Neutrophil Extracellular Traps Induce Human Th17 Cells: Effect of Psoriasis-Associated *TRAF3IP2* Genotype

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Psoriasis lesions are rich in IL-17−producing T cells as well as neutrophils, which release webs of DNA-protein complexes known as neutrophil extracellular traps (NETs). Because we and others have observed increased NETosis in psoriatic lesions, we hypothesized that NETs contribute to increased T helper type 17 (Th17) cells in psoriasis. After stimulating peripheral blood mononuclear cells with anti-CD3/CD28 beads for 7 days, we found significantly higher percentages of $CD3^+CD4^+IL-17^+$ (Th17) cells in the presence versus absence of NETs, as assessed by flow cytometry, IL-17 ELISA, and *IL17A/F* and *RORC* mRNAs. Memory, but not naïve, T cells were competent and monocytes were required for CD3/CD28-mediated Th17 induction, with or without NETs. Th17 induction was enhanced by the T allele of rs33980500 (T/C), a psoriasis risk-associated variant in the *TRAF3IP2* gene encoding the D10N variant of Act1, a key mediator of IL-17 signal transduction. Global transcriptome analysis of CD3/CD28-stimulated peripheral blood mononuclear cells by RNA sequencing confirmed the stimulatory effects of NETs, demonstrated NET-induced enhancement of cytokine gene expression, and verified that the effect of Act1 D10N was greater in the presence of NETs. Collectively, these results implicate NETs and the Act1 D10N variant in human Th17 induction from peripheral blood mononuclear cells, with ramifications for immunogenetic studies of psoriasis and other autoimmune diseases.

INTRODUCTION

IL-17−expressing T cells (T17 cells) are characterized by the production of IL-17 and are considered to have evolved to protect against extracellular bacteria and fungi (Tesmer et al., 2008). However, recent discoveries have shifted focus to the central role of T17 cells in the pathogenesis of autoimmune and inflammatory disorders (Amatya et al., 2017). The role of IL-17 in the pathogenesis of psoriasis is evidenced by genetic (Harden et al., 2015), immunologic (Kryczek et al., 2008; Lowes et al., 2008), and pharmacologic studies (Lowes et al., 2014; Mease, 2015). The clinical effectiveness of drugs targeting IL-23, a cytokine required for the survival and expansion of human T17 cells (Dolgin, 2016; Wilson et al., 2007), further supports the importance of T17 cells as a key target of psoriasis management.

In addition to T17 cells, neutrophils are increased in psoriasis lesions as well, appearing early in developing lesions and accumulating within Munro's microabscesses and spongiform pustules of Kogoj in the epidermis. Cross-talk between neutrophils and T17 cells has been suggested, with T17-associated cytokines increasing the development, recruitment, and lifespan of neutrophils (Kalyan and Kabelitz, 2014; Pelletier et al., 2010; Reich et al., 2015), and neutrophils signaling to T cells via dendritic cells (Amulic et al., 2012) and macrophages (Warnatsch et al., 2015). While the precise role of neutrophils in psoriasis pathophysiology is not well understood, considerable clinical data indicate their in vivo relevance. The drug razoxane, which depresses neutrophil counts in a dose-dependent manner, was highly effective in treating cutaneous and arthritic disease in 35 patients, with an initial response rate of 97% in cutaneous disease (Atherton et al., 1980). Similarly, remission was observed in a psoriatic patient with ticlopidine-induced agranulocytosis, followed by the reappearance of plaques upon neutrophil repletion (Toichi et al., 2000). Dimethylfumarate, a well-known psoriasis treatment, inhibits neutrophil function in vitro and in vivo (Muller et al., 2016). Dapsone (an anti-neutrophil drug) has been successfully used to treat pustular psoriasis (Sheu et al., 2016), as has granulocyte/monocyte leukapharesis (Fujisawa et al., 2014, 2015).

