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IKZF3/Aiolos is associated with, but not sufficient for the expression of IL-10 by CD4+ T cells

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Abstract

The expression of anti-inflammatory IL-10 by CD4+ T cells is indispensable for immune homeostasis as it allows T cells to moderate their effector function. We previously showed that TNFα blockade during T cell stimulation in CD4+ T cell/monocyte co-cultures resulted in maintenance of IL-10 producing T cells and identified IKZF3 as a putative regulator of IL-10. Here, we tested the hypothesis that IKZF3 is a transcriptional regulator of IL-10 using a human CD4+ T cell only culture system. IL-10+CD4+ T cells expressed the highest levels of IKZF3 both *ex vivo* and after activation, compared to IL-10-CD4+ T cells. Pharmacological targeting of IKZF3 with the drug lenalidomide showed that IKZF3 is required for anti-CD3/CD28 mAb-mediated induction of IL-10 but is dispensable for *ex vivo* IL-10 expression. However, overexpression of IKZF3 was unable to upregulate IL-10 at the mRNA or protein level in CD4+ T cells and did not drive the transcription of the *IL10* promoter or putative local enhancer constructs. Collectively, these data indicate that IKZF3 is associated with, but not sufficient for IL-10 expression in CD4+ T cells.

Key points:

Anti-TNF maintains *IL10* expression in CD4+ T cells at the transcriptional level

IKZF3 is enriched in IL-10+CD4+ T cells; degrading IKZF3 disrupts IL-10 production

Overexpression of IKZF3 does not drive *IL10* nor activate local enhancers

Introduction

The production of IL-10 by CD4+ T cells is key for the control of effector function in response to immune challenge (1-3). Even in the absence of pathogens, CD4+ T cell specific deletions of *Il10* lead to a pronounced inflammation in the colonic mucosa in response to commensal gut bacteria (1).

IKZF3 (encoding for the protein Aiolos) is a member of the Ikaros Zinc finger family of transcription factors (4). This gene is expressed by various immune cell types and has been implicated in the function of multiple T helper subsets (5, 6) as well as in controlling CD4/CD8 fate decision in the thymus (7). The expression of IKZF3 in IL-17 producing CD4+ T cells (Th17 cells) is associated with a “non-pathogenic” signature which includes increased IL-10 production (6, 8). IKZF3 has also been shown to interact with known regulators of *IL10* expression including its most closely related family member IKZF1 (encoding Ikaros) (4) which has been shown in mice to directly affect the expression of *Il10* (9).

While IKZF3 has been suggested to act as a transcriptional activator in CD4+ T cells (4, 10), this has mainly been ascribed to its cooperation with other factors such as FOXP3 (11) and BLIMP1 in regulatory CD4+ T cells (Tregs) (12), and with STAT3 in T follicular helper cells (T_{FH}) (13). Studies in multiple cell lines highlight the ability of IKZF3 to repress gene expression, through HDAC and PRC2 recruitment (14-16) as well as by altering chromatin superstructure (17).

Anti-TNFα mAb therapy is commonly used in the treatment of many inflammatory conditions including rheumatoid arthritis (18), inflammatory bowel disease (19) and psoriasis (20). Although the mechanisms governing its therapeutic effects are still not entirely elucidated, multiple effects on the immune system have been reported including induction of an anti-inflammatory CD4+ T cell phenotype (21), modulation of innate immune cell function (22, 23), expansion of Tregs (24), in addition to blocking TNFα proinflammatory signalling. We previously demonstrated that patients with rheumatoid arthritis or ankylosing spondylitis treated with anti-TNFα drugs have increased frequencies of IL-10+ CD4+ T cells in peripheral blood (10). Furthermore, CD4+ T cells from the peripheral blood of healthy volunteers activated in the presence of anti-TNFα therapeutics, had increased frequencies of IL-10+ cells (10, 25). Gene

expression analysis from one of these studies highlighted IKZF3 as a potential regulator of IL-10 expression, at least in Th17 cells (10).

Here we sought to address the hypothesis that IKZF3 is a transcriptional regulator IL-10 production in CD4+ T cells.

Materials and Methods

Cells and cell culture

Peripheral blood was obtained from healthy adult volunteers with written informed consent (Bromley Research Ethics Committee ref 06/Q0705/20). Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation. CD4+ T cells and CD14+ monocytes were isolated by magnetic-activated cell sorting (MACS) using the manufacturer's protocol. CD14+ monocytes were isolated using anti-CD14+ microbeads to ~98% purity (Miltenyi Biotech), and CD4+ T cells were isolated using negative selection ~95% (Miltenyi Biotech).

Cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FCS and 1% penicillin, streptomycin and 10mg/mL L-glutamine (culture medium). CD4+ T cell cultures were stimulated with anti-CD3/CD28 mAb stimulation, by coating tissue culture plate wells with 1.25μg/mL α-CD3 mAb OKT3 (Janssen-Cilag Ltd) in PBS for 3 hours at 37^{o}C. Wells were washed with sterile PBS before adding the cells (10^6/mL) together with 1 μg/mL anti-CD28 mAb (clone CD8.2; BD Biosciences). For co-cultures, $0.5x10^6$ CD14+ peripheral blood monocytes were cultured with $0.5x10^6$ autologous CD4+ T cells in 1ml of culture medium in the presence of 100ng/mLanti-CD3mAb (OKT3). HEK293T cells (gifted from Stuart Neil lab, King's College London, UK) were cultured in DMEM, supplemented with 10% FCS, 1% penicillin, streptomycin and 10mg/mL L-glutamine.

Flow cytometry

For intracellular staining, CD4+ T cells, or CD4+ T cell/monocyte co-cultures were stimulated for 3 hours in the presence of PMA (50ng/mL, Sigma Aldrich), ionomycin (750ng/mL, Sigma Aldrich) and GolgiStop (BD, as per manufacturer's instructions). Cells were washed and stained with CD3-PE Cy7 (UCHT1, Biolegend) and Live/Dead efluor780 (Thermo Fisher). Cells were then fixed in 2% PFA and permeabilised with 0.5% Saponin (Thermo Fisher). Cells were subsequently stained for the following cytokines: IL-10-AlexaFluor488 (JES3-9D7, Biolegend), IL-17A-PE (BL168, Biolegend), IFNγ-Pacific blue (4S.B3, Biolegend) and, TNF-APC (MAb11, Biolegend).

For intranuclear staining of IKZF3, cells were fixed and permeabilised with FOXP3 staining buffer (Biolegend) for 15 minutes at room temperature before being stained for CD3-PE Cy7, IL-10-Alexafluor488, IL-17A-PE, IFNγ-Pacific blue, TNF-BV605 (MAb11, Biolegend) and either, IKZF3-AF647 (EPR9342(B), Abcam) or isotype control (EPR25A, Abcam) for 30 minutes. Standard gating strategy for intracellular cytokine staining is shown in supplemental figure 1A-C

RNA isolation and qPCR

mRNA was isolated using RNEASY mini kit (Qiagen). cDNA was transcribed using a High Capacity cDNA RT Kit (Applied Biosystems) according to the manufacturer's protocol. Realtime PCR was performed using SensiFAST SYBR Green PCR master mix (Bioline) with 10uM of primers (Table 1). Reactions were performed in multiple technical replicates and results calculated using the dCT method.

Actinomycin D assay

CD4+ T cells stimulated with anti-CD3/CD28 mAb cultured in the presence or absence of 1 μg/mL adalimumab for 72 hours. After stimulation the cells were treated for 2 hours with either 1 μg/mL Actinomycin D (Cambridge Biosciences) or equivalent volume of DMSO. Cells were subsequently harvested for RNA and assayed for gene expression by qPCR.

Viral transduction of CD4+ T cells

The plasmids pCSIG-IKZF3-GFP (lenti-IKZF3) and pCSIG-GFP (lenti-EV) were packaged into lentiviral particles by transfecting HEK293T cells with a pCSIG vector, pSPAX2 and pMD2.G. Viral particles were concentrated using PEG-IT (Cambridge Bio) according to manufacturer's instructions.

Primary CD4+ T cells were activated with platebound anti-CD3 and anti-CD28 mAb (2 μg/mL) with 20 U/mL rhIL-2 (Peprotech) for 24 hours at a density of 10^6 cells/mL. Viral supernatants were mixed with TRANSDUX MAX (Cambridge Bioscience), added to the cells and cultured. After 3 days, the cells were supplemented with fresh 10% FCS RPMI and 20 U/mL of rhIL-2 and rested from stimulation for 3 days. These cells were subsequently sorted on live CD3+ GFP+/- cells (Supplemental Figure 1D).

