STAT3 is a critical cell intrinsic regulator of human unconventional T cell numbers and function

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Non-standard abbreviations:
AD-HIES Autosomal dominant hyper IgE syndrome
MAIT - mucosal-associated invariant T
MR1 - MHC-related molecule 1
iNKT – invariant natural killer T
$STAT3^{MUT}$ – STAT3 mutant
TAE - T cell activation and expansion beads

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Abstract

Unconventional T cells such as γδ T cells, natural killer T (NKT) cells and mucosal-associated invariant T (MAIT) cells are a major component of the immune system, however, the signalling pathways that control their development and function remain unclear. Autosomal dominant hyper IgE syndrome (AD-HIES) is a primary immunodeficiency caused by heterozygous dominant negative mutations in *STAT3* and characterised by recurrent infections with *Candida albicans* and *Staphylococcus aureus*. Here, we found that STAT3 deficiency caused a decrease in MAIT and NKT, but not γδ T, cell numbers in the blood. Analysis of *STAT3* mosaic individuals revealed that this effect was cell intrinsic. Surprisingly, the residual STAT3-deficient MAIT cells expressed normal levels of the transcription factor RORγt. Despite this they displayed a deficiency in secretion of IL-17A and IL-17F, but were able to secrete normal levels of cytokines such as IFNγ and TNFα. **Detailed analysis of cytokine receptor expression, STAT activation and individuals with loss of function mutations in *IL12RB1* and *IL21R* revealed that IL-23R/STAT3 signalling was required for maintenance of MAIT cells while IL-21R/STAT3 controlled NKT cell numbers.**

Introduction

Unconventional T cells possess unique properties that set them apart from conventional T cells. These include limited TCR diversity and a pre-activated phenotype that allows them to respond quickly with cytokine production upon activation and recognition of Ags that are not presented by classical MHC molecules. These unconventional T cell populations include γδ T cells, mucosal associated invariant T (MAIT) cells and natural killer T (NKT) cells. These T cells, particularly MAIT and γδ T cells, are a significant component of the human immune system, comprising up to 15% of lymphocytes in peripheral blood and in mouse models have been show to provide protection against multiple pathogens (Chien et al., 2014; Gold and Lewinsohn, 2013). However, our knowledge of their precise role in protective immunity in humans and the molecular mechanisms regulating their development, activation and function is limited.

Several distinct populations of NKT cells have been identified, the best studied of which are the type I NKT (also called invariant or iNKT) cells that in humans typically express an invariant Vα24-Jα18 TCR α-chain paired with the Vβ11 TCR β-chain. These iNKT recognise self, synthetic and bacterial glycolipids presented by CD1d (Rossjohn et al., 2012). The other NKT cell populations (type II and atypical) express a more diverse TCR repertoire (Rossjohn et al., 2012) and do not respond to the prototypical Type I NKT cell ligand, α-galactosylceramide, making them more difficult to identify and thus study. As their name suggests, MAIT cells are enriched in the mucosa. They typically express the invariant Vα7.2-Jα33 TCRα chain and are restricted by the MHC-related molecule 1 (MR1) (Gold and Lewinsohn, 2013). MR1 binds Vitamin B metabolites, which are produced by multiple microorganisms including *C. albicans* and *S. aureus*, and present them to MAIT cells (Kjer-Nielsen et al., 2012). γδ T cells are more diverse than type I NKT or MAIT cells with humans possessing 8 Vδ and 6 Vγ functional TCR chains. The γδTCRs can bind a range of Ags and Ag presenting molecules, including CD1 and MHC-I stress-induced molecules (Bonneville et al., 2010; Chien et al., 2014). Certain chains

seem to be preferentially expressed and paired. Thus, in the blood approximately 70% of γδ T cells express Vγ9Vδ2 and the remainder are mostly Vδ1$^+$ (Bonneville et al., 2010; Chien et al., 2014; Parker et al., 1990). These different δ chain-expressing sub-populations have been associated with immune responses to different pathogens. For example, δ2$^+$ cells can respond to *M. tuberculosis* (Li et al., 1996), while δ1$^+$ cells are involved in responses to *C. albicans* (Fenoglio et al., 2009). Despite differences in TCR gene usage, and recognition of distinct Ag via different MHC-I-like molecules, a common feature of these populations of unconventional T cells is their ability to promptly produce a broad range of effector cytokines such as IFNγ, IL-4, IL-17 and IL-21 following activation (Bonneville et al., 2010; Chien et al., 2014; Dusseaux et al., 2011; Gold and Lewinsohn, 2013; Rossjohn et al., 2012).

Autosomal dominant hyper IgE syndrome (AD-HIES) is a primary immunodeficiency characterised by elevated serum IgE, eczema and susceptibility to a narrow and well-defined spectrum of pathogens. Patients suffer from recurrent skin and lung abscesses caused by *S. aureus* as well as chronic mucocutaneous infections due to *C. albicans* (Chandesris et al., 2012). AD-HIES results from heterozygous loss of function mutations in the gene encoding the transcription factor STAT3 (Holland et al., 2007; Minegishi et al., 2007). STAT3 signals downstream of many key cytokine receptors including those for IL-6, IL-10, IL-21 and IL-23 as well as growth hormones and IFNγ (Kane et al., 2014). Studies in patients with AD-HIES have revealed multiple roles for STAT3 in cells of the adaptive immune system. For example, STAT3 signalling is crucial for the differentiation of naïve CD4$^+$ T cells into Th17 cells (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008). This deficiency in Th17 cells is thought to explain, at least in part, the susceptibility of AD-HIES patients to *C. albicans* and *S. aureus* as IL-17 is crucial for host defence against these pathogens (Cypowyj et al., 2012; Puel et al., 2011).

Human unconventional T cells have been reported to recognise *C. albicans* and *S. aureus*, and mouse models support a role for these cells in immunity against these pathogens. Further, unconventional T cells produce IL-17 and express many cytokine receptors that signal through STAT3 (Constantinides and Bendelac, 2013; Cua and Tato, 2010; Gold and Lewinsohn, 2013). However, the role of STAT3 in the development and function of human unconventional T cells, and how they may contribute to the clinical phenotype of AD-HIES, has not previously been assessed. Thus we investigated the development and function of MAIT, NKT and γδ T cells in patients with mutations in STAT3, **as well as in patients with mutations in the STAT3-activating cytokine receptors IL-12Rß1 and IL-21R.**

Results

Functional STAT3 deficiency causes a decrease in MAIT and NKT cells *in vivo*

To determine whether functional STAT3 deficiency affects the numbers of circulating unconventional T-cells, we analysed peripheral blood mononuclear cells (PBMCs) from AD-HIES patients with *STAT3* mutations and normal controls. This showed a 20-fold reduction in iNKT ($CD3^+V\alpha24^+V\beta11^+$) cells in STAT3 mutant individuals (Fig 1A). Similarly, we observed a 4-fold decrease in the percentage of MAIT cells as identified both by expression of the invariant Vα7.2 TCRα chain and high levels of CD161 (Fig 1B) or by staining with MR1 tetramers loaded with 5-OP-RU, the potent riboflavin metabolites recognized by MAIT cells (Corbett et al., 2014; Reantragoon et al., 2013) (Fig. 1 C). This dramatic decrease in NKT and MAIT cells suggests that STAT3 regulates the generation and/or survival of both of these unconventional T cell populations.

In contrast, the frequency of γδ T cells in the blood was not significantly different between normal controls and STAT3-deficient individuals (Fig. 1 D). As the different TCRδ chains are associated with responses to different pathogens (Chien et al., 2014; Fenoglio et al., 2009; Li et al., 1996), we also examined the relative proportions of $\delta2^+$ and $\delta1^+$ T cells to ascertain whether STAT3 deficiency selectively affects a particular subpopulation of γδ T cells. However, our analysis showed that STAT3 deficiency had no significant effect on the percentage of δ1 or δ2 expressing T cells (Fig. 1 E, F), suggesting that STAT3 is not critical for the development or survival of γδ T cells *in vivo*.

