis. As noted above, TNAP is upregulated in the inflamed intestine, partly due

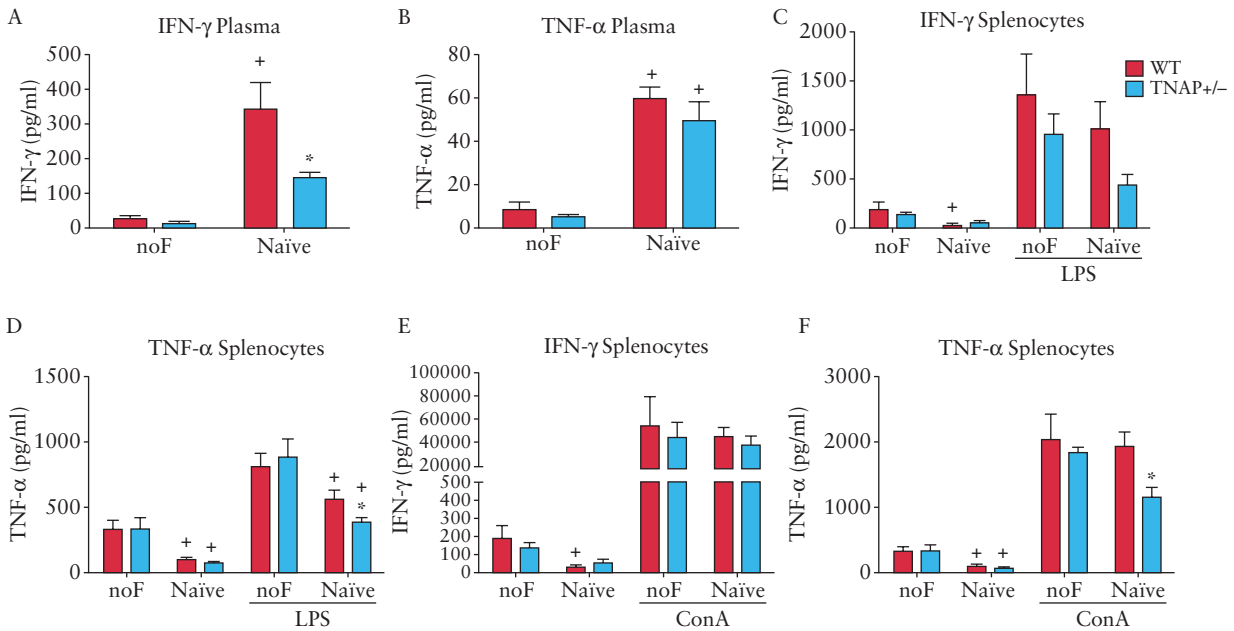

Figure 10. Cytokine levels in T cell transfer colitis. Plasma measurement of [A] IFN-γ and [B] TNF-α. Splenocyte production of [C] IFN-γ and [D] TNF-α when stimulated with LPS [1 μg/ml]. Splenocyte production of [E] IFN-γ and [F] TNF-α production when stimulated with ConA [5 μg/ml]. *$p < 0.05$ vs WT; $^+p < 0.05$ vs noF [control]. LPS, lipopolysaccharide; ConA, concanavalin A; WT, wild type.

