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 TNF-α blockade during T cell stimulation in CD4+ T cell/monocyte cocultures resulted in maintenance of IL-10–producing T cells and identified IKZF3 as a putative regulator of IL-10. In this study, 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 with 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.

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

In this study we sought to address the hypothesis that IKZF3 is a transcriptional regulator of 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 reference 06/Q0705/20). PBMCs were isolated using density gradient centrifugation. CD4+ T cells and CD14+ monocytes were isolated by MACS using the manufacturer’s protocol. CD14+ monocytes were isolated using anti-CD14+ microbeads to >98% purity (Miltenyi Biotec), and CD4+ T cells were isolated using negative selection to >95% purity (Miltenyi Biotec).

Cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10% FCS and 1% penicillin-streptomycin and 10 mg/ml l-glutamine (culture medium). CD4+ T cell cultures were stimulated with anti-CD3/CD28 mAb OKT3 (Janssen-Cilag) in PBS for 3 h at 37˚C. Wells were washed with sterile PBS before adding the cells (1 × 10^6 cells/ml) together with 1 μg/ml anti-CD28 mAb (clone CD83-2; BD Biosciences). For cocultures, 0.5 × 10^6 CD14+ peripheral blood monocytes were cultured with 0.5 × 10^6 autologous CD4+ T cells in 1 ml of culture medium in the presence of 100 ng/ml anti-CD3 mAb (OKT3). HEK293T cells (gifted from the Stuart Neil laboratory, King’s College London, London, U.K.) were cultured in DMEM supplemented with 10% FCS, 1% penicillin-streptomycin, and 10 mg/ml l-glutamine. Where indicated, adalimumab (ADA; obtained from Guy’s Hospital Pharmacy) was added at 1 μg/ml.

Flow cytometry

For intracellular cytokine staining, CD4+ T cells or CD4+ CD25+ T cell/monocyte cocultures were stimulated for 3 h in the presence of PMA (50 ng/ml; Sigma-Aldrich), ionomycin (750 ng/ml; Sigma-Aldrich), and GolgiStop (as per the manufacturer’s instructions; BD Biosciences). Cells were washed and stained with CD3-PE Cy7 (UCHT1; BioLegend) and LIVE/DEAD efluor 780 (Thermo Fisher Scientific). Cells were then fixed in 2% PFA and permeabilized with 0.5% saponin (Thermo Fisher Scientific). Alexa Fluor 647 (EPR9342[B]; Abcam) or isotype control (EPR25A; Abcam) before being stained for CD3-PE Cy7, IL-10–Alexa Fluor 488, IL-17A–PE, and FOXP3 staining buffer (BioLegend) for 15 min at room temperature before being stained for CD3-PE Cy7, IL-17A–PE, IFN-γ–Pacific blue (4S.B3; BioLegend), and TNF–allophycocyanin (Ab1b1; BioLegend).

For intranuclear staining of IKZF3, cells were fixed and permeabilized with FOXP3 staining buffer (BioLegend) for 15 min at room temperature before being stained for CD3-PE Cy7, IL-10–Alexa Fluor 488, IL-17A–PE, IFN-γ–Pacific blue, TNF-BV605 (Ab1b1; BioLegend), and either IKZF3-Alexa Fluor 647 (EPR9342[B]; Abcam) or isotype control (EPR25A; Abcam) for 30 min. Standard gating strategy for intracellular cytokine staining is shown in Supplemental Fig. 1A–C.

RNA isolation and quantitative PCR

mRNA was isolated using an RNeasy Mini Kit (QIAGEN). cDNA was transcribed using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s protocol. Real-time PCR was performed using SensiFAST SYBR Green PCR Master Mix (Bioline) with 10 μM primers (Table I). Reactions were performed in multiple technical replicates, and results were calculated using the Δ cycle threshold method.

Actinomycin D assay

CD4+ T cells were stimulated with anti-CD3/CD28 mAb and cultured in the presence or absence of 1 μg/ml ADA for 72 h. After stimulation, the cells were treated for 2 h with either 1 μg/ml actinomycin D (Cambridge Bioscience) or an equivalent volume of DMSO. Cells were subsequently harvested for RNA and assayed for gene expression by quantitative PCR (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 then concentrated using PEG-it (Cambridge Bioscience) according to the manufacturer’s instructions.