Neutrophils undergo a specialized form of cell death termed *NETosis*, in which protein-binding webs of decondensed chromatin extrude from the neutrophil (Branzk and Papayannopoulos, 2013). It has been proposed that NETs

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Abbreviations: DEG, differentially expressed gene; LPS, lipopolysaccharide; NET, neutrophil extracellular trap; PBMC, peripheral blood mononuclear cells; PMA, phorbol myristate acetate; Th, T helper

eliminate bacteria, fungi, and parasites by physically trapping them in an environment with high local concentrations of antimicrobials (Brinkmann et al., 2004; Papayannopoulos and Zychlinsky, 2009; Remijsen et al., 2011). Others have emphasized their role as "sentinels" of innate immunity (Sorensen and Borregaard, 2016). Although the mechanism is not yet fully understood, nicotinamide adenine dinucleotide phosphate oxidase, myeloperoxidase, neutrophil elastase, and histone citrullination are important for NET formation (Papayannopoulos et al., 2010; Patel et al., 2010; Wang et al., 2009). NETs are prominent within psoriasis lesions (Hu et al., 2016; Lin et al., 2011), and they are markedly increased in neutrophils prepared from blood of psoriasis patients (Hu et al., 2016). NETs are also present in several other autoimmune disorders, including systemic lupus erythematosus (Garcia-Romo et al., 2011), small-vessel vasculitis (Kessenbrock et al., 2009), and rheumatoid arthritis (Khandpur et al., 2013). Based on these observations, we sought to address the possible role of NETs in psoriasis pathogenesis. To this end, we developed and characterized a method for induction of T helper type 17 (Th17) cells from human peripheral blood mononuclear cells (PBMC), and used it as a model to explore the role of NETs as modulators of this process. We also used the model to assess the effect of a missense psoriasis-associated genetic variant in the *TRAF3IP2* gene.

RESULTS

NETs promote the induction of Th17 cells from PBMC

To assess the role of NETs in T17 induction, neutrophils isolated from healthy donors were plated on poly-L-lysine—coated wells incubated in the absence or presence of 5 ng/ml *E. coli* lipopolysaccharide (LPS) to induce NETosis. PBMC, isolated from the same blood samples, were seeded on these NET-coated wells, or on poly-L-lysine—coated wells without NETs, with or without anti-CD3/anti-CD28 beads to effect polyclonal T-cell activation. After 7 days of culture, flow cytometric analysis revealed a significant ($P = 0.0088$), 1.6-fold increase in the percentage of $CD3^+CD4^+IL\text{-}17^+$ cells (i.e., Th17 cells) cells when CD3/CD28-stimulated PBMC were exposed to NETs that formed in the absence of LPS, increasing to 2.4-fold in the presence of LPS ($P = 4.3 \times 10^{-7}$) (Figure 1a and Supplementary Figure S1a online). $CD3^+CD8^+IL\text{-}17^+$ cells (Tc17) cells were nearly absent (data not shown). ELISA assays revealed increased secreted IL-17A protein by co-culture with NETs, reaching significance for LPS-treated NETs (1.9-fold, $P = 0.009$) compared to CD3/CD28 stimulation alone (Figure 1b and Supplementary Figure S1b online). We also observed corresponding increases in *IL17A, IL17F,* and *RORC* mRNA levels (Figure 1c and Supplementary Figure S1c). Th17 cells are known to express CD161, also known as the killer cell lectin—like receptor B1 (Kleinschek et al., 2009). As shown in Figure 1d and Supplementary Figure S1d (online), cell surface CD161 expression in $CD3^+CD4^+$ T cells was increased 7-fold in co-cultures with spontaneous NETs ($P = 8.1 \times 10^{-5}$) and 9.2-fold in co-cultures with LPS-induced NETs ($P = 4 \times 10^{-4}$) compared to CD3/CD28 stimulation alone. CD3/CD28 stimulation was also effective in increasing the percentage of $CD3^+CD4^+IFN\text{-}\gamma$—expressing Th1 cells. However, unlike Th17 cells, there was no further increase in the percentage of Th1 cells as a function of NET exposure (Supplementary Figure S1e online).