A cell intrinsic requirement for STAT3 in unconventional T cells

While our results clearly show that *STAT3* mutations result in a decrease in iNKT and MAIT cells, it does not necessarily follow that there is a cell intrinsic requirement for STAT3 in the survival or development of these cells. Indeed, the reduction in these cells could result from a defect in supporting cells that require STAT3 or as a result of the prolonged chronic infection associated with AD-HIES. To delineate whether the reduction in iNKT and MAIT cells in AD-HIES is due to a cell-intrinsic function of STAT3, we took advantage of two individuals who have been identified to have an

intermediate AD-HIES phenotype resulting from somatic mosaicism at one *STAT3* allele (Hsu et al., 2013; Siegel et al., 2011). In these individuals approximately half of their immune cells express the mutant *STAT3* genotype while the other half express the wild type (WT) genotype and thus have normal functioning STAT3 (Hsu et al., 2013; Siegel et al., 2011). Samples from these individuals offer the unique opportunity to examine STAT3 deficient and normal unconventional T cells in the same environment, thereby allowing the comparison of cell extrinsic and intrinsic effects of *STAT3* mutations. Thus, if STAT3 has a critical intrinsic role in development or survival of unconventional T cells, cells with the WT genotype will have a distinct selective advantage and would therefore comprise a far greater proportion of the circulating T cells. To test this hypothesis, unconventional T cell populations were sorted from total PBMCs from the two *STAT3* mosaic individuals and the frequency of expression of the WT and mutant *STAT3* alleles determined by qPCR. In these mosaic individuals it has previously been observed that conventional $CD4^+$ and $CD8^+$ memory T cells have a cell intrinsic dependency on STAT3; in contrast naïve T cells do not (Siegel et al., 2011). Therefore, we included these cell types as controls. As expected the population of naïve T cells were composed of approximately equal numbers of cells expressing each genotype while the memory T cell populations were composed almost entirely of cells with the WT genotype (Fig 2A). Strikingly, the unconventional T cell populations were composed almost entirely of WT cells (Fig. 2A). We confirmed the enrichment of the WT allele in these populations from one mosaic individual by Sanger sequencing of genomic DNA at the position of the 1145G>A SNP (Fig. 2 B). Traces from the healthy control showed only the WT G at positing 1145 whereas the trace from a AD-HIES patient harbouring this same mutation in the germline showed equal G and A peaks consistent with the heterozygous mutations in AD-HIES. While traces from the unconventional T cells showed the same genotype as the WT control with no A peak visible at the mutation point, naïve $CD4^+$ and $CD8^+$ T cells were clearly mosaic at this nucleotide, confirming the results of the qPCR. Together these results unequivocally demonstrate that STAT3 functions as a crucial intrinsic mediator of unconventional T cell frequency.

STAT3-deficient MAIT cells express normal levels of RORγt and PLZF

Given the role of STAT3 in regulating the numbers of iNKT cells and MAIT cells we considered whether STAT3 might control expression of transcription factors important for the development of these unconventional T cells. PLZF is a transcription factor highly expressed by both MAIT and NKT cells, which controls the development and pre-activated phenotype of these cells (Eidson et al., 2011; Kovalovsky et al., 2008; Savage et al., 2008). Thus, we determined whether the levels of this molecule were altered in STAT3-deficient MAIT cells. Consistent with previous results (Savage et al., 2008), intracellular staining revealed that MAIT cells from normal controls expressed significantly more PLZF than other T cells (Fig 3A). Strikingly, STAT3-deficient MAIT cells expressed similar levels of PLZF as normal controls. Due to the paucity of iNKT cells in STAT3 deficient patients, we were not able to determine the levels of PLZF in these cells.

RORγt is a transcription factor key to the differentiation of Th17 cells and IL-17 production (Ivanov et al., 2006), however, is also highly expressed by MAIT cells (Dusseaux et al., 2011). Furthermore, induction of RORγt expression in $CD4^+$ T cells is dependent on STAT3 such that patients with AD-HIES lack $CD4^+$ Th17 cells *in vivo* and naïve $CD4^+$ T cells from these individuals fail to upregulate RORγt and produce Th17-type cytokines following *in vitro* polarisation under Th17 culture conditions (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008). Thus we reasoned that STAT3 deficiency might also ablate RORγt expression in MAIT cells leading to their decrease in AD-HIES patients. However, intracellular staining revealed that healthy control and STAT3-deficient MAIT cells expressed similar levels of RORγt (Fig 3B). **This surprising finding that STAT3-deficient MAIT cells expressed normal levels of RORγt was confirmed by qPCR for *RORC* transcripts (Fig 3C) as well as by intracellular staining for RORγt using a different clone (data not shown).** We also observed no significant difference in expression of other transcriptional regulators of T cell differentiation, such as Eomesodermin and Tbet, between STAT3-deficient and control MAIT cells (data not shown). Thus it is unlikely that the decreased frequency of MAIT cells (and by extension

NKT cells) is due to a deregulation in either PLZF or RORγt. Rather it is likely that the deficiency of NKT and MAIT cells stems from defects in survival or expansion of these cells.

Furthermore these results suggest that, unlike $CD4^+$ Th17 differentiation, RORγt is acquired independently of STAT3 in MAIT cells. In mice, populations of $ROR\gamma t^+$ $IL\text{-}17^+$ NKT and γδ T cells have been described that appear to be pre-programmed in the thymus (Coquet et al., 2008; Rachitskaya et al., 2008; Shibata et al., 2011) and at least for γδ T cells this has been shown to occur independently of STAT3 signalling (Shibata et al., 2011). Thus, it is possible that a similar STAT3–independent RORγt upregulation occurs during thymic development of human MAIT cells.

STAT3 is required for IL-17 secretion by unconventional T cells

The normal expression of RORγt by STAT3-deficient MAIT cells, coupled with normal levels of other markers of IL-17-producing cells such as CD161 and CCR6 (Fig 1B and data not shown) led us to investigate whether these cells were also capable of expressing normal levels of IL-17. To address this, $\delta 2^-$ and $\delta 2^+$ γδ T cells, MAIT cells and **total** $CD4^+$ and $CD8^+$ T cells were sorted from patients and normal controls and stimulated with PMA/ionomycin. iNKT cells could not be analysed due to their rarity both in normal controls but more strikingly in AD-HIES patients. Consistent with previous reports (Dusseaux et al., 2011), both MAIT cells and $CD4^+$ T cells from normal donors produced low levels of IL-17A. Importantly, this IL-17A production was abrogated by STAT3 deficiency (Fig. 4A) suggesting that STAT3 plays a crucial role in inducing IL-17 not only in conventional $CD4^+$ T cells but also in MAIT cells. This reflected a specific defect in producing IL-17A, rather than a general dysfunction in cytokine secretion because production of other cytokines such as IFNγ, TNFα, Granzyme A and B and IL-2 (Fig 4B-D and data not shown) was comparable for STAT3 mutant and normal T cell subsets. We also analysed cytokine secretion by γδ T cell populations to determine whether a human equivalent of the thymically-programmed IL-17 producing population could be identified. However we detected little IL-17 production from either γδ T cell population (Fig. 4A).

Whilst we found that all T cells secreted high levels of IFNγ and Granzyme B (Fig 4B, D) in response to PMA/ionomycin the levels of IL-17A and IL-17F secreted were very low to absent (Fig. 4A and data not shown). The STAT3 activating cytokines IL-6 and IL-23, in combination with TGFβ and IL-1β, can induce the differentiation of $CD4^+$ and γδ T cells to an IL-17-secreting phenotype (Acosta-Rodríguez et al., 2007; Ness-Schwickerath et al., 2010; Wilson et al., 2007). To determine whether these cytokines also promoted IL-17 production from human MAIT cells, and if STAT3 plays a role in this polarisation/**enhancement** in unconventional T cells, sorted T cells ($\delta2^-$ and $\delta2^+$ γδ T, MAIT and as controls **total** $CD4^+$) were cultured for 5 days with T cell activation and expansion beads (TAE; beads expressing anti-CD3, anti-CD28 and anti CD2 mAb) alone or together with Th17-polarising stimuli. The cells were also cultured under Th1 polarising condition as a control for intact responsiveness to alternative differentiation stimuli. As shown previously (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008) Th17 culture conditions induced high levels of IL-17A and IL-17F secretion from $CD4^+$ T cells and this response was ablated in STAT3 deficient cells (Fig 5A,B). The Th17 culture also stimulated high levels of IL-17F and a lower level of IL-17A by MAIT cells and this response was severely reduced in STAT3-deficient cells (Fig 5A, B). The level of IL-17 secretion by γδ T cells also appeared to be reduced in STAT3 deficient cells however this difference was not statistically significant, possibly due to the large variability amongst γδ T cells even from normal donors.