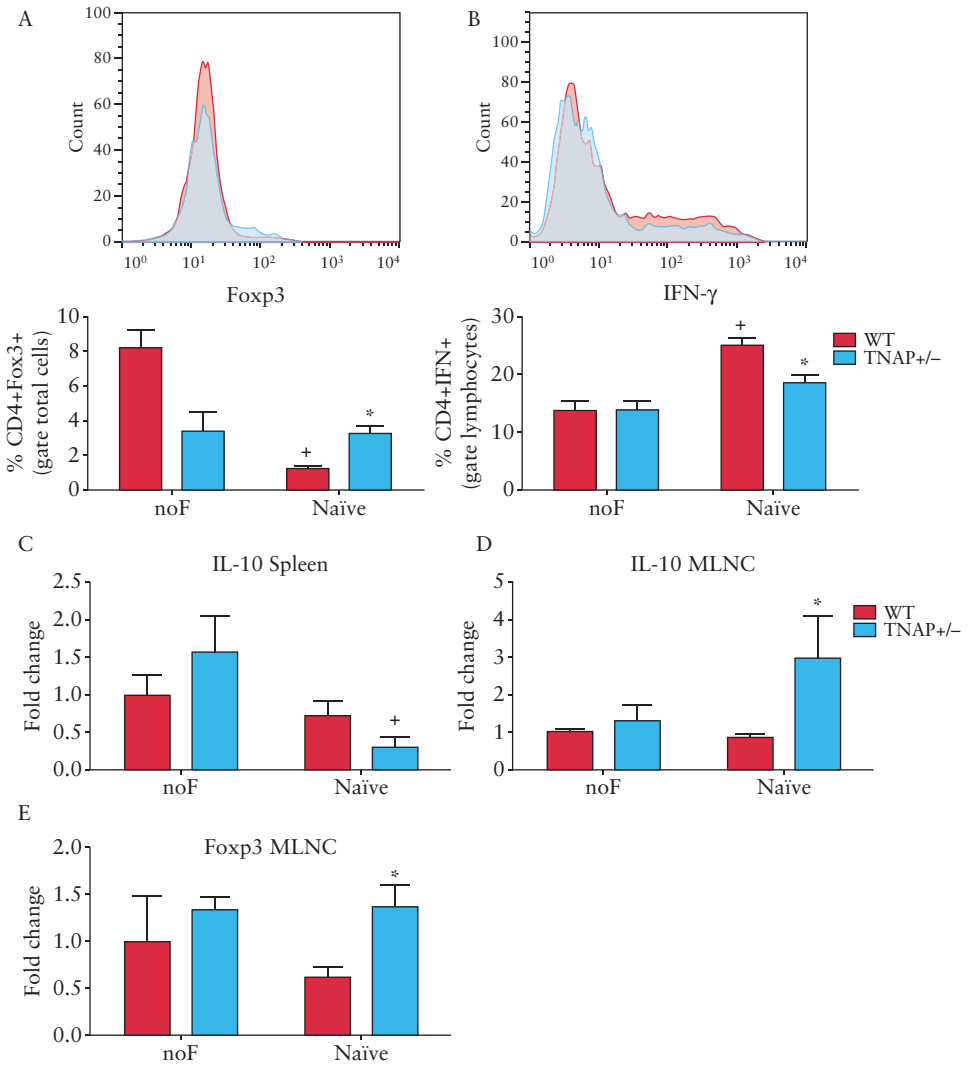

Figure 11. T cell lineage derived from transferred colitic mice. MLNC [A] Foxp3 histogram [CD4⁺ gate] and %CD4⁺Foxp3⁺ [gate total cells]. [B] IFN-γ histogram [CD4⁺ gate] and %CD4⁺ IFN-γ⁺ [gate lymphocytes]. [C] Spleen expression of IL-10 by RT-qPCR. [D] IL-10 and [E] Foxp3 expression in MLNC by RT-qPCR. *$p < 0.05$ vs WT; ⁺$p < 0.05$ vs noF [control]. MLNC, mesenteric lymph node cells; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WT, wild type.

to infiltrating lymphocytes and neutrophils. We hypothesised that TNAP expressed by T cells is relevant to T cell function and colitis, based on the above plus the observation that TNAP is involved in B cell biology.[19] We used TNAP$^{+/-}$ mice as a model. Our results show that TNAP$^{+/-}$ T cells exhibit a lower proliferative response to a-CD3/a-CD28, albeit not to ConA, and depressed production and release of pro-inflammatory cytokines, particularly of IFN-γ. These results are quite remarkable because TNAP$^{+/-}$ mice have been regarded as essentially normal by the investigators who developed TNAP KO mice [and in the process the heterozygous mice as well],[25,26] and indeed by ourselves. In sharp contrast with KO mice, which are not viable and die shortly after birth, the absence of a single allele simply results in a decrease of activity in some but not all target tissues, so that there was no major loss of activity in any of the organs analysed, with the exception of the kidney which lost half of AP activity.[32] This suggests that AP activity is mostly insensitive, in principle, to the TNAP 'gene dose'. However, differential sensitivity to the specific AP inhibitor levamisole *in vitro* indicates that enzymatic characteristics are changed, possibly due to altered glycosylation, as the three TNAP isoforms differ precisely in the glycosylation of the protein.[23,24] In turn, protein glycosylation may be affected by protein turnover, which may result from the lack of one of the Alpl alleles. Such a mechanism has been previously documented. Thus in experimental colitis, TNAP activity and sensitivity to levamisole are both increased in epithelial cells due to changes at the post-translational level.[24] Similarly, gene knockdown resulting in almost complete [85–97%] abolition of mRNA in adipocytes only translates into a 15–47% reduction in activity.[18] Thus our results suggest that the lack of one Alpl allele alters TNAP glycosylation, although enzymatic activity [as measured using p-nitrophenylphosphate as substrate] is generally preserved.