Cells were sorted and rested overnight at a density of 10^5 cells/mL then stained for IL-10, IL-17A, IFNγ and IKZF3.

Plasmids and cloning

The selected regions of the human *IL10* locus (indicated in Table 2) were amplified by PCR using the BAC RP11-262N9 (Thermo Fisher) as a template, and TOPO cloned into TOPO Blunt II (Invitrogen). These were then sequenced to confirm 100% conformity to the reference sequence. These regions of interest were subcloned into a pGL4.26 vector (Promega).

FLAG-cMAF-pCMV was a gift from Paul Lavender (King's College London, UK). and HA-IKZF3 was PCR cloned from a pCMV sport vector purchased from Source BioScience.

Luciferase assay

HEK293T cells were seeded at a density of 200,000 cells/mL in 96 well plates. The next day, each well was transfected with 1 µg of PEI (Sigma Aldrich) mixed with 0.2 µg of experimental pGL4.26, 0.01 µg of control pRL4 and 0.2 µg of transcription factor-pCSIG or empty vector. After 18 hours of transfection, the cell culture media was replaced and left for a further 48 hours before harvesting the cells.

Luciferase assays were performed using the Dual-glo luciferase kit (Promega) according to manufacturer's instructions and data collected on a Tecan Spark 10M. Firefly luciferase activity was normalised to Renilla luciferase activity for each sample to control for transfection efficiency, and further normalised to the empty vector control.

Statistical analysis

Statistical analysis was performed using Graphpad Prism version 8. Wilcoxon test was used for comparisons between 2 groups unless otherwise stated. Significant p values are reported as $p<0.05$ *, $p<0.01$ **, $p<0.001$ ***, $p<0.0001$ ****.

RESULTS

TNFα blockade maintains *IL10* transcription in CD4+ T cells

We previously observed a transient increase in the frequency of IL-10+ CD4+ T cells when PBMC were stimulated with anti-CD3 mAb, which was maintained in the presence of TNFα blockade (10, 25). Since we aimed to utilise a reductionist CD4+ T cell culture in our experiments, we first sought to determine the kinetics of IL-10 expression in cultures of anti-CD3/CD28 mAb stimulated CD4+ T cells rather than PBMC cultures. CD4+ T cells were purified and stimulated with platebound anti-CD3 and soluble anti-CD28 mAb for 1-3 days, with or without the anti-TNFα antibody adalimumab (ADA), before being restimulated with PMA and ionomycin for intracellular cytokine staining (representative gating strategies are shown in Supplemental Figure 1). We observed a transient increase in the frequency of IL-10+ cells when CD4+ T cells were stimulated with anti-CD3/CD28 mAb, which was maintained by TNFα blockade (Figure 1A, B). To rule out a possible artefact due to the PMA/ionomycin restimulation, we examined the expression of *IL10* mRNA levels by qPCR in CD4+ T cells stimulated with anti-CD3/CD28 mAb with or without anti-TNF. We observed a similar pattern, namely a transient increase of *IL10* expression upon stimulation, which was maintained in the presence of ADA at day 3 (Figure 1C). We also observed IL-10 secretion in the cell culture supernatant upon 3 days of anti-CD3/CD28 mAb stimulation which was significantly increased in the presence of anti-TNFα (Figure 1D).

IL10 mRNA has been shown previously to be controlled at the post transcriptional level (26). In order to determine whether *IL10* mRNA was stabilised by TNFα blockade, we performed an Actinomycin D assay on CD4+ T cells stimulated with anti-CD3/CD28 mAb for 3 days. This assay is frequently used to determine the relative stability of mRNA species between treatments or cell types (27). The treatment of cells with actinomycin D inhibits mRNA transcription. Once blocked, unstable mRNA transcripts are degraded by cellular machinery over time and not replenished. Comparing mRNA levels between actinomycin D and vehicle control treated cells, gives an indication of mRNA stability. *IL10* mRNA in activated CD4+ T cells was sensitive to the addition of Actinomycin D and therefore unstable, similar to *MYC* and unlike the more stable mRNA *IL2RA* (Figure 1E). We did not observe a significant difference between control

and ADA-treated CD4+ T cells. These results indicate that the increase in *IL10* mRNA is due to active transcription.

IKZF3 is enriched in IL-10 producing CD4+ T cells

Our previous gene expression analysis indicated that IKZF3 was upregulated in Th17 cells in response to TNFα blockade and could bind at the *IL10* locus in these cells (10). To examine whether IKZF3 was associated with IL-10 production in CD4+ T cells, we performed a combined intracellular cytokine staining and an intranuclear stain for IKZF3 to determine the expression of IKZF3 within CD4+ T cells expressing IL-10, IL-17A, IFNγ or TNFα either *ex vivo* or after 3 days of anti-CD3/CD28 mAb stimulation. IKZF3 was expressed at higher levels in IL-10+ CD4+ T cells compared to the total CD4+ T cell population and the IL-17A+ and TNFα+ subsets *ex vivo* (Figure 2A, B). Upon anti-CD3/CD28 mAb stimulation, a significant increase was observed in IKZF3 expression in IL-10-expressing cells compared to the total CD4+ and the TNFα+ cell populations. However, there was no longer a significant difference between IL-10+ and IL-17A+ CD4+ T cells (Figure 2C, D). Since IL-10 can be expressed by multiple cytokine producing CD4+ T cell subsets (especially after stimulation), we compared IKZF3 expression in the IL-17A+, TNFα+ and IFNγ+ cells that co-produced IL-10 and those that did not (Figure 2E, F). In all subsets analysed, a significantly higher expression of IKZF3 was observed in IL-10 co-producing CD4+ T cells compared to cells that did not produce IL-10 (Figure 2F).

In our previous work, we observed an increase in IKZF3 expression in Th17 cells following TNFα blockade using a CD14+ monocyte/CD4+ T cell co-culture system. In order to determine if the increase in IKZF3 upon TNFα blockade occurred in the absence of monocytes and in all T cell subsets, we compared IKZF3 expression in CD4+ T cells cultured alone versus CD4+ T cells co-cultured with CD14+ monocytes in the absence or presence of ADA (Supplemental Figure 2). We previously established that IL-10 expression is increased upon TNF blockade in both culture systems (10, 25). In agreement with our previous results, upon T cell stimulation in the presence of CD14+ monocytes and anti-TNF, IKZF3 expression was increased in the total CD4+ T cell population, as well as in the IL-10+ and IL-17A+ subsets (Supplemental Figure 2A). In the absence of CD14+ monocytes, IL-10+ CD4+ T cells

had high expression of IKZF3 in both control and ADA treated samples, but TNFα blockade did not alter IKZF3 expression in these cells (Supplemental Figure 2B, C). These data indicate that in CD4+ T cell only cultures, the anti-TNFα mediated increase of IL-10 can occur in the absence of a concomitant increase in IKZF3 expression.

IKZF3 degradation by lenalidomide does not alter IL-10 expression *ex vivo* but disrupts anti-CD3/CD28 mAb-mediated IL-10 production

We sought to determine whether IKZF3 is required for IL-10 expression. We first attempted to deplete IKZF3 from CD4+ T cells using siRNA in primary CD4+ T cells. However, this approach did not work due to the stability of the IKZF3 protein (as shown by cycloheximide assays, data not shown) and its upregulation upon anti-CD3/CD28 stimulation (required to render the cells transfectable or transducable, data not shown). As an alternative approach, we employed the thalidomide derivative lenalidomide (Lena), which has been shown to induce the proteasomal degradation of IKZF3 (and IKZF1) and is used therapeutically in treating multiple myeloma (28-30).

Treatment of CD4+ T cells with lenalidomide overnight led to a dose-dependent decrease in IKZF3 protein levels as shown by Western blot (Figure 3A) and flow cytometry (Figure 3B). CD4+ T cells were then treated with lenalidomide for 24 hours, in the absence of T cell activation, followed by intracellular cytokine staining. Whilst a significant reduction in the levels of IKZF3 was observed, the frequency of IL-10+ cells within CD4+ T cells was slightly increased (Figure 3C). *Ex vivo* treatment of CD4+ T cells with lenalidomide had no effect on IL-17A, IFNγ expression or viability (Supplemental Figure 3A). In contrast, when CD4+ T cells were treated with lenalidomide for 3 days in the presence of anti-CD3/CD28 mAb stimulation, a strong reduction in both IKZF3 expression and the frequency of IL-10+ CD4+ T cells was observed (Figure 3D). These data indicate that while IL-10 production in unstimulated CD4+ T cells is not lenalidomide-sensitive, the anti-CD3/CD28-mAb mediated increase in IL-10 expressing cells is lenalidomide-sensitive, and thus by extrapolation, potentially regulated by IKZF3. Lenalidomide treatment for 72 hours also resulted in statistically significant increases in IFNγ+ and TNFα+ frequencies, a decrease in IL-17A+ frequencies, and a slight decrease in cell viability (median viability: 88.9%-

82.75%, control vs lenalidomide respectively, Supplementary Figure 3B). Treatment of CD4+ T cells with lenalidomide also consistently increased secretion of IL-2 by CD4+ T cells after 3 days of anti-CD3/CD28 mAb stimulation (Supplemental Figure 3C, n=5).