We also examined secretion of IFNγ and Granzyme B (Fig 5 C,D). Interestingly, in contrast to the normal secretion of IFNγ observed from STAT3-deficient $\delta2^+$ γδ T and MAIT cells in response to PMA/ionomycin stimulation (Fig 4B), both of these cells types displayed decreased IFNγ secretion in the non-polarising culture containing TAE beads alone (Fig 5C). However, this impairment was rescued in the Th1 and Th17 cultures. The $\delta2^+$ γδ T also showed decreased Granzyme A and B expression (Fig. 5D and data not shown) in the Th0 cultures which once again was rescued by addition of IL-12. It is not clear what the mechanism underlying this defect in IFNγ and Granzyme secretion is as the normal production in response to PMA/ionomycin (Fig. 4) coupled with normal expression of T-

bet (data not shown) suggests that the in vivo differentiation of these cells to a Th1-like phenotype is intact. Instead it may reflect the requirement for a STAT3-dependent response to endogenously produced STAT3-activating cytokines in the Th0 cultures. To determine whether STAT3 deficiency also affected T cell survival or expansion we enumerated the number of live cells after 5 days of culture (Fig. 5E). We saw no differences in the number of cells recovered between normal controls and STAT3 deficient cultures for any cell type (Fig. 5E). However, whilst we observed a significant increase in cell numbers over the starting 20,000 cells in cultures of $CD4^+$ and $\delta2^-$ γδ T cells, consistent with substantial proliferation, very few cells were recovered from cultures of $\delta2^+$ T and MAIT cells. **Flow cytometric analysis of forward scatter of cells from these cultures also demonstrated that only $CD4^+$ and $\delta2^-$ γδ T cells showed characteristics of cells undergoing blastogenesis and proliferation (data not shown). Indeed CFSE labelling of the different populations confirmed that $CD4^+$ and $\delta2^-$ γδ T cells, but neither MAIT nor $\delta2^+$ γδ T cells, underwent proliferation following 5 days in culture (data not shown).** Remarkably, although we recovered approximately 30-40-fold less cells from cultures of MAIT cells compared to $CD4^+$ T cells, the level of IFNγ and IL-17F secretion was only 2- and 4-fold lower respectively, suggesting that MAIT cells have a much higher potential for producing these cytokines than $CD4^+$ T cells. **Indeed, intracellular cytokine staining of cells from day 5 cultures revealed that a substantially higher (3-5 fold) percentage of MAIT than $CD4^+$ T cells were positive for IFNγ and/or IL-17A production (data not shown).**

Together these results reveal that STAT3 deficiency leads to a loss of IL-17 production by all T cell populations. It is of particular interest that although STAT3-deficient MAIT cells expressed many features of IL-17 producing cells including CD161 and CCR6 and most importantly high levels of the Th17 master regulator RORγt they were still unable to secrete appreciable levels of IL-17. This suggests that while RORγt can be upregulated independently of STAT3 in these cells, possibly during thymic development, the action of RORγt alone is not sufficient to drive transcription of the *IL17A* and *IL17F* genes and thus induce their secretion. This is presumably due to a requirement for STAT3 to act directly at the promoters of these genes (Durant et al., 2010).

These studies also provide further insight into the clinical phenotype of AD-HIES, which is characterised by recurrent infection with *C. albicans* and *S. aureus* (Kane et al., 2014). Control of both of these infections has been associated with the actions of IL-17A and IL-17F (Cypowyj et al., 2012; Puel et al., 2011). Indeed, previous work demonstrated a lack of IL-17 producing $CD4^+$ T cells in STAT3 deficient individuals (de Beaucoudrey et al., 2008; Ma et al., 2008; Milner et al., 2008) suggesting this defect in $CD4^+$ T cell differentiation contributed to the lack of control of pathogen infection. Importantly, MAIT cells can be activated by *C. albicans* and *S. aureus* as both of these microorgansims generate the riboflavin metabolites that are specifically recognised by these cells (Kjer-Nielsen et al., 2012). Further, γδ T cells have also been implicated in responses to these organisms (Cho et al., 2010; Cua and Tato, 2010; Ness-Schwickerath et al., 2010). Thus our results that STAT3-deficient MAIT and γδ T cells are also unable to produce IL-17 broaden these findings and suggest that functional and numerical defects in unconventional T cells also contribute to the infectious susceptibility observed in AD-HIES. Together these data reinforce the significance of unconventional T cells and IL-17 in the immune system and underscores the essential role STAT3 plays in their action.

IL-21 and IL-23 activate STAT3 in human MAIT cells and are required for the maintenance of innate-like T cells

STAT3 can be activated by multiple cytokines including IL-6, IL-10, IL-21 and IL-23 (Kane et al., 2014). Thus we sought to identify which of these cytokine signalling pathways may be active in MAIT and NKT cells. We assessed expression of IL-6R, IL-12Rß1 and IL-21R and found prominent expression of IL-12Rß1 and IL-21R on unstimulated MAIT and NKT cells; however these cells lacked detectable expression of IL-6R (Fig 6A). This suggested that these cells could potentially respond to IL-12, IL-23 (both of which share IL12-Rß1) and IL-21. To test this, we next examined STAT phosphorylation in sort-purified MAIT cells that were pre-activated for 48 hours. IL-12 induced phosphorylation of only STAT4, and IL-21 induced phosphorylation of STAT3 (Fig 6B). However, IL-23 induced robust activation of both STAT3 and STAT4, implying

IL-12Rß1 is being utilised by IL-23 - rather than IL-12 - to initiate STAT3-dependent signalling in human MAIT cells. To determine whether signalling through IL-21R/STAT3 or IL-23R/STAT3 was important for controlling MAIT and iNKT cells numbers we enumerated these populations in patients with loss-of function mutations in *IL21R* or *IL12RB1* (Fig 6C,D). We also examined *IFNGR1*-deficient individuals as an additional control for immune deficient patients. This revealed intact numbers of MAIT and iNKT cells in *IFNGR1*-deficient patients, but striking deficiencies of MAIT cells in individuals with *IL12RB1* mutations (Fig. 6C) and of iNKT cells in *IL21R*-deficient patients (Fig 6D). Indeed, the proportions of MAIT and iNKT cells in these patients approximated those in STAT3-deficient individuals, implicating IL-23 and IL-21 as the major cytokines responsible for the STAT3-dependent maintenance of human MAIT and iNKT cells, respectively (Fig 6C, D). Importantly, ~25% of patients with *IL12RB1* mutations present with Candida infections (de Beaucoudrey et al., 2010) consistent with a role for MAIT cells in protective immunity to Candida. Together, these data identify for the first time the distinct cytokine-induced STAT3-signalling pathways responsible for regulating MAIT and iNKT cells in humans. Importantly, identification of these pathways potentially paves the way for manipulating the frequencies of these cell types to modulate immune responses in the setting of infection with specific pathogens.

Methods

Human blood samples.

Buffy coats from normal donors were obtained from the Australian Red Cross Blood Service. Peripheral blood was also collected from patients with mutations in *STAT3* (Chandesris et al., 2012; Deenick et al., 2013; Holland et al., 2007), ***IL12RB1*** **(de Beaucoudrey et al., 2010),** ***IL21R*** **(Deenick et al., 2013; Ives et al., 2013; Kotlarz et al., 2013; Stepensky et al., 2014) or** ***IFNGR1*** **(Dorman et al., 2004)** and from individuals with somatic mosaicism for *STAT3* mutations (Hsu et al., 2013; Siegel et al., 2011). PBMCs were prepared by centrifugation using Ficoll-Paque. Approval for this study was obtained from human research ethics committees of the St. Vincent's Hospital and Sydney South West Area Health Service (Australia), the Rockefeller University Institutional Review Board (New York), and the National Institute of Allergy and Infectious Diseases Intramural Institutional Review Board in Bethesda, MD. All participants gave written informed consent in accordance with the Declaration of Helsinki.

Flow cytometry reagents

PerCp-Cy5.5 anti-CD161, PE anti-RORγt, PE anti-γδ TCR, biotinylated anti-γδ TCR, PerCp-Cy5.5 anti-CD45RA and APC anti-αβ TCR antibodies were purchased from eBioscience. A647 anti-PLZF, APC-Cy7 anti-CD4, PE-Cy7 anti-CD8, BV421 anti-CD3, BV421 anti-CD161, **PE anti-STAT4 (pY693), PE anti-IL-6R, PE anti-IL-12Rß1, PE anti-IL21R, PE mouse IgG1 isotype control, PerCP-Cy5.5 anti-STAT3 (pY705)** and FITC anti-Vδ2 TCR antibodies were purchased from BD. FITC anti-Vα24 TCR and PE **and biotinylated** anti-Vβ11 antibodies were purchased from Beckman Coulter. FITC anti-Vδ1 TCR was purchased from ThermoFisher Scientific. APC or APC-Cy7 anti-Vα7.2 and PE-Cy7 anti-αβ TCR antibodies were purchased from Biolegend. FITC anti-CCR7 antibody

was purchased from R&D systems. MR1–5-OP-RU tetramers have been described previously (Corbett et al., 2014; Reantragoon et al., 2013).