Despite the attenuated response to stimulation of purified T cells from TNAP$^{+/-}$ mice, no change in the capacity of Th0 cells for polarisation was noted, as the *in vitro* polarisation experiments yielded similar outcomes in the different conditions. Rather, T cell proliferation was affected. Although basal mRNA levels and enzymatic activity, including sensitivity to levamisole inhibition, were comparable in T cells isolated from TNAP$^{+/-}$ and WT mice [see Supplementary Figure 3, available at *ECCO-JCC* online], we found a differential response to stimulation *in vivo*, since TNAP was significantly induced

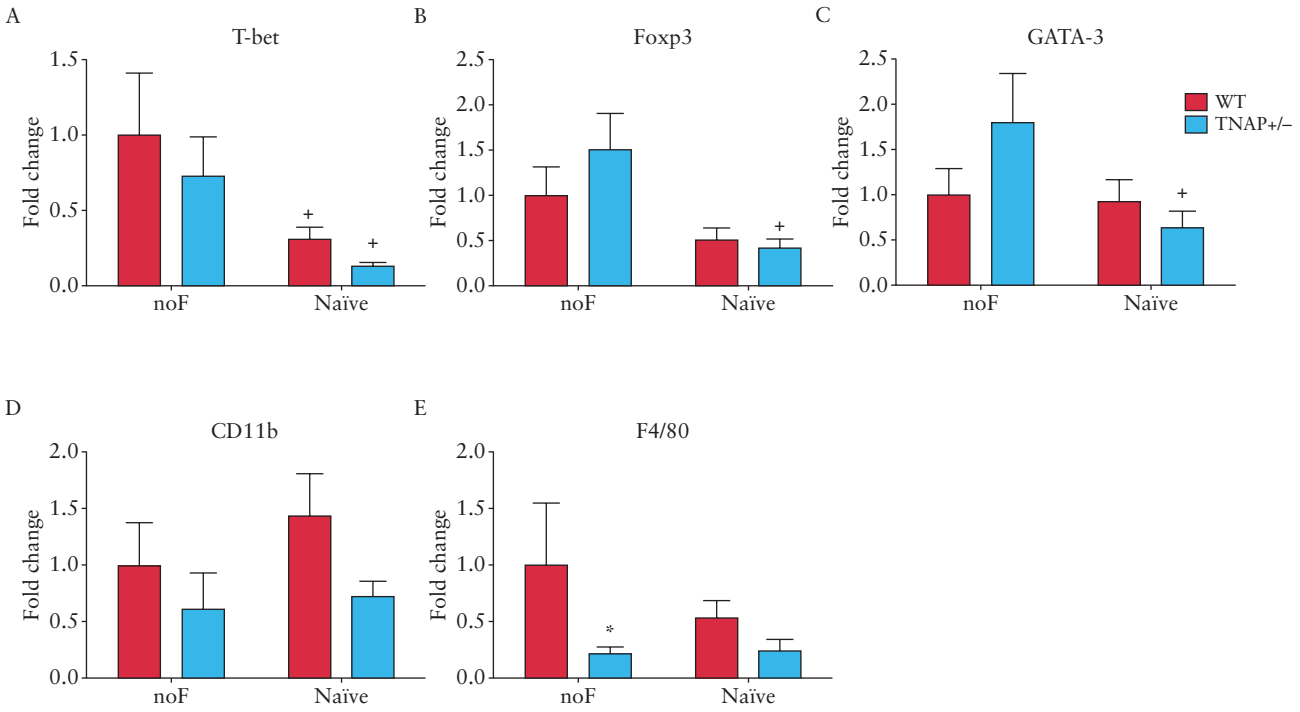

Figure 12. Cell lineage analysed by RT-qPCR in colitic mice. Spleen mRNA levels of [A] T-bet, [B] Foxp3, [C] Gata3, [D] CD11b, and [E] F4/80 are shown. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs noF [control]. RT-qPCR, reverse-transcription quantitative polymerase chain reaction; WT, wild type.