IKZF3 is not sufficient to drive expression of *IL10* in CD4+ T cells at the mRNA or protein level

We next sought to determine whether IKZF3 was sufficient to drive IL-10 expression in CD4+ T cells. To overexpress IKZF3, we activated CD4+ T cells and transduced the cells with an IKZF3-IRES-GFP lentiviral construct (Lenti-IKZF3) or an empty vector (Lenti-EV) encoding only GFP (Figure 4A). After transduction, live GFP+ cells were sorted for mRNA isolation or rested and stimulated with PMA/ionomycin for intracellular cytokine staining. Whilst cells transduced with IKZF3 showed a significant increase in *IKZF3* transcript, *IL10* mRNA levels were low and not consistently increased by IKZF3 overexpression (Figure 4B). Also, at the protein level, IKZF3 transduced cells did not show a consistent increase in IL-10 producing cells, compared to the empty vector (Figure 4C, D). A considerable proportion of cells was able to produce IFNγ or IL-17A indicating that the transduction protocol had not affected the capacity of the cells to produce cytokines. Together, these data indicate that IKZF3 overexpression is not sufficient to drive IL-10 expression in CD4+ T cells.

IKZF3 is insufficient to drive the expression of enhancer or promoter elements of *IL10*

Our previous work showed that IKZF3 is able to bind evolutionary conserved regions at the *IL10* locus in Th17 cells. To determine whether IKZF3 can drive transcription of *IL10* via these regions, we identified 10 putative enhancer sites at the *IL10* locus (Figure 5A), as defined by accessible chromatin (31), high H3K4me1 and low CpG methylation (from the BLUEPRINT consortium (32)). We cloned these regions and a 1.5kb region of the *IL10* promoter upstream of a Firefly luciferase open reading frame (pGL4). These vectors were then co-transfected with a control Renilla luciferase vector together with the plasmids: lenti-IKZF3 (Figure 5B) or lenti-MAF (Supplemental Figure

4B, C), a known regulator of *IL10* (33). To validate that our constructs were functional, we stained HEK293T cells transfected with lenti-IKZF3, lenti-MAF or lenti-EV for IKZF3 or cMAF by flow cytometry (Supplemental Figure 4A, B) and observed at least a 10-fold increase in expression in the relevant conditions.

The luciferase experiments showed that IKZF3 has limited capacity to drive transcription of the *IL10* constructs (Figure 5B). An induction of reporter gene expression in response to IKZF3 transfection was only seen for enhancer 10, whilst reporter gene expression for most other constructs decreased in a dose-dependent manner upon increasing amounts of IKZF3. In contrast, transfection with cMAF, a known transcriptional regulator of *IL10* (34, 35), significantly upregulated multiple enhancers compared to the empty vector (Supplemental Figure 4C).

Discussion

Regulation of IL-10 expression is a multi-layered process at the levels of transcription (33, 36), post-transcriptional stability (37, 38) and translation (39). In the innate immune system, IL-10 has been shown to be temporally regulated through regulation of transcript stability, such as through the p38/TTP axis (26, 37).

We found that *IL10* mRNA was maintained at higher levels in the presence of anti-TNFα mAb. This increase in IL-10+ producing CD4+ T cells does not appear to be attributable to changes in cell survival or increased cell proliferation after TNF blockade, as we showed recently (40).

We also show that *IL10* mRNA in anti-CD3/CD28-mAb activated primary human CD4+ T cells is an unstable transcript. This may represent a mechanism by which CD4+ T cells, which can transiently produce IL-10 on stimulation, eventually prevent its expression via negative feedback, similar to macrophages (41).

In order to understand what drives the transcriptional regulation of *IL10* we focussed on IKZF3. Our previous work with a CD4+ T cell:CD14+ monocyte co-culture system showed increased IKZF3 expression upon TNFα blockade in Th17 cells which correlated with increased IL-10 expression. In our current study using a T cell reductionist system, we saw no change in IKZF3 expression in cytokine producing

CD4+ T cell subsets upon TNFα blockade while still observing an increase in IL-10+CD4+ T cell frequency. We did observe a generally higher level of IKZF3 expression in IL-10 producing CD4+ T cells *ex vivo* and after CD3/CD28 stimulation. An association between IKZF3 and IL-10 producing CD4+ T cells has been noted by other studies in human Th17 clones (42), as well as mouse Th1 (5) and Th17 cells (8). This association may indicate common transcriptional regulators under steady-state conditions, but not upon TNFα blockade. In our study, IKZF3 was highly expressed in IL-17A+IL-10+ CD4+ T cells. The expression of IKZF3 and IL-10 in "non-pathogenic" Th17 cells with a reduced capacity to drive experimental autoimmune encephalomyelitis, has been previously noted (6, 8).

Similar to our findings with CD4+ T cells cultured without monocytes, another study found that memory CD4+ T cells activated by anti-CD3/CD28 mAb in the presence of the TNFα inhibitor drug etanercept, in the absence of monocytes, showed an increased expression of *IL10* upon TNFα blockade which was not accompanied by changes in IKZF3 expression (43).

IKZF3 (and IKZF1) has been previously described as a negative regulator of *Il2* expression in CD4+ T cells (6, 29), and our findings that IL-2 secretion is increased upon lenalidomide treatment support that observation. The expression of IL-10 and a reduced capacity to produce IL-2 is a known hallmark of Tregs. Therefore, high IKZF3 expression in the IL-10+ population might be indicative of a high proportion of Tregs. However, CyToF data from our lab did not reveal a higher expression of IKZF3 in $CD25^{high}$ $CD127^{low}$ Tregs compared to $CD25^{low}$ $CD127^{high}$ effector T cells *ex vivo* (data not shown). Furthermore, upon TNFα blockade we did not observe an increase in FOXP3+Tregs (10).

Studies have implicated IL-2 signalling to be required for IL-10 expression by multiple Thelper subsets *in vitro* (44). Therefore, one could expect that blocking IKZF3, which is an *IL2* transcriptional repressor would lead to an increase in IL-10 production and frequency. However, we instead observed a significant reduction in the frequency of IL-10+CD4+ T cells in the presence of lenalidomide.

From our data, lenalidomide also seems to have effects on the frequency of IL-17A, TNFα and IFNγ producing cells. The reduction in IL-17A+ CD4+ T cells could be due to the increase of IL-2 in the cell culture supernatants, which has been shown to

inhibit the differentiation of Th17 cells in humans (45, 46). Similarly, IL-2 has been shown to increase the expression of IFNγ in human CD4+ T cells (47, 48) and TNFα expression in mouse CD8+ T cells (49). It should be noted however, that expression of IFNγ and TNFα can be suppressed by IL-10 (50). Therefore, the decrease in IL-10 expression accompanying lenalidomide treatment could boost the induction of IFNγ and TNFα producing cells.

It should be considered that the effect of lenalidomide on IL-10 production in CD4+ T cells may be due to off target effects. Lenalidomide has been shown to downregulate several proteins including transcription factors (51-53). Therefore, the reduction in anti-CD3/CD28 mAb-induced IL-10 production, may stem from another lenalidomide sensitive protein, rather than IKZF3. IKZF1 has previously been shown to be affected by lenalidomide, and is capable of binding similar motifs to IKZF3. However, we previously observed no effect of anti-TNF on CD4+ T cell expression of IKZF1 (10) and do not see the same association of IKZF1 with IL-10 *ex vivo.*

In order to determine whether IKZF3 expression was sufficient to drive IL-10 expression, we overexpressed this protein in primary CD4+ T cells to determine its ability to drive *IL10* mRNA and protein expression, as well as in the HEK293T cell line to determine if it could drive expression of putative *IL10* enhancers or promoters. In both experimental approaches, we found that IKZF3 overexpression was not sufficient to drive the expression of IL-10. IKZF3 may require co-factors to promote transcription such as BLIMP1 (12) and STAT3 (13) which have been shown to interact with IKZF3. ENCODE data show that DNA binding motifs of these factors are in similar locations to IKZF family consensus motifs at the *IL10* locus. It may be that these co-factors are not available in transduced CD4+ T cells or in HEK293T cells to facilitate *IL10* mRNA or reporter expression.