Cell sorting

PBMCs were stained with antibodies and sorted as below using a BD FACS ARIA or ARIA III. For SNP analysis MAIT cells were sorted as $CD3^+Vα7.2^+CD161^{hi}γδ^-$, NKT cells as $CD3^+Vα24^+Vβ11^+$, γδ T cells as $CD3^+Vα7.2^-γδ^+$ $δ2^{+/-}$, $CD4^+$ and $CD8^+$ naive T cell populations as $CD45RA^+CCR7^+$ with the remaining cells sorted as memory cells. For cell culture MAIT cells were sorted as $CD3^+Vα7.2^+CD161^{hi}γδ^-$, γδ T cells as $CD3^+Vα7.2^-γδ^+$ and either $δ2^+$ or $δ2^-$, **total** $CD4^+$ T cells as $CD3^+Vα7.2^-γδ^-CD8^-CD4^+$ and **total** $CD8^+$ T cells as $CD3^+Vα7.2^-γδ^-CD4^-CD8^+$.

Cell culture

Sorted cells were cultured at 20,000 cells per well in a total volume of 100µl media (RPMI 1640 supplemented with 10% FCS,10 mM HEPES pH 7.4, 0.1 mM non-essential amino acid, 1 mM sodium pyruvate 60 µg/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml bovine apo-transferrin [all from Life Technologies, Invitrogen] and 20 µg/ml NormocinTM [InvivoGen, San Diego, CA, USA]). The cells were stimulated with T-cell activation and expansion (TAE) beads (anti-CD2/CD3/CD28; Miltenyi Biotec; concentration of 1 bead: 2 cells) alone (Th0 culture) or under Th1 (IL-12 [20 ng/ml; R&D systems]) or Th17 (TGFβ [2.5ng/mL], IL-1β [20 ng/ml; Peprotech], IL-6 [50 ng/mL; PeproTech], IL-23 [20 ng/ mL; eBioscience]) polarizing conditions for 5 days. In the PMA/ionomycin stimulation experiments cells were cultured with 100 ng/ml PMA and 750 ng/ml ionomycin (both from Sigma Aldrich) for 3 days before harvesting supernatants. Cytokine secretion was measured by cytometric beads array (CBA; BD) as per the manufacturer's instructions. The absolute number of cells in culture at each time was determined by adding a known number of CaliBRITE beads (BD) to cultures before harvest, which can then be distinguished by flow cytometry based on forward and side scatter.

Intracellular transcription factor staining

Cells were stained for cell surface markers to identify γδ T and MAIT cells. The cells were then permeabilised using the BD transcription factor buffer set and stained with PLZF or RORγt-specific mAbs.

SNP Assay

Genomic DNA was extracted from sorted cells by QIAGEN DNeasy mini spin column kit in accordance with manufacturer guidelines. Percent mosaicism was determined in different cell populations using the TaqMan SNP genotyping assay (Life Technologies) as described previously (Hsu et al., 2013). Briefly this qPCR based assay utilised primers for the region of DNA containing the disease-causing point mutation, together with specific probes that recognise either the WT or disease SNPs. The difference between the cycle threshold for each probe was used to determine the proportion of the two alleles in each sample. In each assay a normal control and an AD-HIES sample with the same point mutation were used to normalise the data with the normal control taken as 0% and the AD-HIES sample taken as 100%.

DNA sequencing

Exons 12-14 of *STAT3* were amplified from genomic DNA using specific primers (Forward: TAG TTT AAA GAA ATG CCC AGG AGC ACA GAG and Reverse: TTG GCC TAA GTG ACT TTT TGG AAT AAC TAC AGC). Sanger sequencing of the amplified product was performed by Garvan Molecular Genetics.

qPCR

RNA was isolated from sorted populations using RNeasy kit (Qiagen, Hilden, Germany). For quantitative PCR, total RNA was reverse transcribed with oligo-dT. Expression of genes was

determined using real-time PCR with the LightCycler 480 Probe Master Mix and System (Roche, Mannheim, Germany) as described previously (Ives et al., 2013).

Expression of phospho-STATs

Sorted MAIT cells ($CD3^+V\alpha7.2^+CD161^+$) and non-MAIT T cells ($CD3^+V\alpha7.2^-CD161^-$) were stimulated with TAE beads for 2 days, then rested for two hours in media before being stimulated with IL-12 (50ng/ml), IL-21 (100ng/ml) or IL-23 (100ng/ml) or media alone for 20 minutes. Cells were fixed, permeabilized and stained with anti–phospho- STAT3 (pY705), and STAT4 (pY693) mAbs (Deenick et al., 2013; Ives et al., 2013).

Statistical analysis. Significant differences between datasets were determined using either the unpaired Student's t test when comparing two variables or two-way ANOVA with Sidak's multiple comparison test for more than two variables (Prism; GraphPad Software).

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Figure Legends

Figure 1. Mutations in STAT3 result in decreased NKT and MAIT cell numbers.

PBMCs from normal controls or patients with *STAT3* mutations ($STAT3^{MUT}$) were stained for the presence of (A) iNKT cells ($TCRV\alpha24^+$ $V\beta11^+$), (B, C) MAIT cells [$CD3^+V\alpha7.2^+$ $CD161^+$ (B) or $CD3^+$ cells binding the MR1–rRL-6-CH_2OH tetramers (C)], (D) total γδ T cells, as well as the (E) $\delta2^+$ and (F) $\delta1^+$ subsets. Representative staining of total lymphocytes (A,C,D), $CD3^+$ T cells (B) or γδ T cells (E,F) is shown on the left. Numbers represent the mean ± SEM of the percentage of lymphocytes (A-D) or γδ T cells (E,F). Graphs show combined data with each symbol representing a single normal control (n=11-78) or patient (n=7-23).

Figure 2. Circulating unconventional T-cells in individuals mosaic for STAT3 mutations all express the wild type genotype.

(A) qPCR was carried out on DNA from sorted T cell populations from individuals with *STAT3* mosaicism using probes specific for the wild type or disease-causing genotype. Different symbols represent the two different individuals examined. (B) Exons 12-14 of the *STAT3* gene were amplified from genomic DNA from total PBMCs from a normal donor (WBC) or a STAT3-deficient patient (AD-HIES), or from the indicated populations of sorted cells from a mosaic individual with the 1145G>A *STAT3* mutation. The chromatograms depict heterozygosity of the STAT3-deficient patient at this SNP and different degrees of mosaicism in distinct cell lineages in the mosaic individual.

Figure 3. STAT3-deficient MAIT cells express normal levels of RORγt and PLZF

PBMCs from normal controls or $STAT3^{MUT}$ patients were stained with mAbs to identify MAIT cells or total T cells and then expression of PLZF (A) and RORγt (B) in these cells was determined by intracellular staining. Representative histogram plots of staining for MAIT or T cells ($CD3^+V\alpha7.2$-$\gamma\delta^-$).

Graphs show mean ± SEM (n=6-10) of the MFI corrected relative to the MFI of MAIT cells from normal controls in each experiment. **(C) Expression of *RORC* transcripts was determined by qPCR of sorted T cell populations (n=5-9).**

Figure 4. STAT3-deficient T cells are impaired in their ability to produce IL-17A

Unconventional T cell populations ($\delta2^-$ and $\delta2^+$ γδ T cells and MAIT cells) as well as total $CD4^+$ and $CD8^+$ T cells were isolated from total PBMCs from normal controls or $STAT3^{MUT}$ patients by cell sorting and stimulated with PMA/ionomycin for 72 hours. Culture supernatants were then harvested and analysed by cytometric bead array to determine secretion of (A) IL-17A, (B) IFNγ, (C) TNFα and (D) Granzyme B. Graphs show mean ± SEM (n=**5**-7), * $p<0.05$.

Figure 5. STAT3-deficient MAIT cells do not respond to Th17 polarising stimuli

Sorted $\delta2^+$ and $\delta2^-$ γδ T, MAIT and **total** $CD4^+$ T cells from normal controls or $STAT3^{MUT}$ patients were cultured under Th0 (TAE beads), Th1 (TAE + IL-12) and Th17 (TAE + IL-1β/IL-6/IL-23/TGFβ) conditions. After 5 days, secretion of IL-17A (A), IL-17F (B), IFNγ (C) and granzyme B (D) was determined by CBA. (E) Absolute cell numbers in each culture were also determined at this time. Graphs show mean ± SEM (n=4-**9**), * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 6. IL-21 and IL-23 activate STAT3 and are required for the maintenance of iNKT and MAIT cells

(A) PBMCs from normal controls were stained for expression of IL-6R, IL-12ß1 and IL-21R on $CD4^+$ T, iNKT or MAIT cells. Histograms show representative staining (grey – isotype control, red – cytokine receptor). Graphs show fold increase in MFI over isotype control (mean ± SEM, n=4-9). (B) Sorted MAIT ($CD3^+Vα7.2^+CD161^+$) and non-MAIT ($CD3^+Vα7.2^-CD161^-$) T cells were stimulated with TAE, rested, then stimulated with various cytokines for 20 minutes. After

this time, the cells were stained intracellularly for phospho-STAT activation. Histograms show representative staining (grey – unstimulated, red – cytokine stimulated). Graphs show fold increase in MFI over unstimulated control (mean ± SEM, n=3).