Primary CD4+ T cells were activated with plate-bound anti-CD3 and anti-CD28 mAb (2 μg/ml) with 20 U/ml recombinant human IL-2 (PeproTech) for 24 h at a density of 1 × 10^6 cells/ml. Viral supernatants were mixed with TransDux MAX (Cambridge Bioscience), added to the cells, and cultured. After 3 d, the cells were supplemented with fresh 10% FCS RPMI 1640 and 20 U/ml recombinant human IL-2 and rested from stimulation for 3 d. These cells were subsequently sorted on live CD3+ GFP+ cells (Supplemental Fig. 1D). Cells were rested overnight at a density of 1 × 10^6 cells/ml, then stained for IL-10, IL-17A, IFN-γ, and IKZF3.

Plasmids and cloning

The selected regions of the human IL10 locus (indicated in Table II) were amplified by PCR using the BAC RP11-262S9 (Thermo Fisher Scientific) as a template, and TOPO-cloned into pCR-Blunt II-TOPO (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-MAF-pCMV was a gift from P. Lavender (King’s College London), 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. On the next day, each well was transfected with 1 μg of polyethylenimine (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 h of transfection, the cell culture media was replaced and left for a further 48 h before harvesting the cells.

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

Statistical analysis

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

Results

TNF-α blockade maintains IL10 transcription in CD4+ T cells

We previously observed a transient increase in the frequency of IL-10+ CD4+ T cells when PBMCs were stimulated with anti-CD3 mAb, which was maintained in the presence of TNF-α blockade (10, 25). Because we aimed to use 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 plate-bound anti-CD3 and soluble anti-CD28 mAb for 1–3 d with or without the anti-TNF-α Ab ADA before being restimulated with PMA and ionomycin for intracellular cytokine staining (representative gating strategies are shown in Supplemental Fig. 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 TNF-α blockade (Fig. 1A, 1B). To rule out a possible artifact 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 (Table I). 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 (Fig. 1C). We also observed IL-10 secretion in the cell culture supernatant upon 3 d of anti-CD3/CD28 mAb stimulation that was significantly increased in the presence of anti-TNF-α (Fig. 1D).

IL10 mRNA has been shown previously to be controlled at the posttranscriptional level (26). To determine whether IL10 mRNA was stabilized by TNF-α blockade, we performed an actinomycin D assay on CD4+ T cells stimulated with anti-CD3/CD28 mAb for
3 d. 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 (Fig. 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 TNF-α blockade and could bind at the IL10 locus in these cells (10). To examine whether

Table I. qPCR primer sequences used in this study

| Gene Name | Forward Primer Sequence | Reverse Primer Sequence |
|-----------|-------------------------|-------------------------|
| UBC       | 5’-CGGGAATTTGGTTGGCACTTCTTGG-3’ | 5’-CGTGCTGTTACTGACTGCA-3’ |
| GAPDH     | 5’-GTCAGCAGATGTCTTGACAC3’ | 5’-AAGCTCTACCCCTGGAAC3’ |
| B2M       | 5’-GTATGACCTGGCATGCAC-3’ | 5’-AATGCAGCTACTGGCAGCA-3’ |
| IL10      | 5’-GCTTACCATGTCTGAGATC-3’ | 5’-TGACAGTTGACCTGAGGG-3’ |
| IKZF3     | 5’-AGCCGCAACACTGGAAAGA-3’ | 5’-TGGGCCTGCTACCATGGCT-3’ |
| IL2RA     | 5’-ACAAGCTCTGCAACTGGAAC-3’ | 5’-AGCCCTGTATCCCTGGACAG-3’ |
| MYC       | 5’-TAGTGAAAACCAGGCTCCC-3’ | 5’-GGCCGAGCCTGAACTTCTT-3’ |
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, IFN-γ, or TNF-α, either ex vivo or after 3 d of anti-CD3/CD28 mAb stimulation. IKZF3 was expressed at higher levels in IL-10⁺ CD4⁺ T cells compared with the total CD4⁺ T cell population and the IL-17A⁺ and TNF-α⁺ subsets ex vivo (Fig. 2A, 2B). Upon anti-CD3/CD28 mAb stimulation, a significant increase was observed in IKZF3 expression in IL-10⁺ expressing cells compared with the total CD4⁺ and the TNF-α⁺ cell populations. However, there was no longer a significant difference between IL-10⁺ and IL-17A⁺ CD4⁺ T cells (Fig. 2C, 2D). Because IL-10 can be expressed by multiple cytokine-producing CD4⁺ T cell subsets (especially after stimulation), we compared IKZF3 expression in the IL-17A⁺, TNF-α⁺, and IFN-γ⁺ cells that coproduced IL-10 and those that did not (Fig. 2E, 2F). In all subsets analyzed, a significantly higher expression of IKZF3 was observed in IL-10⁺-coproducing CD4⁺ T cells compared with cells that did not produce IL-10 (Fig. 2F).