The effect of IKZF3 on most of the luciferase reporters is consistent with its reported function as a transcriptional repressor and indicates that IKZF3 is unable to directly drive *IL10* expression, even when enhancers and promoters are accessible to bind (6, 15). These reporters were based on ATAC-seq data (31) which should be reflective of the accessible regions in CD4+ T cells *ex vivo*. Changes to chromatin by anti-CD3/CD28 mAb stimulation, however, could reveal other enhancers which IKZF3 can bind to drive expression.

It should be noted that IKZF3 has a number of splice variants which have varying abilities to drive gene expression (15). Our data suggest that CD4+ T cells predominantly express the largest isoform of ~70 kDa, and this is the isoform we cloned in our overexpression studies. This isoform has previously been shown to drive gene expression in mouse T_{FH}-like cells and it is therefore possible that this isoform could drive transcription in human CD4+ T cells (13). However, we cannot rule out that other IKZF3 isoforms may differentially affect *IL10* expression.

In summary, this study shows that IKZF3 expression is associated with IL-10+CD4+ T cells at the protein level, and that pharmacological inhibition of IKZF3 disrupts the ability of CD4+ T cells to produce IL-10. However, the expression of IKZF3 is not sufficient to drive IL-10 protein or mRNA expression. We also note that while TNFα blockade does lead to increased *IL10* mRNA expression this is not necessarily attributable to differential expression of IKZF3. Further work is required to establish the transcription factors modified by TNFα blockade which lead to increased *IL10* expression, and whether such transcriptional regulation occurs in patients treated with TNFα inhibitors.

Author Contributions

M.L.R. designed and performed experiments, analysed the data and wrote the manuscript; V.F and C.A.R designed and performed experiments and analysed data, S.L, A.A, A.O'B, K.J.A.S and G.A.M.P performed experiments; J.S and P.L provided plasmids and contributed to technical discussions; L.S.T supervised the study, contributed to the designing of experiments, data interpretation and manuscript writing.

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Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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633 **Legends**

634 **Figure 1. TNFα blockade maintains the expression of IL-10 in CD4+ T cells**
635 **which involves active transcription**.(A, B) Primary CD4+ T cells from healthy
636 donors were stimulated with anti-CD3/CD28 mAb for 0, 1, 2 or 3 days in the absence
637 (blue bars) or presence (red bars) of 1 μg/mL adalimumab. Cells were restimulated
638 with PMA and ionomycin and assessed for IL-10 expression. Representative (A) and
639 cumulative (B, n=7) data showing the frequency of IL-10 expressing cells within
640 CD4+ T cells. (C) *IL10* mRNA expression was analysed by qPCR after 1, 2 or 3 days
641 in culture without restimulation (n=6). Data in B, C analysed by 2-Way ANOVA. (D)
642 Quantification of IL-10 in cell culture supernatants from CD4+ T cells stimulated as
643 above for three days (n=7, Wilcoxon test). (E) After 3 days of culture as above,
644 CD4+ T cells were treated with either DMSO or 1μg/mL Actinomycin D for 120
645 minutes to block transcription. mRNA abundance was assessed by qPCR (n=5, 2-
646 way ANOVA with multiple comparisons, comparing DMSO and actinomycin D
647 treatment conditions within each group, as well as actinomycin D treated cells
648 between cells stimulated in the absence or presence of 1 μg/mL adalimumab).

649

650 **Figure 2. IKZF3 is associated with IL-10 producing CD4+ T cells.** (A, B) Primary
651 CD4+ T cells from healthy donors were stimulated with PMA and ionomycin, and
652 assessed for frequency of cytokine producing cells and IKZF3 expression.
653 Representative (A) and cumulative data shown (B, n=8). (C-F) CD4+ T cells were
654 stimulated with anti-CD3/CD28 mAb for 3 days and subsequently restimulated with
655 PMA and ionomycin, and assessed for frequency of cytokine producing cells and
656 IKZF3 expression. Expression of IKZF3 was calculated within total populations of
657 cytokine producing cells (C, D) or within- IL-10+ or IL10- subsets, within those
658 populations (E, F). Representative (C) and cumulative (D, n=13) data of total
659 cytokine producing populations are shown. Representative (E) and cumulative (F,
660 n=8-11) data for IKZF3 expression within IL-10+ and IL-10- subsets are shown. Data
661 in B and D, analysed by ANOVA, data in F analysed by Wilcoxon test.

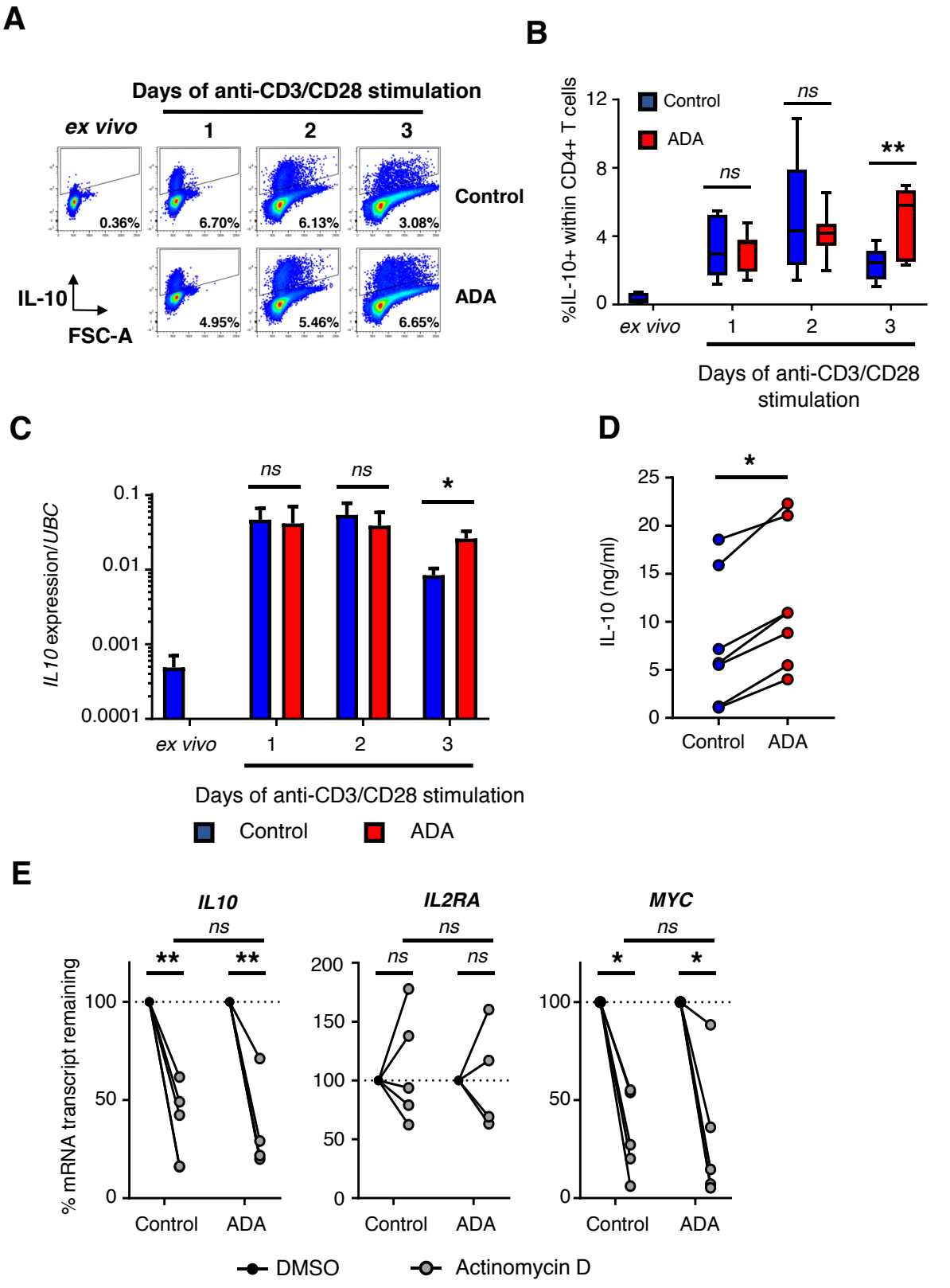
662

663 **Figure 3. Lenalidomide disrupts the anti-CD3/CD28 mAb mediated induction of**
664 **IL-10+ CD4+ T cells but does not affect ex vivo IL-10 production.** (A, B) Primary