(C, D) PBMCs from patients with mutations in *IL12RB1, IL21R* or *IFNGR1* were stained for the presence of (C) MAIT cells ($CD3^+V\alpha7.2^+$ $CD161^+$) or (D) iNKT cells ($TCRV\alpha24^+$ $V\beta11^+$). Data from normal controls and $STAT3^{MUT}$ patients from Figure 1 are included for comparison. Each point represents a different individual, * $p<0.05$, **** $p<0.0001$.

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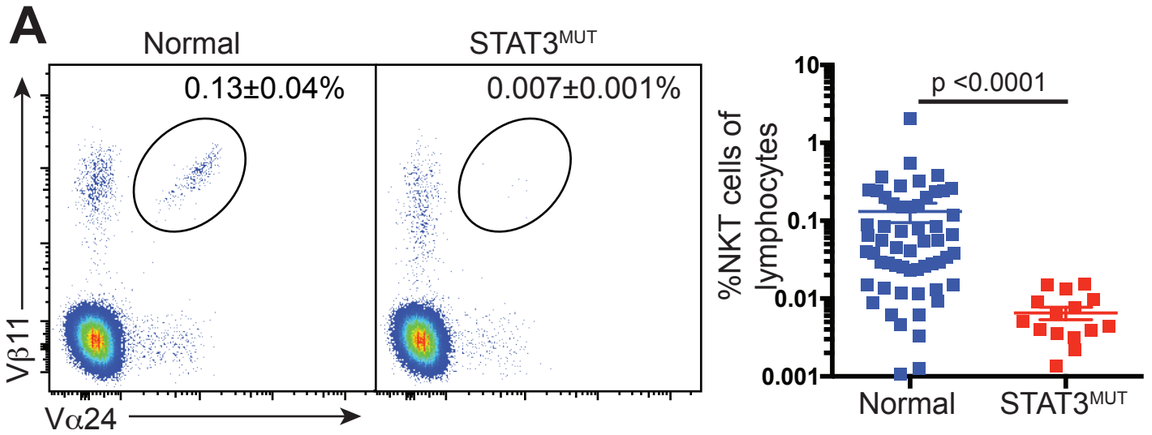
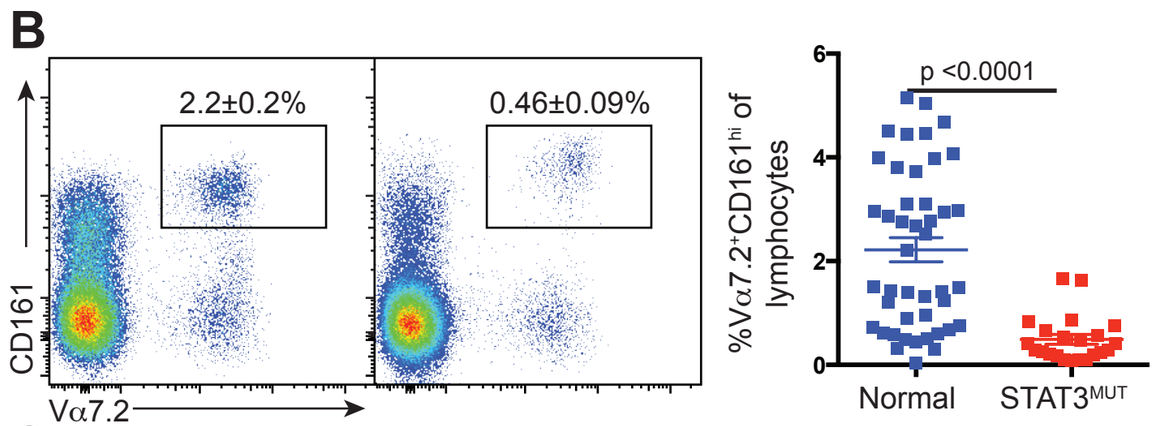
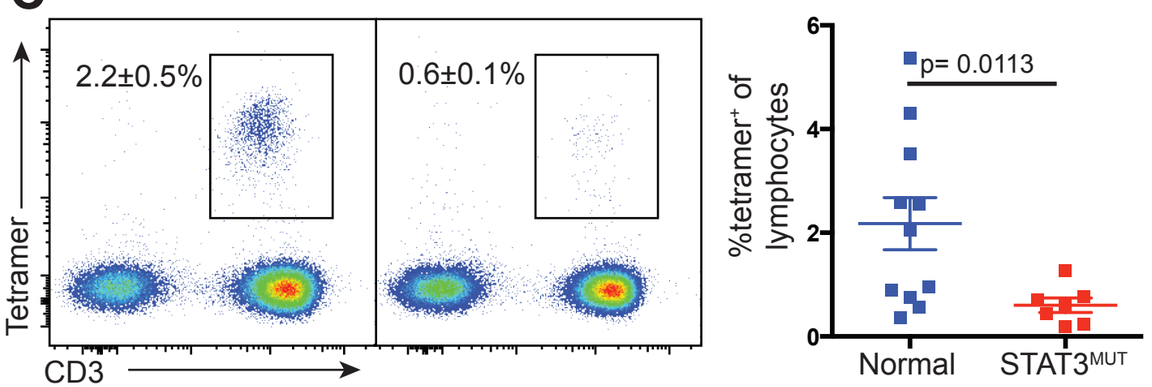
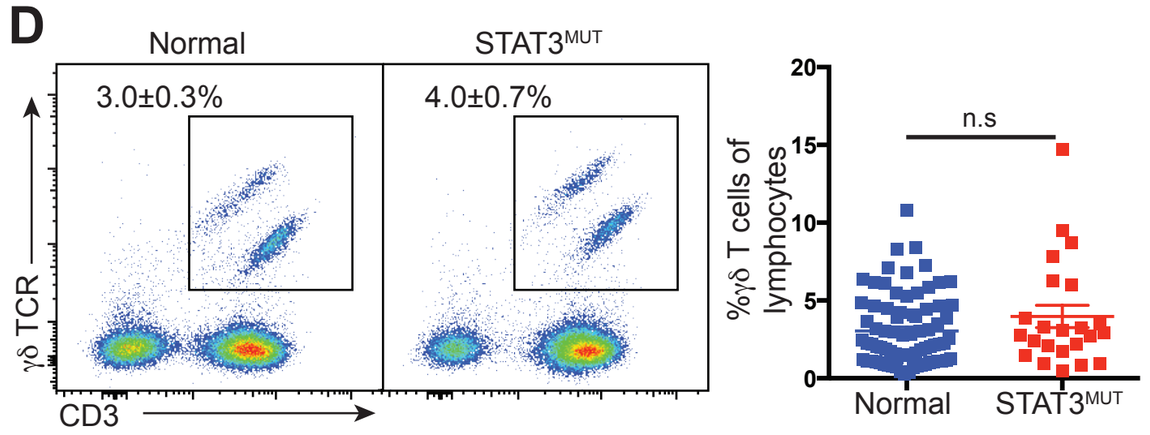
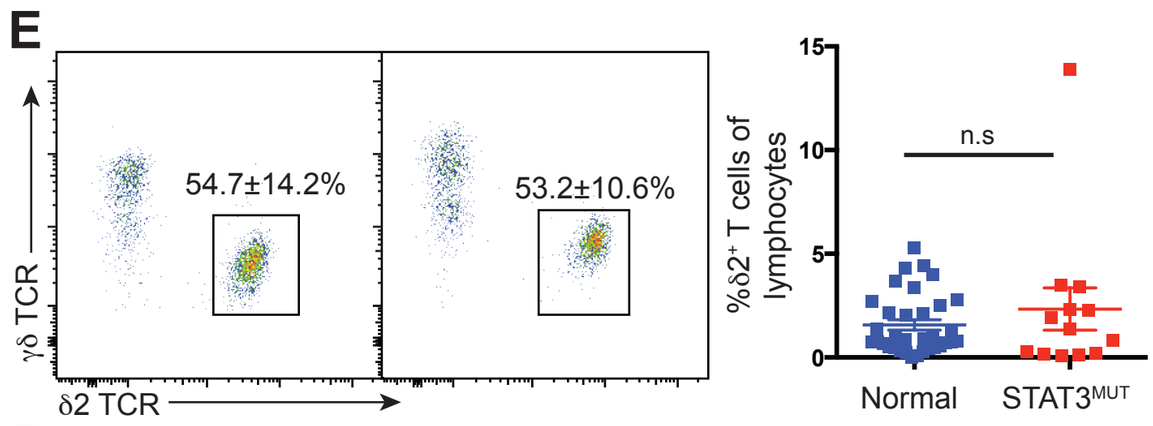
Figure 1