NETs presented a similar morphology in the presence or absence of LPS (Supplementary Figure S2a online), with the effect of LPS being most evident at early time points. Quantitation of DNA released from NETs revealed that treatment with 5 ng/ml LPS for 4 hours did not significantly increase NETosis (Supplementary Figure S2b). We also generated NETs using phorbol myristate acetate (PMA), another frequently used stimulus for NET formation (Khan et al., 2017). Interestingly, while 25 nM PMA was highly effective in inducing NETosis (Supplementary Figure S2b), the resulting NETs were completely ineffective in inducing Th17 cells (Supplementary Figure S2c).

Given that LPS has been reported to enhance Th17 induction from PBMC (Evans et al., 2007), one concern was that LPS might be retained on the NETs, thereby influencing myeloid cell activation. To address this question, we measured LPS levels after washing of NETs using the limulus amebocyte lysate endotoxin assay. We found that LPS was undetectable in media immediately after NET washing, as well as on day 7 (all <0.01 ng/mL, n = 3). Additionally, we found that addition of 5 ng/ml LPS for the duration of the 7-day assay did not increase the percentage of Th17 cells, whether in the presence or absence of NETs (Supplementary Figure S3a online). To ask whether the effects of NETs on Th17 induction might also be observed using live, intact neutrophils as opposed to NETs, we cultured CD3/CD28-stimulated PBMC in the presence or absence of autologous neutrophils for 7 days. We found no significant difference in comparison to CD3/CD28-stimulated PBMC cultured without neutrophils (Supplementary Figure S3b). The purified neutrophils were viable at the time of addition, as demonstrated by Trypan blue exclusion (6.0% ± 1.2% of neutrophils were Trypan blue-positive, n = 3).

As shown in Figure 2a, the $IL\text{-}17^+$ population was limited to 0.4% of the $CD4^+$ T-cell population at the beginning of the culture and was not significantly increased after 7 days of culture in the absence of CD3/CD28 stimulation. Moreover, NETs alone did not significantly increase the proportion of Th17 cells in the absence of CD3/CD28 stimulation. We also analyzed Th17 cell divisions after 7 days of culture using a dye dilution assay. There was no significant difference across the three CD3/CD28-stimulated conditions when considered as a group (Figure 2b). On average, fewer than two divisions of $IL17^+$ T cells took place over the culture period, which would not appear to be sufficient to achieve the observed percentages of Th17 cells.

NET-induced Th17 induction is monocyte-dependent and requires cell-to-cell contact