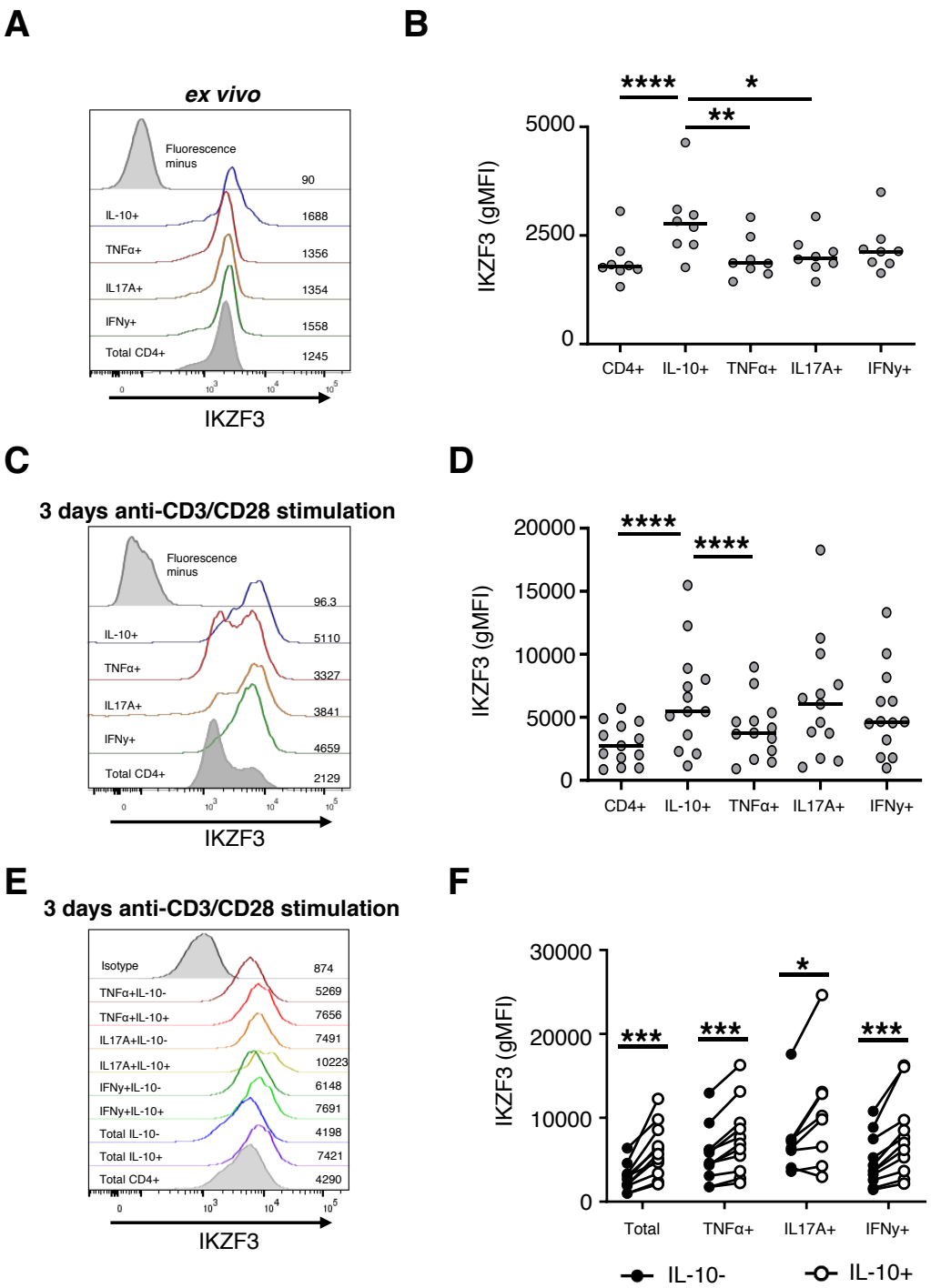
CD4+ T cells from healthy donors were treated with 0.1, 1 or, 10 μM of lenalidomide overnight and examined for IKZF3 expression by Western blot (A) or flow cytometry (B). (C) CD4+ T cells were treated with 1 μM lenalidomide overnight and then stimulated with PMA and ionomycin and assessed for IKZF3 expression and frequency of IL-10+ CD4+ T cells (n= 13). (D) CD4+ T cells were stimulated with anti-CD3/CD28 mAb for 3 days in the presence of 1 μM lenalidomide and subsequently restimulated with PMA and ionomycin and assessed for IKZF3 expression and frequency of IL-10+CD4+ T cells (n=13). Data in C and D analysed by Wilcoxon test.

Figure 4. IKZF3 overexpression in CD4+ T cells is not sufficient to drive the expression of IL10. (A-D) Primary CD4+ T cells from healthy donors were transduced with an IKZF3-IRES-GFP (Lenti-IKZF3) or GFP only (Lenti-EV) lentivirus. 7 days post transduction cells were sorted on GFP expression. (A) Representative GFP expression. (B) CD4+ T cells transduced with lenti-IKZF3 or Lenti-EV were sorted on GFP expression and mRNA expression of *IKZF3* and *IL10* was quantified by qPCR (n=6). (C, D) Cells transduced with Lenti-IKZF3 or Lenti-EV were sorted on GFP expression, rested overnight, then restimulated with PMA and ionomycin and assessed for frequency of IL-10, IFNγ or IL-17A producing cells. Representative (C) and cumulative (D, n=6-8) data shown. Data in B and D analysed by Wilcoxon test.

Figure 5. IKZF3 is insufficient to drive transcriptional activity via the *IL10* promoter or local enhancers. (A) ChIP-seq (from the Blueprint consortium) and ATAC-seq data (from *Buenrostro* et al. 2015) from *ex vivo* human CD4+ T cells for the *MAPKAPK2:IL10:IL19* locus was used to identify regions of putative enhancers (yellow vertical bars, numbered 1-10) as well as 1.5kb promoter region of *IL10* (vertical green bar, labelled Promo). (B) These promoter and enhancer regions were then cloned upstream of a luciferase reading frame and transfected along with an expression plasmid encoding IKZF3 (Lenti-IKZF3) or empty vector control (Lenti-EV) into HEK293T cells and assessed 48 hours later for luciferase activity (n=4). Data analysed by 2-Way ANOVA.



Ridley et al. Figure 1



Ridley et al. Figure 2

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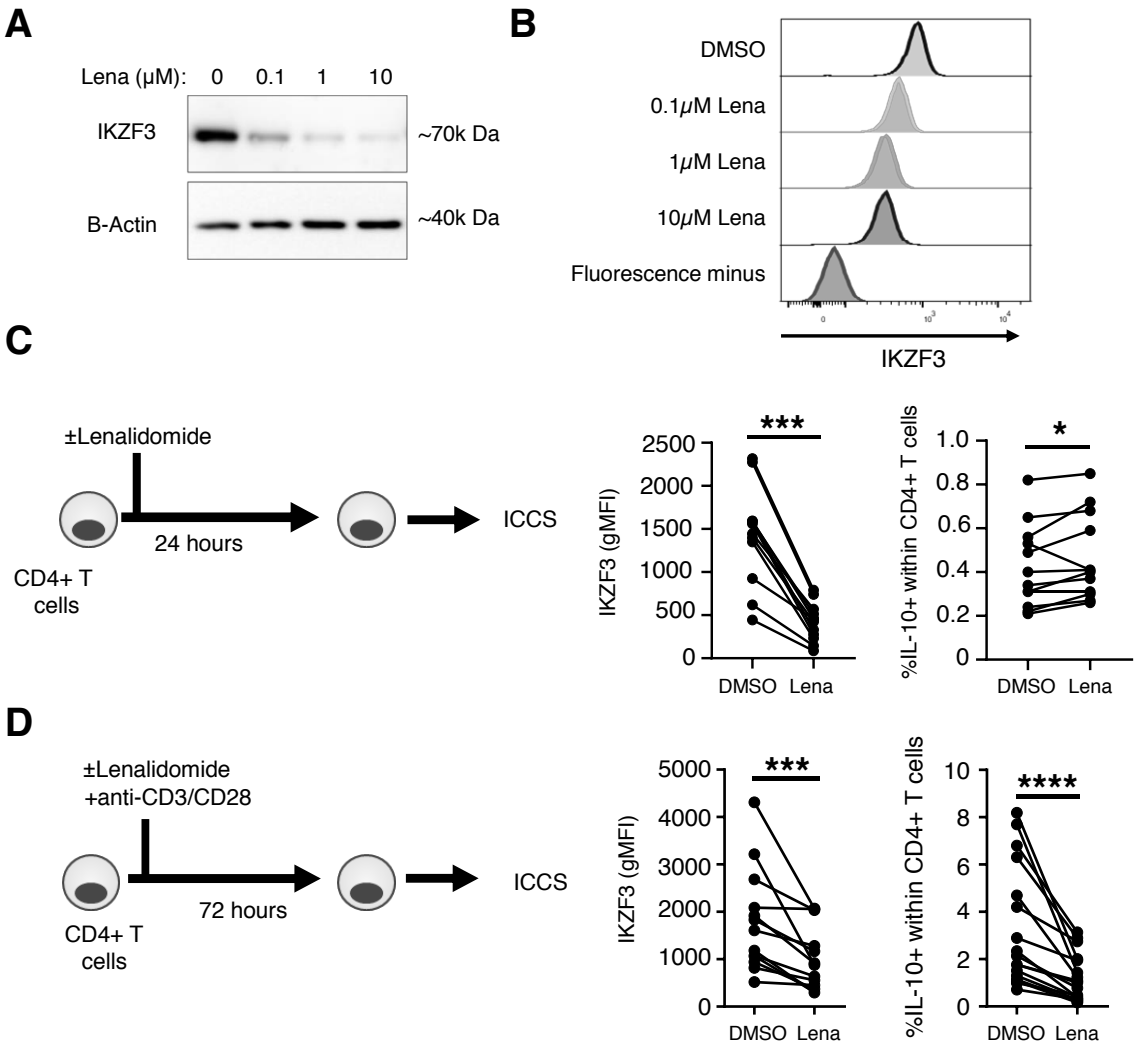