A
Normal
STAT3MUT
0.13±0.04%
0.007±0.001%
Vβ11
Vα24
%NKT cells of lymphocytes
p <0.0001
10
1
0.1
0.01
0.001
Normal
STAT3MUT


B
2.2±0.2%
0.46±0.09%
CD161
Vα7.2
%Vα7.2+CD161hi of lymphocytes
p <0.0001
6
4
2
0
Normal
STAT3MUT


C
2.2±0.5%
0.6±0.1%
Tetramer
CD3
%tetramer+ of lymphocytes
p= 0.0113
6
4
2
0
Normal
STAT3MUT


D
Normal
STAT3MUT
3.0±0.3%
4.0±0.7%
γδ TCR
CD3
%γδ T cells of lymphocytes
n.s
20
15
10
5
0
Normal
STAT3MUT


E
54.7±14.2%
53.2±10.6%
γδ TCR
δ2 TCR
%δ2+ T cells of lymphocytes
n.s
15
10
5
0
Normal
STAT3MUT

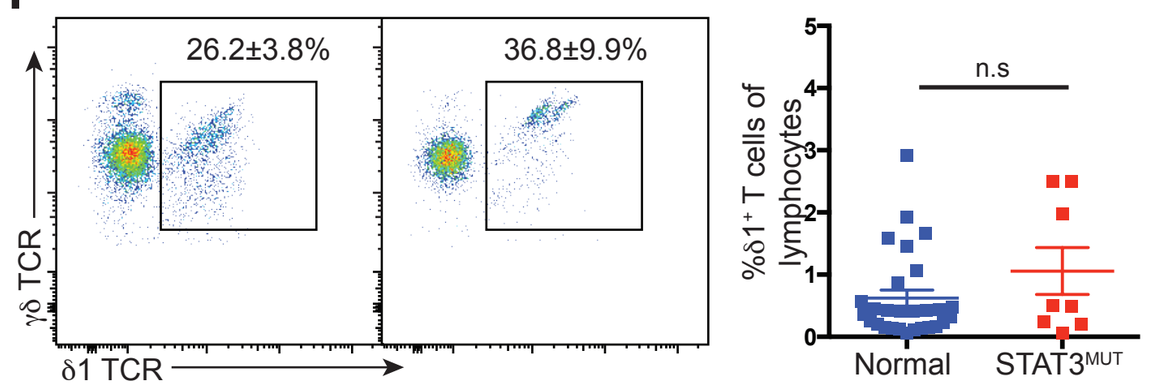
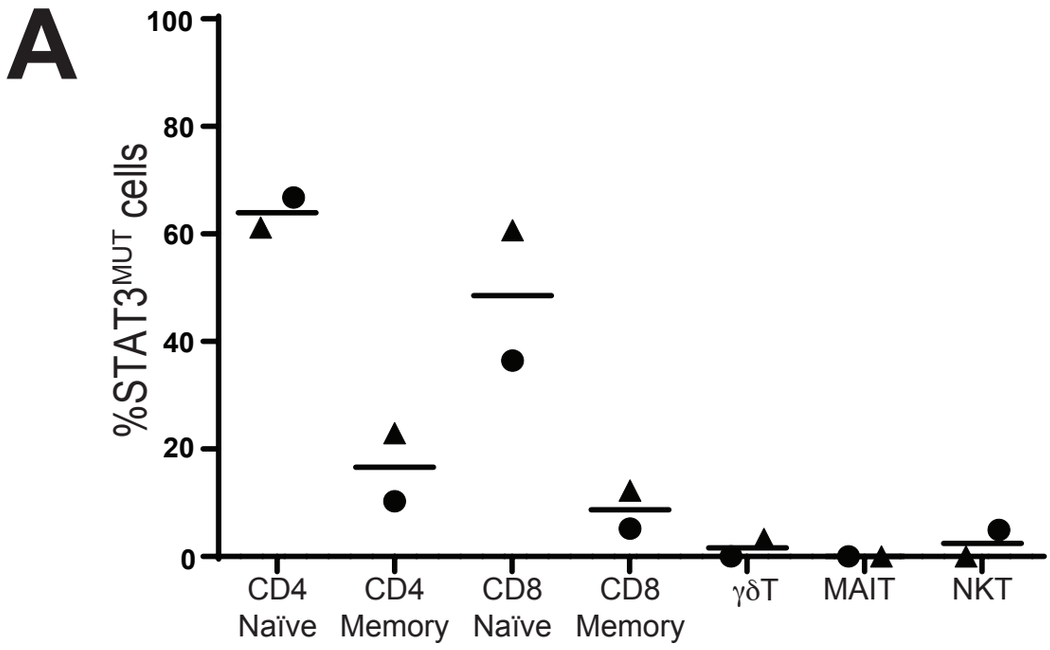

F
26.2±3.8%
36.8±9.9%
γδ TCR
δ1 TCR
%δ1+ T cells of lymphocytes
n.s
5
4
3
2
1
0
Normal
STAT3MUT

Figure 2


A
100
80
60
40
20
0
%STAT3MUT cells
CD4 Naïve
CD4 Memory
CD8 Naïve
CD8 Memory
γδT
MAIT
NKT

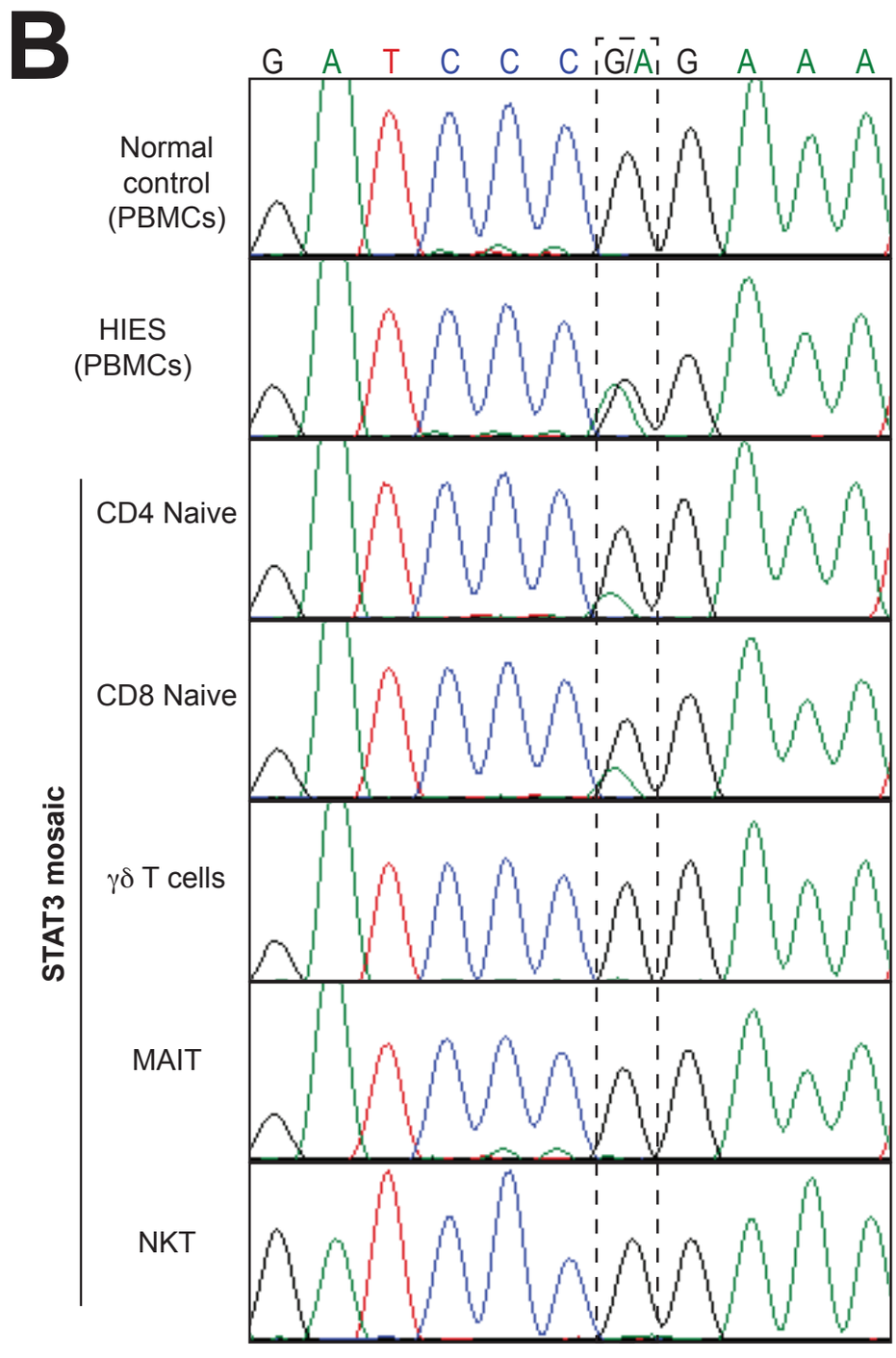

B
G A T C C C G/A G A A A
Normal control (PBMCs)
HIES (PBMCs)
STAT3 mosaic
CD4 Naive
CD8 Naive
γδ T cells
MAIT
NKT