We used immunomagnetic bead purification to characterize the monocyte dependence of Th17 induction by depletion experiments. As shown in Figure 3a, depletion of monocytes from PBMC abrogated the induction of Th17 cells after CD3/CD28 activation, whether in the presence or absence of NETs. This monocyte requirement was also observed in ELISA assays for IL-17 protein (Figure 3b) and quantitative reverse transcriptase PCR assays for *IL-17A, IL-17F,* and *RORC*
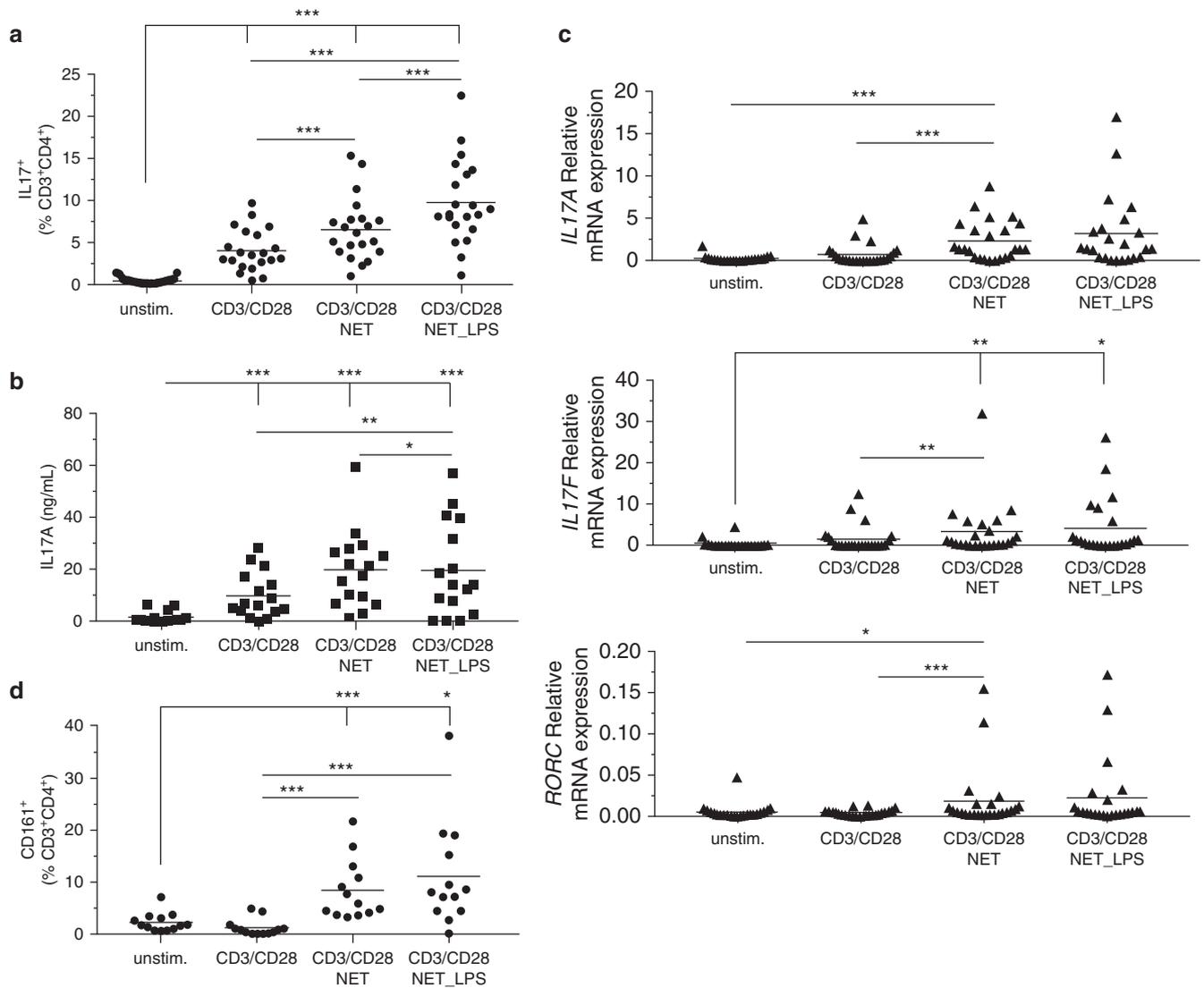
Figure 1. Neutrophil extracellular trap promote CD3/CD28 induction of Th17 cells from peripheral blood mononuclear cells. Unstimulated peripheral blood mononuclear cells, and peripheral blood mononuclear cells activated with anti-CD3/CD28 beads in presence of neutrophil extracellular trap or lipopolysaccharide-induced neutrophil extracellular traps, were incubated for 7 days followed by (**a**) flow cytometry to determine the percentage of IL-17$^+$ T cells; (**b**) ELISA to determine secreted IL-17A protein; (**c**) quantitative reverse transcription PCR to determine *IL-17A, IL-17F*, and *RORC* mRNA levels; and (**d**) flow cytometry to determine the percentage of CD161$^+$ T cells. Means are indicated by horizontal lines. Statistics: one-way repeated measures analysis of variance was performed on transformed data (**a** and **d**, logit; **b**, log (ln); **c**, log$_2$), with all *P* values corrected to maintain experiment-wise type I error rate for the tested factor. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Graphical results for the transformed data and a summary of statistically significant outcomes are presented in Supplementary Figure S1. LPS, lipopolysaccharide; NET, neutrophil extracellular trap.

mRNAs (Figure 3c). We performed several experiments to ask whether the observed change in the Th17 population was due to differentiation of naïve T cells or the induction or expansion of Th17 cells from an existing population. As shown in Figure 3d, depletion of memory T cells greatly diminished acquisition of a Th17 phenotype, whereas depletion of naïve T cells had no effect. We then performed Transwell experiments to ask whether the Th17 induction was due to soluble factors or contact between cells. We found that the Th17 induction did not occur when the CD4$^+$ T cells or the monocytes were not in contact with the other cell types (Figure 3e). Taken together, these results suggest that Th17 cells are being expanded and/or induced from pre-existing memory T cells, rather than by differentiation of naïve T cells, and that the presence of monocytes as well as physical contact between the different cell types involved is necessary.