Ridley et al. Figure 3

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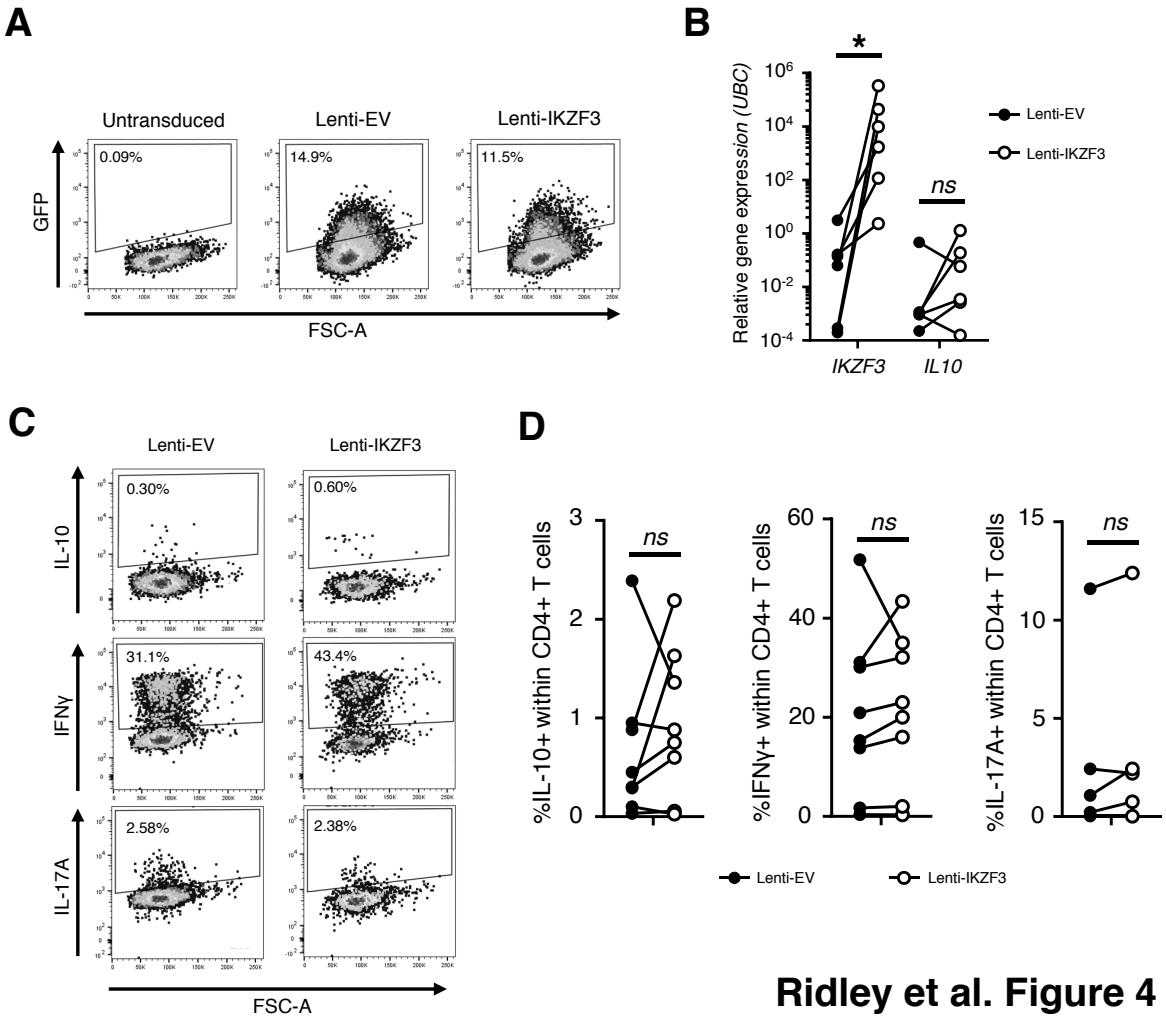

Ridley et al. Figure 4

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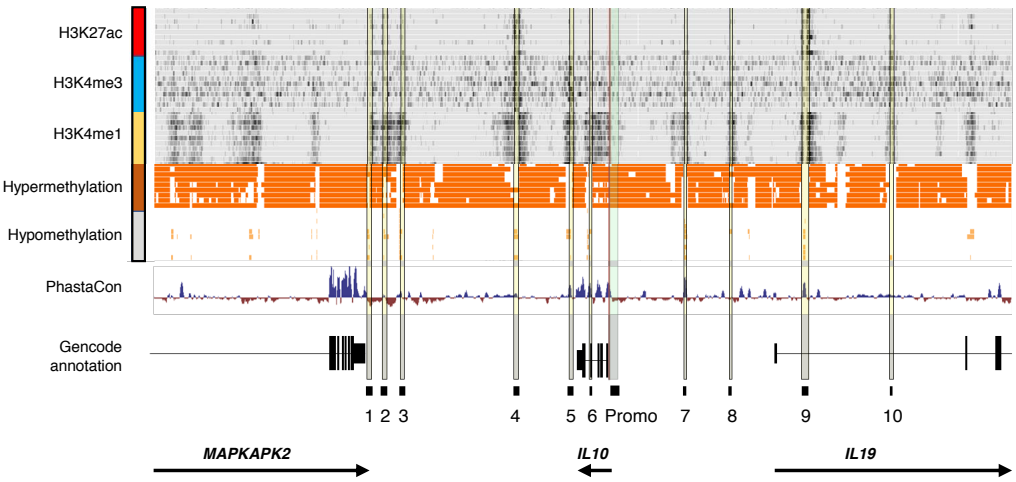
A



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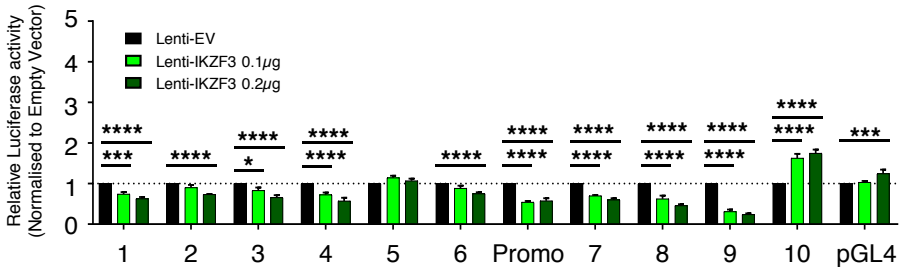