In our previous work, we observed an increase in IKZF3 expression in Th17 cells following TNF-α blockade using a CD14⁺ monocyte/CD4⁺ T cell coculture system. To determine whether the increase in IKZF3 upon TNF-α 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 cocultured with CD14⁺ monocytes in the absence or presence of ADA (Supplemental Fig. 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 Fig. 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 TNF-α blockade did not alter IKZF3 expression in these cells (Supplemental Fig. 2B, 2C). These data indicate that in CD4⁺ T cell–only cultures, the anti–TNF-α–mediated increase of IL-10 can occur in the absence of a concomitant increase in IKZF3 expression.

**FIGURE 2.** IKZF3 is associated with IL-10-producing CD4⁺ T cells. (A and B) Primary CD4⁺ T cells from healthy donors were stimulated with PMA and ionomycin and assessed for frequency of cytokine-producing cells and IKZF3 expression. Representative gMFI of IKZF3 expression shown on the right of each histogram (A) and cumulative data (n = 8) shown (B). (C-F) CD4⁺ T cells were stimulated with anti-CD3/CD28 mAb for 3 d and subsequently restimulated with PMA and ionomycin and assessed for frequency of cytokine-producing cells and IKZF3 expression. Expression of IKZF3 was calculated within total populations of cytokine-producing cells (C and D) or within IL-10⁺ or IL-10⁻ subsets within those populations (E and F). Representative gMFI of IKZF3 expression shown on the right of each histogram (C and D) or data of total cytokine-producing populations are shown. Representative gMFI of IKZF3 expression shown on the right of each histogram (E) and cumulative (n = 8–11) (F) data for IKZF3 expression within IL-10⁺ and IL-10⁻ subsets are shown. Data in (B) and (D) were analyzed by ANOVA, data in (F) was analyzed by Wilcoxon test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
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 small interfering RNA in primary CD4⁺ T cells. However, this approach did not work because of 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 transducible; data not shown). As an alternative approach, we employed the thalidomide derivative lenalidomide, 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 (Fig. 3A) and flow cytometry (Fig. 3B). CD4⁺ T cells were then treated with lenalidomide for 24 h in the absence of T cell activation followed by intracellular cytokine staining. Although a significant reduction in the levels of IKZF3 was observed, the frequency of IL-10⁺ cells within CD4⁺ T cells was slightly increased (Fig. 3C). Ex vivo treatment of CD4⁺ T cells with lenalidomide had no effect on IL-17A and IFN-γ expression or viability (Supplemental Fig. 3A). In contrast, when CD4⁺ T cells were treated with lenalidomide for 3 d 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 (Fig. 3D). These data indicate that, whereas IL-10 production in unstimulated CD4⁺ T cells is not lenalidomide-sensitive, the anti-CD3/CD28 mA–mediated increase in IL-10–expressing cells is lenalidomide-sensitive and thus, by extrapolation, potentially regulated by IKZF3. Lenalidomide treatment for 72 h also resulted in statistically significant increases in IFN-γ and TNF-α frequencies, a decrease in IL-17A⁺ frequencies, and a slight decrease in cell viability (median viability: 88.9–82.75%, control versus lenalidomide, respectively; Supplemental Fig. 3B). Treatment of CD4⁺ T cells with lenalidomide also consistently increased secretion of IL-2 by CD4⁺ T cells after 3 d of anti-CD3/CD28 mAb stimulation (Supplemental Fig. 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 (Fig. 4A). After transduction, live GFP⁺ cells were sorted for mRNA isolation or rested and stimulated with PMA/ionomycin for intracellular cytokine staining. Although cells transduced with IKZF3 showed a significant increase in IKZF3 transcript, IL10 mRNA levels were low and not consistently increased by IKZF3 overexpression (Fig. 4B). Also, at the protein level, IKZF3-transduced cells did not show a consistent increase in IL-10–producing cells compared with the empty vector (Fig. 4C, 4D). A considerable proportion of cells was able to produce IFN-γ 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 IL-10