Figure 3

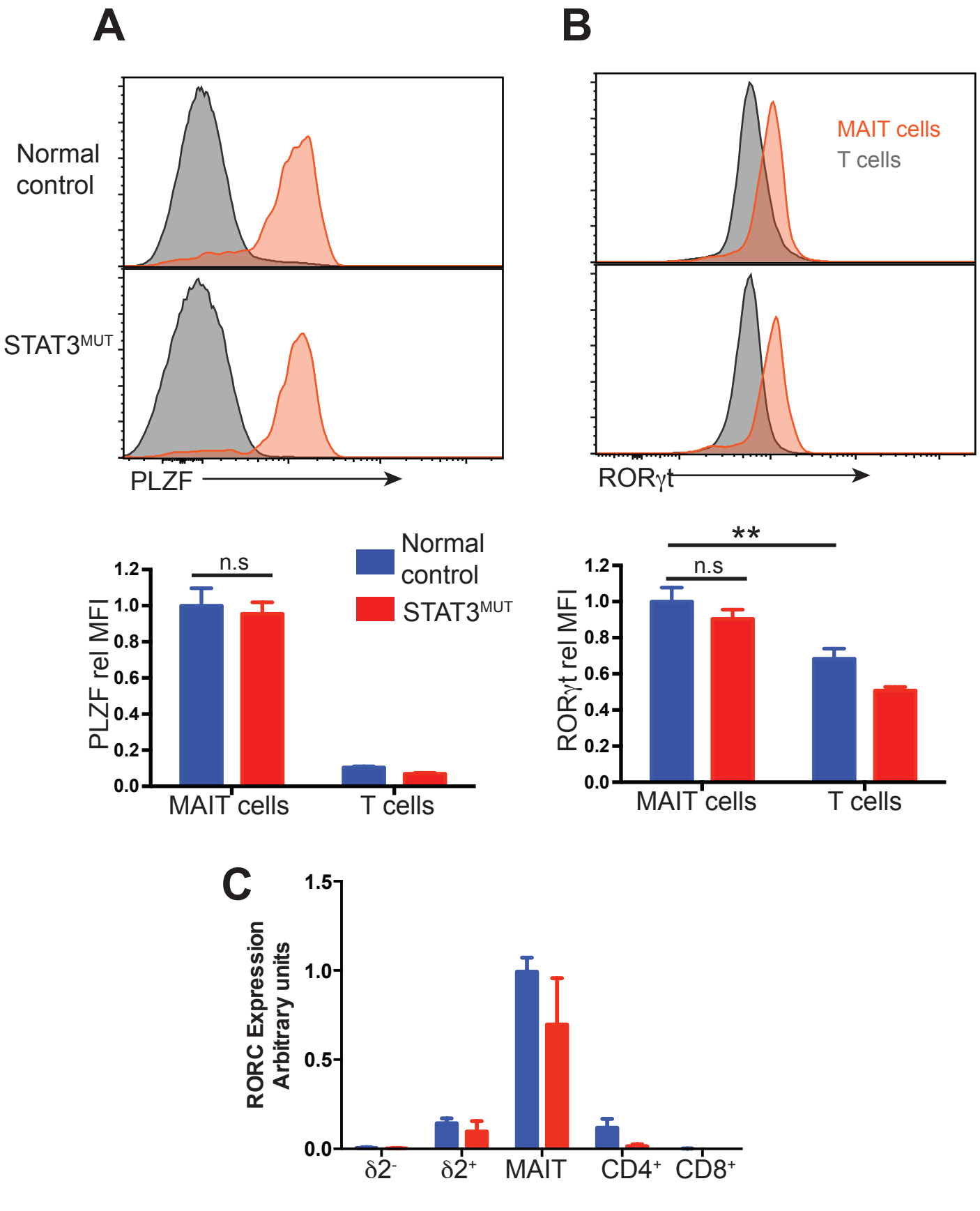

A
B
Normal control
STAT3MUT
PLZF
MAIT cells
T cells
RORγt
Normal control
STAT3MUT
n.s
PLZF rel MFI
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MAIT cells
T cells
**
n.s
RORγt rel MFI
C
RORC Expression Arbitrary units
1.5
1.0
0.5
0.0
δ2-
δ2+
MAIT
CD4+
CD8+

Figure 4

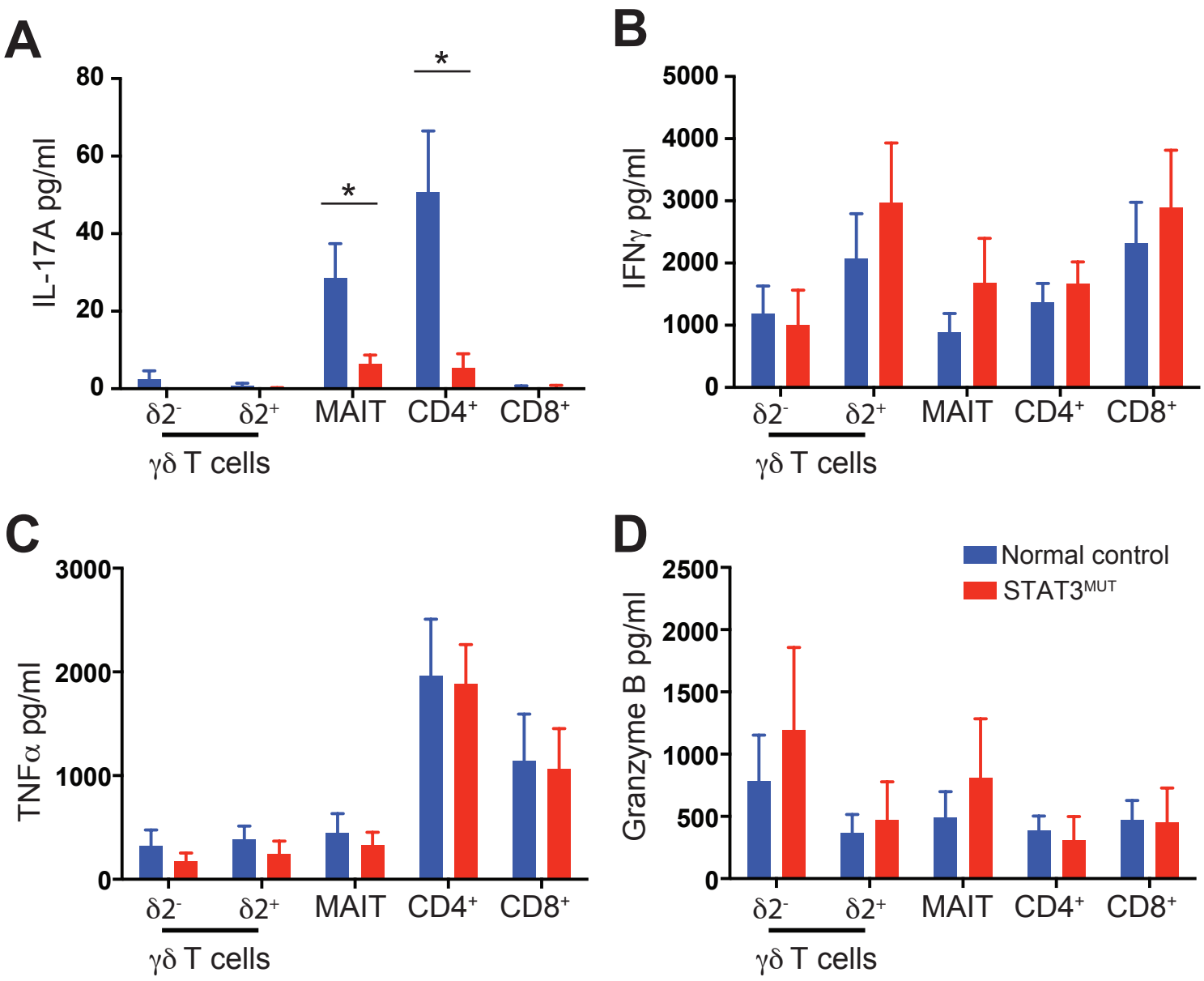

A
IL-17A pg/ml
80
60
40
20
0
*
*
δ2-
δ2+
MAIT
CD4+
CD8+
γδ T cells
B
IFNγ pg/ml
5000
4000
3000
2000
1000
0
δ2-
δ2+
MAIT
CD4+
CD8+
γδ T cells
C
TNFα pg/ml
3000
2000
1000
0
δ2-
δ2+
MAIT
CD4+
CD8+
γδ T cells
D
Granzyme B pg/ml
2500
2000
1500
1000
500
0
Normal control
STAT3MUT
δ2-
δ2+
MAIT
CD4+
CD8+
γδ T cells