Ridley et al. Figure 5

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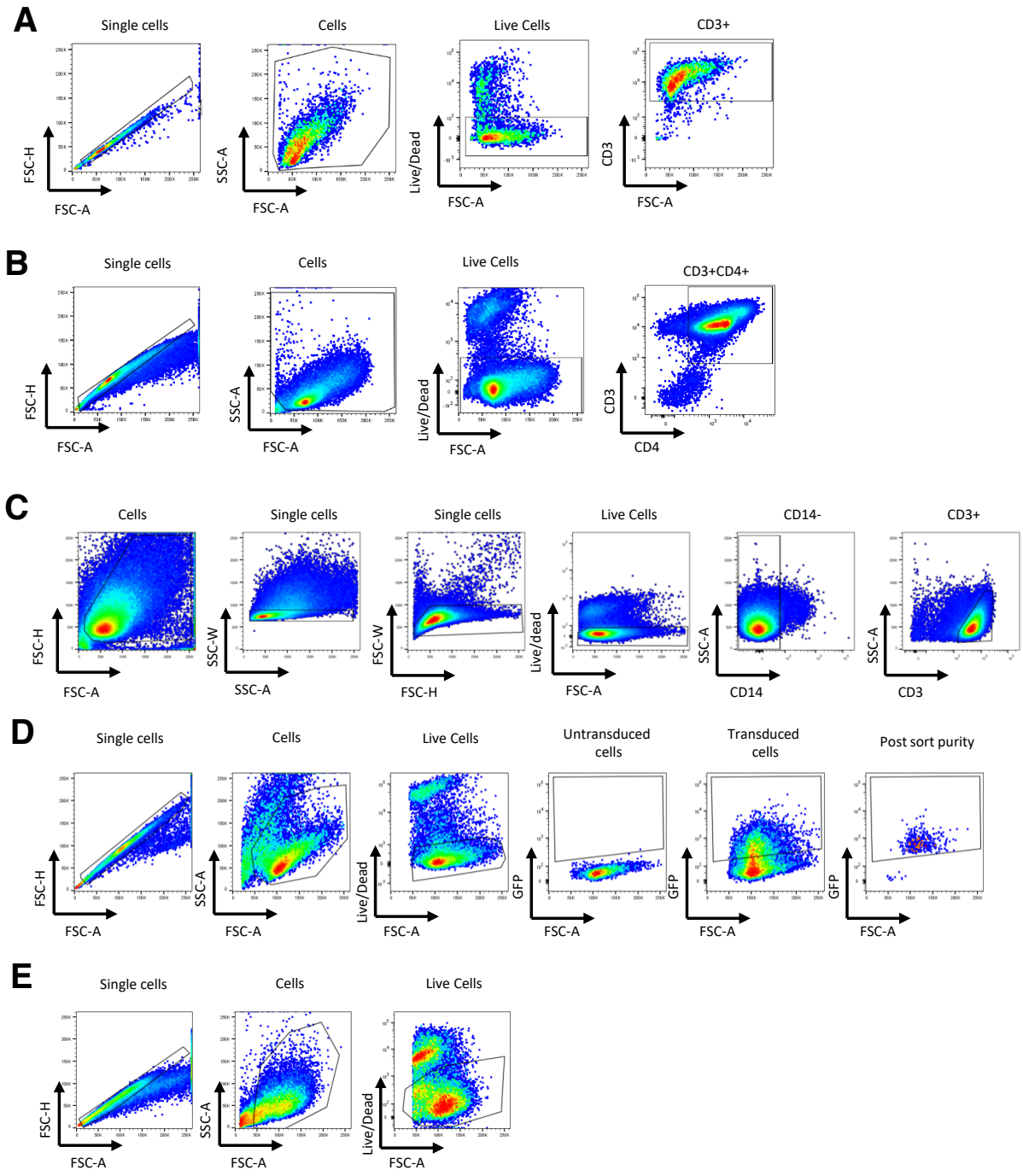
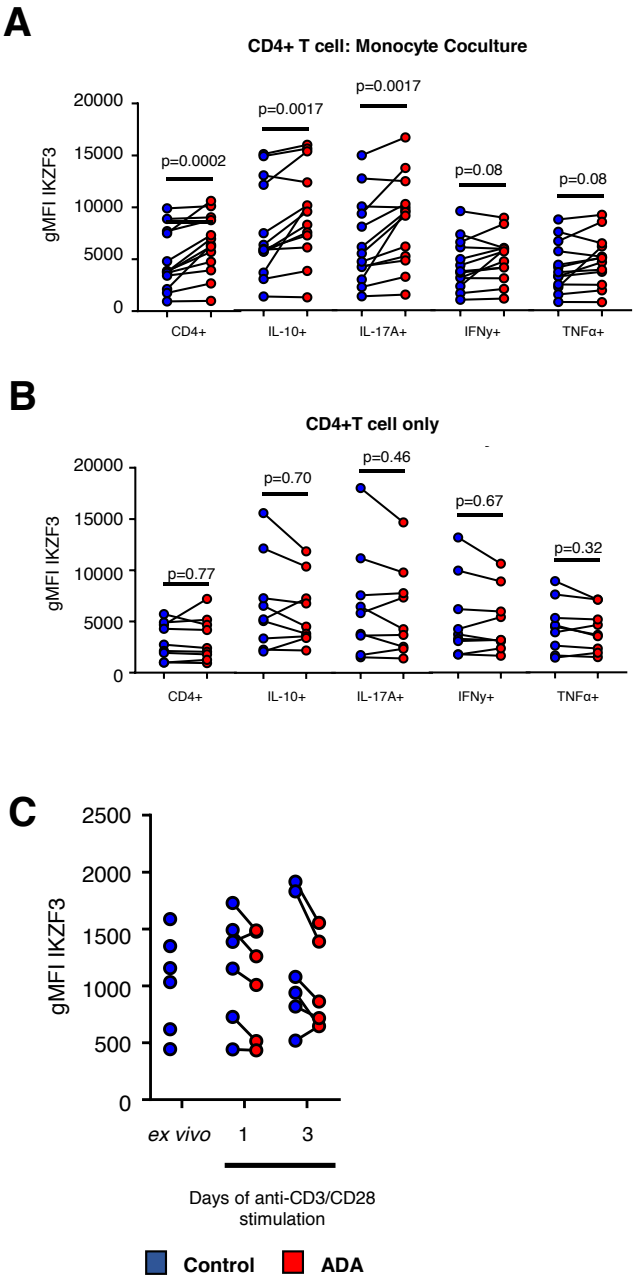


Supplemental Figure 1. Gating strategy for flow cytometry data. (A, B) CD4+ T cells were isolated from the peripheral blood of healthy donors and gated on live (A) CD3+ events or (B) CD3+ CD4+. This gating strategy was used in Figures 1A and B, 2A-F, 3B-D, Supplemental Figure 2B, Supplemental Figure 3A and B. (C) CD4+ T cells were isolated from the peripheral blood of healthy donors and cultured at a 1:1 ratio with autologous CD14+ monocytes and gated on Live CD14- CD3+ Events. This gating strategy was used in Supplemental Figure 2A. (D) CD4+ T cells were stimulated and transduced with lentiviral particles for 7 days and then sorted on Live GFP+ CD3+ events by a FACS ARIA. The depicted gating strategy was used in the analysis of Figure 4C and D. (E) HEK293T cells were transfected with plasmids encoding IKZF3 or cMAF as well as GFP. These cells were subsequently stained for viability, IKZF3 and cMAF and gated on Live cell events. This gating strategy was used in Supplemental Figure 4A and B.



Supplemental Figure 2. TNF blockade does not alter IKZF3 expression in CD4+ T cells in the absence of monocytes. (A) CD4+ T cells and CD14+ monocytes from healthy donors were cultured together at a 1:1 ratio for 3 days with anti-CD3 mAb in the absence (blue circles) or presence (red circles) of 1 μg/mL adalimumab. Cells were restimulated with PMA and ionomycin and assessed for cytokine and IKZF3 expression (n=13). (B) Primary CD4+ T cells were stimulated with anti-CD3/CD28 mAb for 3 days in the absence or presence of 1μg/mL adalimumab. Cells were restimulated with PMA and ionomycin and assessed for cytokine and IKZF3 expression (n= 9). (C) Primary CD4+ T cells were stimulated with anti-CD3/CD28 mAb for 0, 1 or 3 days in the absence or presence of 1μg/mL adalimumab and assessed for IKZF3 expression (n=6). Data in A and B analysed by Wilcoxon test, data in C analysed by Friedman test.