Effect of *TRAF3IP2* variation on NET-induced Th17 induction

We and others reported that a coding variant of the *TRAF3IP2* gene encoded by single nucleotide polymorphism rs33980500T/C is associated with increased risk for psoriasis (Ellinghaus et al., 2010; Huffmeier et al., 2010). *TRAF3IP2* encodes Act1, an adaptor with ubiquitin ligase activity that connects the IL-17 receptor to downstream signaling pathways (Qian et al., 2007). rs33980500 T causes an amino acid change from aspartic acid to asparagine residue at position 10 (Act1 D10N) that interferes with binding to TRAF2, TRAF3, and TRAF6 (Wang et al., 2013).

We investigated the effects of NETs on Th17 induction and IL-17 production in donors carrying each of the three genotypes for the Act1 D10N variant. In the presence of spontaneous NETs, both the percentage of IL-17$^+$ T cells (Figure 4a)
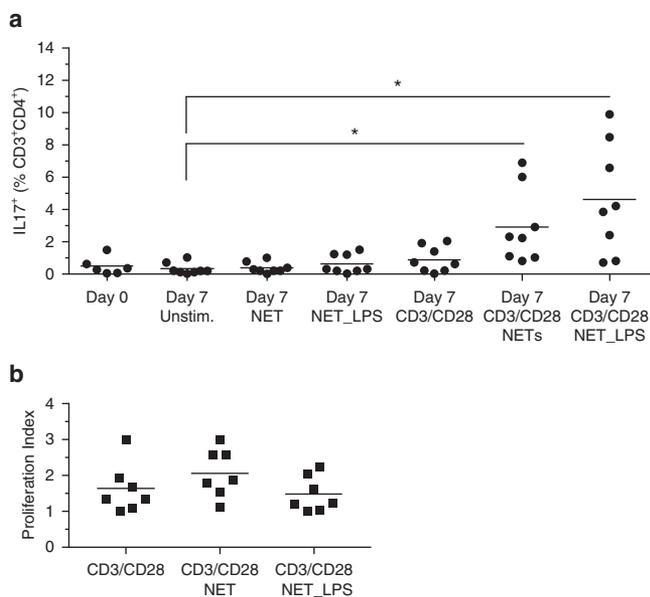
a

b

Figure 2. Induction of Th17 cells by neutrophil extracellular traps cannot be fully explained by expansion of T cells. (**a**) Percentage of Th17 cells in the starting population (day 0) compared to 7 days of culture, either in the presence of neutrophil extracellular traps alone, with CD3/CD28 stimulation alone, or both. Neutrophil extracellular trap were prepared with or without 5 ng/ml lipopolysaccharide, as indicated below the Figure. Statistics: *$P = 0.05$ by one-way repeated measures analysis of variance of untransformed data, with Dunnett's test for multiple comparisons. (**b**) Th17 proliferation index. Peripheral blood mononuclear cells were labeled at the time of seeding with carboxyfluorescein succinimidyl ester and the number of Th17 cell divisions were measured as a proliferation index after 7 days of culture as described in Materials and Methods. Statistics: analysis of untransformed data by one-way analysis of variance with Tukey's correction. LPS, lipopolysaccharide; NET, neutrophil extracellular trap.

and IL-17A release into the culture supernatant (Figure 4b) were significantly more pronounced for Act1 D10N homozygotes compared to wild-type homozygotes, with significant linear trend tests as a function of D10N genotype (0, 1, or 2 alleles) for both the percentage of Th17 cells ($P = 0.037$) and IL17A secretion ($P = 0.0035$). There was also a strong main effect of Act1 genotype on IL-17 secretion across all stimulation conditions ($P = 0.0045$), with a fold-change of 4.25 for Act1 D10N versus wild-type ($P = 0.0078$) (Supplementary Figure S4 online). As shown in Figure 4c and Supplementary Figure S4c, there was a trend towards increased IL17A, IL17F, and RORC mRNAs as a function of Act1 D10N genotype, with a significant main effect of Act1 genotype for IL17A mRNA.