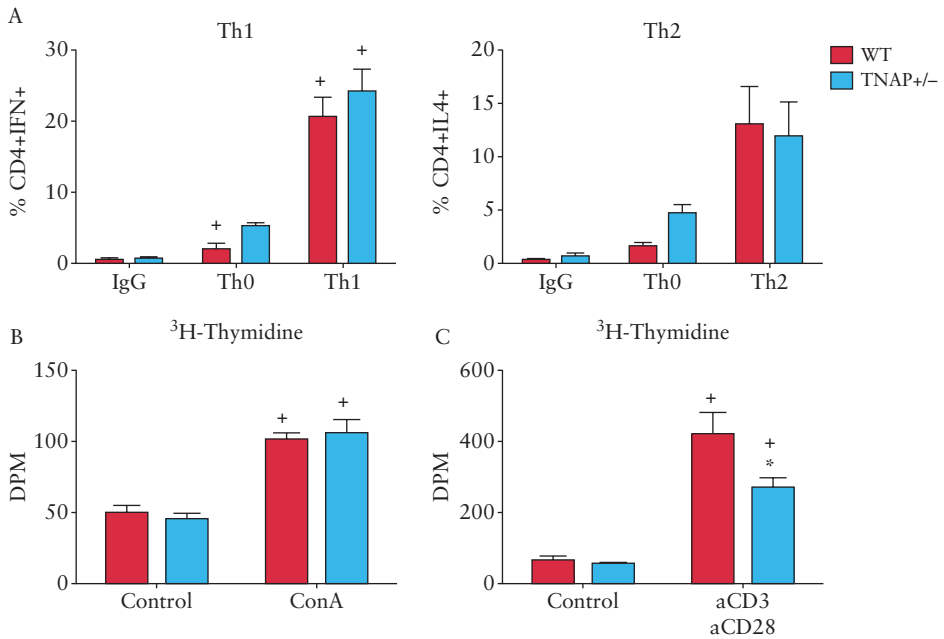

Figure 13. T cell differentiation and proliferation assay. [A] Th1 and Th2 polarisation. [B] ^{3}H-thymidine proliferation assay in T cells stimulated with ConA [5 μg/ml]. [C] ^{3}H-thymidine proliferation assay in T cells stimulated with a-CD3/a-CD28. *$p < 0.05$ vs WT; $^{+}p < 0.05$ vs control. ConA, concanavalin A; WT, wild type.

in T cells of WT mice receiving anti-CD3 antibody, but not in those from TNAP$^{+/-}$ mice. Similarly, TNAP was induced in LPS-stimulated splenocytes but much less so in TNAP$^{+/-}$ cells. This induction was replicated in a-CD3/a-CD28-stimulated T cells *in vitro*, but in this case AP activity was not significantly different [$p = 0.1$] in WT and TNAP$^{+/-}$ cells. In turn, the AP activity in WT T lymphocytes was clearly levamisole-sensitive, whereas that of TNAP$^{+/-}$ cells was not, consistent with our findings in other organs. TNAP expression in T lymphocytes has been previously documented,[21] and the resulting enzymatic activity is reportedly comparable to that of liver, i.e. relatively low, resistant to inhibition by levamisole, which actually activates enzymatic activity slightly at high concentrations [see Supplementary Figure 3].[23] Latheef *et al.*[21] failed to detect an increase in TNAP gene expression or activity in murine T cells after ConA stimulation *in vitro*. The reason for this discrepancy is unknown but may be related to a relatively low degree of stimulation [compared with a-CD3/a-CD28].