Figure 5

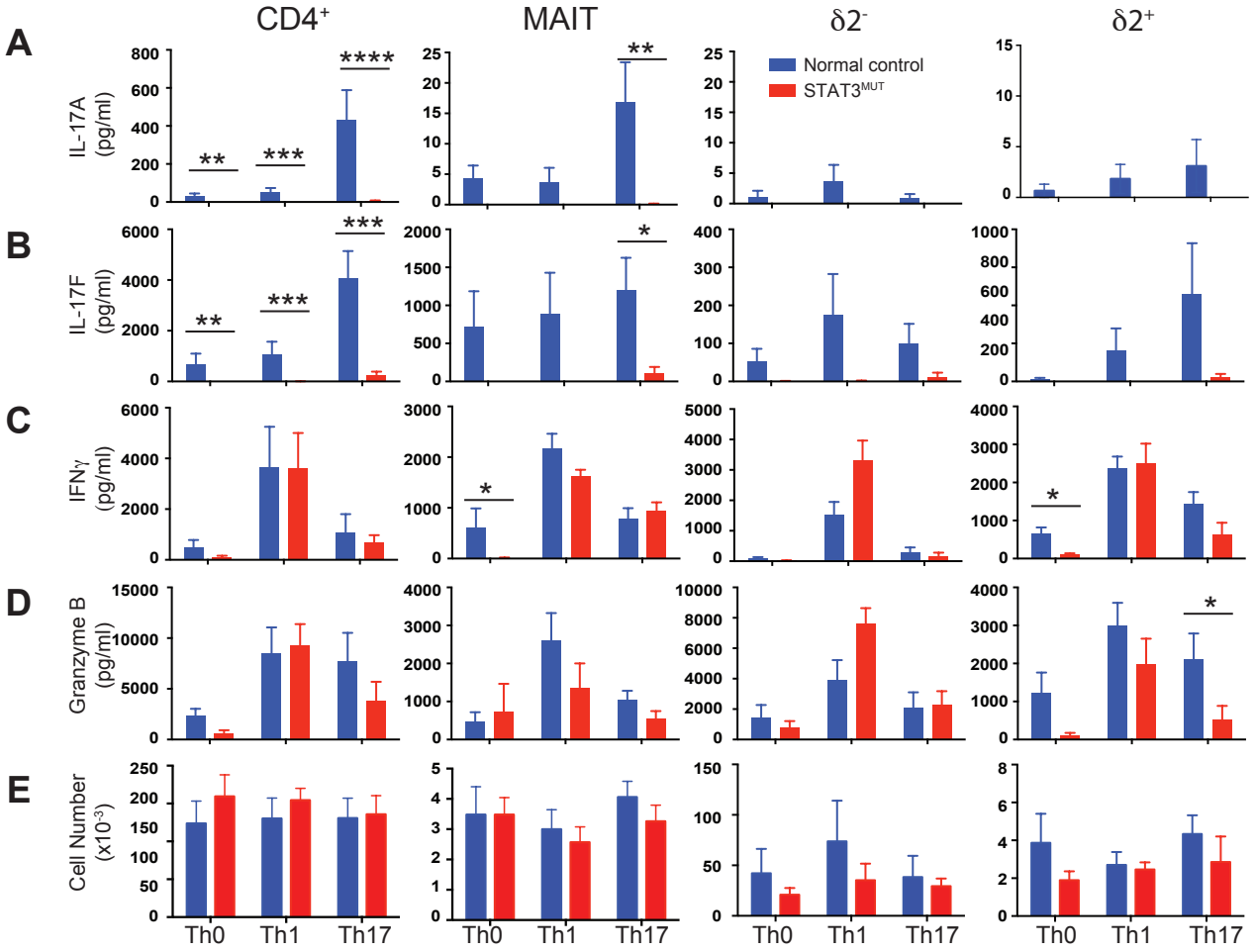
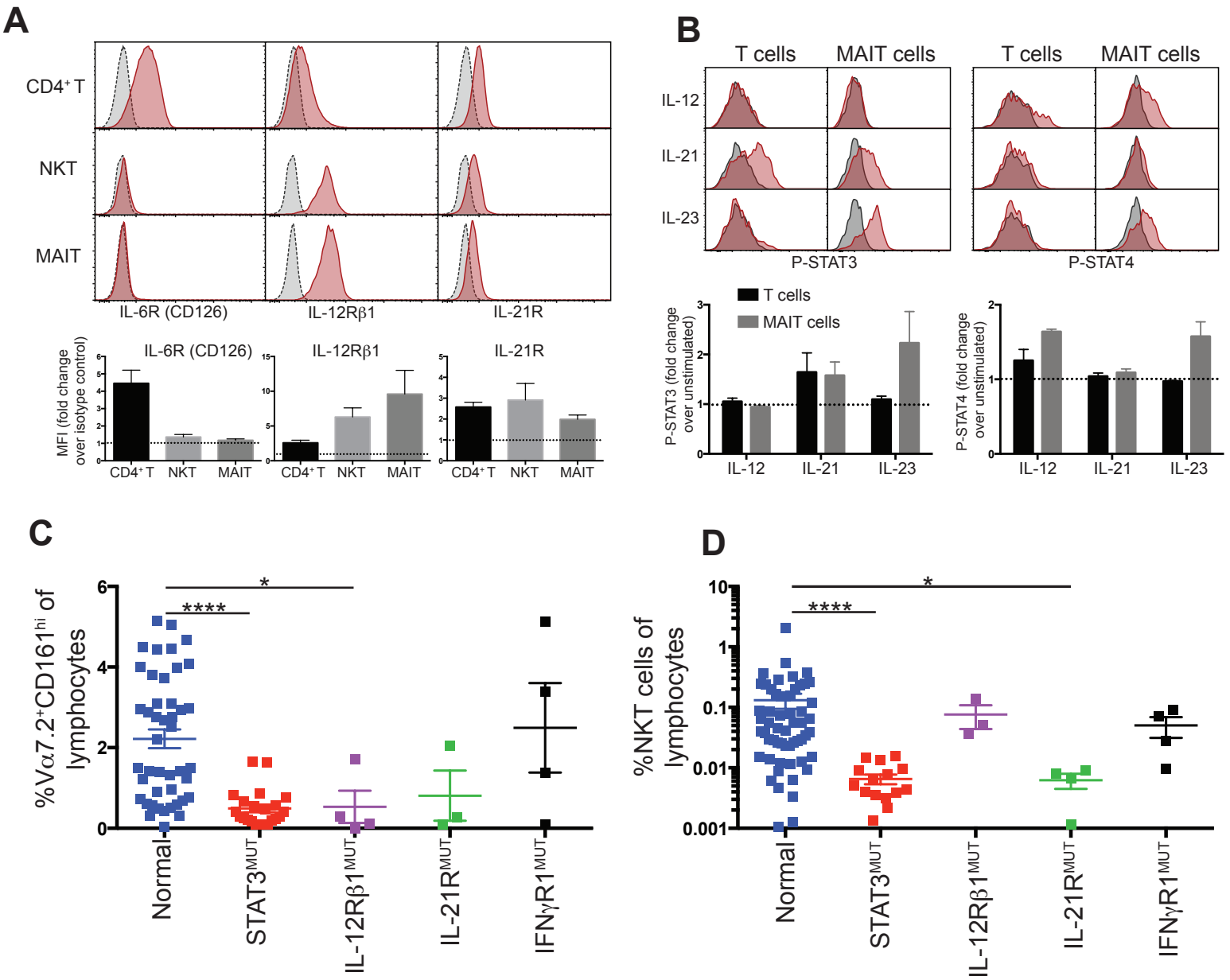

CD4+
MAIT
δ2-
δ2+
Normal control
STAT3MUT
A
IL-17A (pg/ml)
B
IL-17F (pg/ml)
C
IFNγ (pg/ml)
D
Granzyme B (pg/ml)
E
Cell Number (x10-3)
Th0
Th1
Th17

Figure 6


A
CD4+ T
NKT
MAIT
IL-6R (CD126)
IL-12Rβ1
IL-21R
MFI (fold change over isotype control)
CD4+ T
NKT
MAIT
B
T cells
MAIT cells
IL-12
IL-21
IL-23
P-STAT3
P-STAT4
P-STAT3 (fold change over unstimulated)
P-STAT4 (fold change over unstimulated)
C
%Vα7.2+CD161hi of lymphocytes
Normal
STAT3MUT
IL-12Rβ1MUT
IL-21RMUT
IFNγR1MUT
D
%NKT cells of lymphocytes