Our previous work showed that IKZF3 is able to bind evolutionarily 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 (Fig. 5A), as defined by accessible chromatin (31), high H3K4me1, and low CpG density. We next used chromatin immunoprecipitation (ChIP)–seq analysis of human CD4⁺ T cells to determine whether IKZF3 physically interacts with these 10 sites in vivo. As shown in Figure 5B, a significant enrichment of IKZF3 was observed only at one site (site 1) and not at any of the other sites (Supplemental Fig. 5A, n = 3). To determine whether IKZF3 could drive expression of IL10 via site 1, we stimulated CD4⁺ T cells with an anti-CD3/CD28 antibody and measured IL10 mRNA levels by qPCR. As shown in Figure 5C, 5D, 5E, and 5F, no significant increase in IL10 mRNA levels was observed in IKZF3-transduced cells (Fig. 5C) and no significant increase in IL10 production was observed when CD4⁺ T cells were treated with lenalidomide (Fig. 5D). These results indicate that IKZF3 is insufficient to drive expression of IL10 via site 1.
methylation [from the BLUEPRINT consortium (32)]. We cloned these regions (genomic coordinates of cloned regions defined in Table II) and a 1.5-kb region of the IL10 promoter upstream of a Firefly luciferase open reading frame (pGL4). These vectors were then cotransfected with a control Renilla luciferase vector together with the plasmids lenti-IKZF3 (Fig. 5B) or lenti-MAF (Supplemental Fig. 4B, 4C), 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 Fig. 4A, 4B) 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 (Fig. 5B). An induction of reporter gene expression in response to IKZF3 transfection was only seen for enhancer 10, whereas 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 with the empty vector (Supplemental Fig. 4C).

**Discussion**

Regulation of IL-10 expression is a multilayered process at the levels of transcription (33, 36), posttranscriptional 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–TNF-α 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). To understand what drives the transcriptional regulation of IL10, we focused on IKZF3. Our previous work with a CD4+ T cell:CD14+ monocyte coculture system showed increased IKZF3 expression upon TNF-α 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 TNF-α blockade, although we did observe 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 TNF-α blockade. In our study, IKZF3 was highly expressed in IL-17A + IL-10+ CD4+ T cells. The expression of IKZF3 and IL-10 in nonpathogenic 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 TNF-α inhibitor drug etanercept, in the absence of monocytes, showed an increased expression of IL10 upon TNF-α blockade that 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, mass cytometry data from our laboratory did not reveal a higher expression of IKZF3 in CD25high CD127low Tregs compared with

Table II. UCSC Genome Browser hg19 coordinates for regions used in cloning putative IL10 enhancer and promoter regions

| Region Name | Region Coordinates (hg19) | Forward Primer Sequence | Reverse Primer Sequence |
|-------------|--------------------------|-------------------------|-------------------------|
| Chr1        |                          | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 2           | 2069010301–206910900     | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 3           | 206912926–206913525      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 4           | 20690851–206931750       | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 5           | 206939626–206940225      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 6           | 206942701–206943150      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 7           | 206945773–206947172      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 8           | 206957626–206958150      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 9           | 206964826–206965275      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |
| 10          | 206976226–206977725      | 5′-TTGCTAGCTTTTGGCCAATGAGGAGACATCAGATCAGAATCA-3′ | 5′-AATTAGGAGAATGAGGAGACATCAGATCAGAATCA-3′ |

FIGURE 5. IKZF3 is insufficient to drive transcriptional activity via the IL10 promoter or local enhancers. (A) Chromatin immunoprecipitation sequencing (from the BLUEPRINT consortium) and assay for transposase-accessible chromatin using sequencing (ATAC-seq) data (from Ref. 31) 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 1.50-kb promoter region of IL10 (vertical green bar labeled 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 h later for luciferase activity (n = 4). Data were analyzed by two-way ANOVA. *p < 0.05, **p < 0.001, ***p < 0.0001.
CD25low CD127high effecter T cells ex vivo (data not shown). Furthermore, upon TNF-α blockade we did not observe an increase in FOXP3+ Tregs (10).

Studies have implicated IL-2 signaling to be required for IL-10 expression by multiple Th subsets in vitro (L. Gabryśová, E.H. Mann, L. Bradley, J.I. MacRae, C. Whicher, C.M. Hawrylowicz, D. Anastasiou, and A. O’Garra, manuscript posted on bioRxiv). 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+, TNF-α-, and IFN-γ-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 (6, 44, 45). IL-2 has also been shown to increase the expression of IFN-γ in human CD4+ T cells (46, 47) and TNF-α expression in mouse CD8+ T cells (48). It should be noted, however, that expression of IFN-γ and TNF-α can be suppressed by IL-10 (49). Therefore, the decrease in IL-10 expression accompanying lenalidomide treatment could boost the induction of IFN-γ- and TNF-α-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 (50–52). 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.

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 whether 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 cofactors to promote transcription, such as BLIMP1 (12) and STAT3 (13), which have been shown to interact with IKZF3. Encyclopedia of DNA Elements (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 cofactors 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 assay for transposase-accessible chromatin using sequencing (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 that IKZF3 can bind to drive expression.

It should be noted that IKZF3 has a number of splice variants that have various 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 follicular helper cell–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, whereas TNF-α 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 TNF-α blockade that lead to increased IL10 expression and whether such transcriptional regulation occurs in patients treated with TNF-α inhibitors.

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Disclosures

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