The shift in T cell function had significant consequences *in vivo*. Administration of anti-CD3 antibodies to mice results in stimulation of T cells in the short term with enhanced release of pro-inflammatory cytokines, a rapid depletion of blood T lymphocytes, and a long-lasting immunosuppresant effect.[33,34] In WT mice, this resulted in lymphopenia and marked upregulation of IFN-γ, IL-4, IL-5, and other cytokines. In contrast, TNAP$^{+/-}$ mice exhibited an attenuated lymphopenia and cytokine production, consistent with reduced T cell activation and/or enhanced T cell depletion in this context. Next, we assessed the impact of T cell TNAP on T cell-dependent colitis using the adoptive T cell transfer model of colitis. Transfer of TNAP$^{+/-}$ T cells resulted in colitis of reduced severity compared with WT cells, consistent with a lower activation of the adaptive immune system. One striking feature of this experiment was the observation that Rag1$^{-/-}$ mice transferred with the noF unprocessed [rather than naïve] T cell population had a dramatically lower number of lymphocytes, monocytes, and neutrophils in their blood compared with the recipients of WT cells, the relative proportions staying the same. This was not the case with mice receiving CD4^{+} CD62L^{+} cells, whose haemogram was basically normal, except for a minor decrease in percentage of lymphocytes. Since Rag1$^{-/-}$ mice are devoid of both mature T and B lymphocytes, they normally show a greatly reduced lymphocyte count in the blood, so that those measured in the haemogram of colitic mice correspond chiefly to the expansion of transferred cells. Therefore, our results suggest that the unfractionated TNAP$^{+/-}$ T cells fail to colonise Rag1$^{-/-}$ mice, consistent with a low proliferative capacity, whereas this is compensated in mice receiving the TNAP$^{+/-}$ naïve cells by their reactivity toward the microbiota and the resulting colitis.

Our results do not allow us to establish the mechanism accounting for the phenotype of TNAP$^{+/-}$ T lymphocytes. However, they indicate that activated T cells undergo a shift in TNAP expression, with increased activity and augmented sensitivity to levamisole, features that are attenuated in TNAP$^{+/-}$ cells. The fact that treatment of WT T cells with three different AP inhibitors recapitulates the depressed cytokine production and proliferative response observed in TNAP$^{+/-}$ T lymphocytes strongly suggests that this phenotype is directly related to inhibition of enzymatic activity. Consistent with the other organs of TNAP$^{+/-}$ mice, a difference in TNAP glycosylation may be inferred in T cells, since inhibition by levamisole is glycosylation-dependent; resistance to inhibition by this probe in activated TNAP$^{+/-}$ T cells and recapitulation of the phenotype by levamisole and the other inhibitors would suggest that the liver type of TNAP predominates in these conditions. However, confirmatory studies are warranted in this regard. In particular, we did not assess the expression of other AP forms, which may also affect both AP activity and sensitivity to levamisole.

Increased TNAP may arguably modulate T cell function by dephosphorylating relevant signalling mediators, such as nucleotides, thereby modulating purine receptor signalling,[14] or osteopontin. Our results suggest that changes in substrate specificity brought about by differential glycosylation may account for altered T cell activation, but this remains speculative until the corresponding *in vitro* experiments are carried out. It is intriguing, however, that Spp1 [encoding osteopontin] mRNA levels are markedly different in WT and TNAP$^{+/-}$ T cells. Osteopontin is one of the physiological targets of TNAP and its activity, which includes immunomodulatory effects and is regulated by phosphorylation.[35–37] Among T cells, basal osteopontin expression appears to be low except in the case of memory cells. Secreted osteopontin has both pro-inflammatory and anti-inflammatory roles, although the former appear to predominate.[35] Also worth noting is the observation that Klf4 and Spib, involved in T cell proliferation, tend to be downregulated in TNAP$^{+/-}$ T cells. Whatever the mechanism, TNAP has been previously shown to be involved in cell proliferation[14] and adipokine expression[18] in neural and adipose cells in culture, respectively. Experiments are under way to characterise the role of TNAP induction in T cell function and T cell-dependent colitis.

Our results offer clear evidence that TNAP modulates T lymphocyte function and specifically T cell-dependent colitis. T cells exhibit a depressed response to activation stimuli if they express a levamisole-resistant TNAP [TNAP$^{+/-}$ T cell] or if treated with TNAP inhibitors, suggesting that levamisole-sensitive TNAP is required for full activation. Such modulation of TNAP properties is attributable to changes in glycosylation.

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Conflict of Interest

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Author Contributions

CHC and RGB were primarily responsible for acquisition of data and statistical analysis. OMA and FSM did the study concept and design. CHC and RGB drafted the manuscript. All authors participated in the analysis and interpretation of data and general revision of the paper.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online.

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