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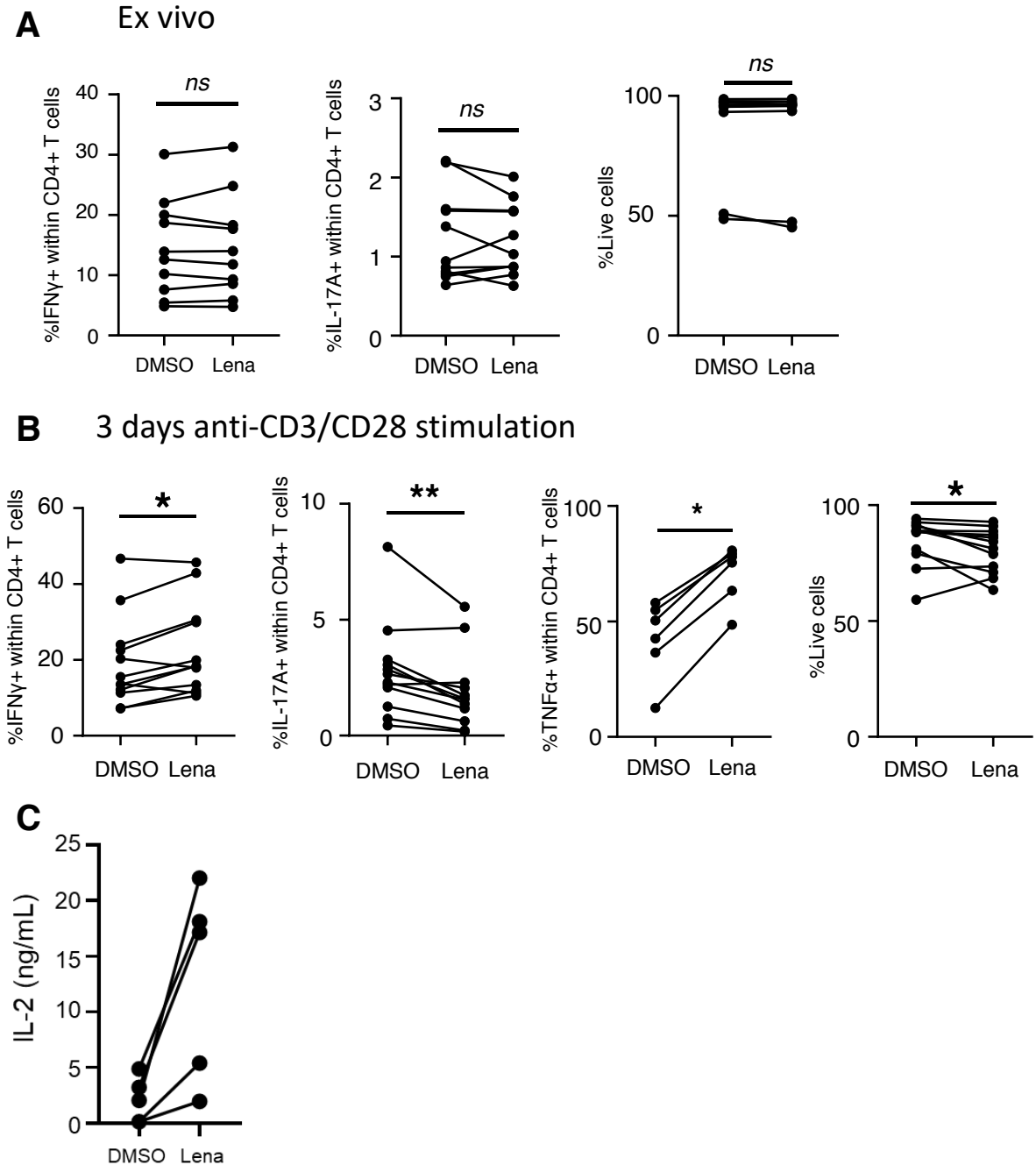
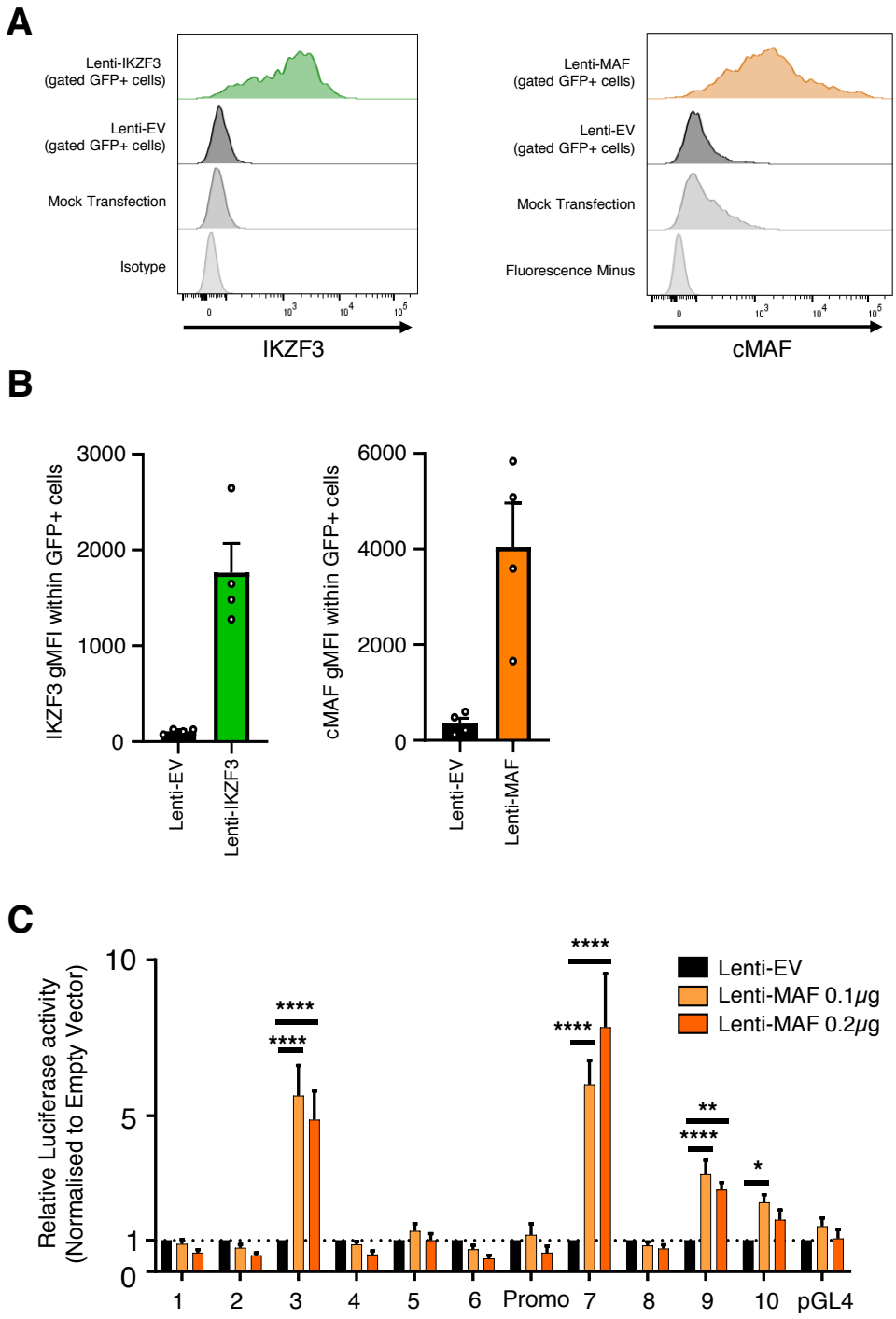


Supplemental Figure 3. The effect of lenalidomide on CD4+ T cell cytokine production and viability. (A) CD4+ T cells were treated with 1 µM lenalidomide overnight and then stimulated with PMA and ionomycin and assessed for frequencies of IFNγ+, IL-17A+ and live CD4+ T cells (n=10-11). (B and C) CD4+ T cells were stimulated with anti-CD3/CD28 mAb for 3 days in the presence of 1 µM lenalidomide or DMSO control and (B) subsequently restimulated with PMA and ionomycin and assessed for IFNγ+, IL-17A+, TNF+ and live CD4+ T cells (by staining with Live/Dead discriminator dye). (C) Cell culture supernatants of CD4+ T cells treated with DMSO or lenalidomide were assessed for IL-2 secretion (n=5). Data analysed by Wilcoxon test.



Supplemental Figure 4. Overexpression of proteins in HEK293T cells. (A,B) HEK293T cells were transfected with 2 µg of Lenti-IKZF3, Lenti-EV or Lenti-MAF for 48 hours and assessed for IKZF3 or cMAF expression by flow cytometry. (A) Representative histograms and (B) cumulative data (n=4) are shown. (C) HEK293T cells were transfected with the putative IL10 enhancer and promoter reporter plasmids in addition to Lenti-EV or Lenti-MAF (n=4). Data in C analysed by 2-way ANOVA with multiple comparisons.

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