Using a global approach that did not focus exclusively on Th17 cells, we next applied RNA sequencing to globally assess differentially expressed genes (DEGs) as a function of NET exposure and Act1 D10N genotype. For this analysis, we compared Act1 D10N homozygotes to wild-type homozygotes, and focused on "spontaneous" NETs to avoid confounding due to LPS exposure. As shown in Figure 5a, analysis of DEGs in CD3/CD28-stimulated PBMC showed that NETs had a stimulatory effect on global gene expression, with 1,343 genes yielding $P_{adj} \leq 0.05$ and |log$_2$ fold-change | ≥ 1 (840 up and 503 down) (Supplementary Table S1 online).

Functional enrichment analysis of this data set found significant enrichment for the Gene Ontology term *cytokine activity* (4.3-fold, false discovery rate = 3.3×10^{-19}) among others (Supplementary Table S2 online). On average, expression of DEGs mapping to this Gene Ontology term was markedly increased as a function of NET exposure (53 upregulated genes averaging 5.9-fold versus 2 downregulated genes averaging 0.28-fold) (Supplementary Table S3 online) with further increases for the Act1 D10N variant (Figure 5b). The Th17-related genes IL17A, IL17F, and RORC analyzed by quantitative reverse transcriptase PCR in Figures 1, 3, and 4 were among the "cytokine activity" genes whose expression was increased by NETs and further upregulated in Act1 D10N homozygotes (Figure 5b). Genomewide, Act1 D10N homozygotes presented a skewed distribution of fold-change values with respect to wild-type homozygotes under conditions of NET exposure, with the effect of Act1D10N being significantly more prominent in the presence of NETs ($P < 1 \times 10^{-16}$ by Wilcoxon signed-rank test) (Figure 5c).

DISCUSSION

Here we describe a method for induction of human Th17 cells from PBMC, and use it to explore the role of NETs and the Act1D10N variant as modulators of this process. We found that CD3/CD28 stimulation of PBMC significantly increased the percentage of Th17 cells (Figure 1a) and IL-17A secretion (Figure 1b), both of which were further enhanced by NETs. IL17A, IL17F, and RORC mRNAs were also increased by NETs, relative to CD3/CD28 stimulation alone (Figure 1c). Indicative of a specific requirement for NETosis, the effect of NETs could not be mimicked by intact viable neutrophils (Supplementary Figure S3b). Moreover, NETs alone could not induce Th17 cells in the absence of CD3/CD28 stimulation (Figure 2a). NET exposure also markedly increased the proportion of Th17 cells expressing CD161, a lectin-like cell surface receptor coupled to STAT3 and mTOR activation (Bai et al., 2014) (Figure 1d). CD161 defines a distinctive transcriptional and functional profile shared across multiple T-cell subsets, including mucosal-invariant T cells (Fergusson et al., 2014), suggesting that NETs may promote "innate-like" immune responses.

It is important to ask whether our results reflect differentiation of Th17 cells from naïve T cells, preferential expansion of existing Th17 cells, or induction of Th17 cells from "precommitted" memory T-cell subsets. Using depletion experiments, we confirmed observations (Evans et al., 2007; van Beelen et al., 2007) that the induction of human Th17 cells from CD3/CD28-stimulated PBMC is much more efficient using memory than naïve CD4$^+$ T cells (Figure 3d). Given that only ~0.4% of CD4$^+$ T cells are IL-17$^+$ directly ex vivo (Figure 2a), and that replication-competent IL-17−expressing CD4$^+$ T cells averaged fewer than two divisions over 7 days, as determined by dye dilution assays (Figure 2b), we consider it unlikely that the observed increase in the percentage of Th17 cells can be explained solely by proliferation. T-cell polarity has been shown to be flexible in human CD4$^+$ T cells (Cosmi et al., 2014), and emerging data suggest that Th17 cells may be derived from IL-17−negative helper T cells by metabolic reprogramming (Binger et al., 2017). Hence, in this report we utilize the non-specific term *induction*, rather
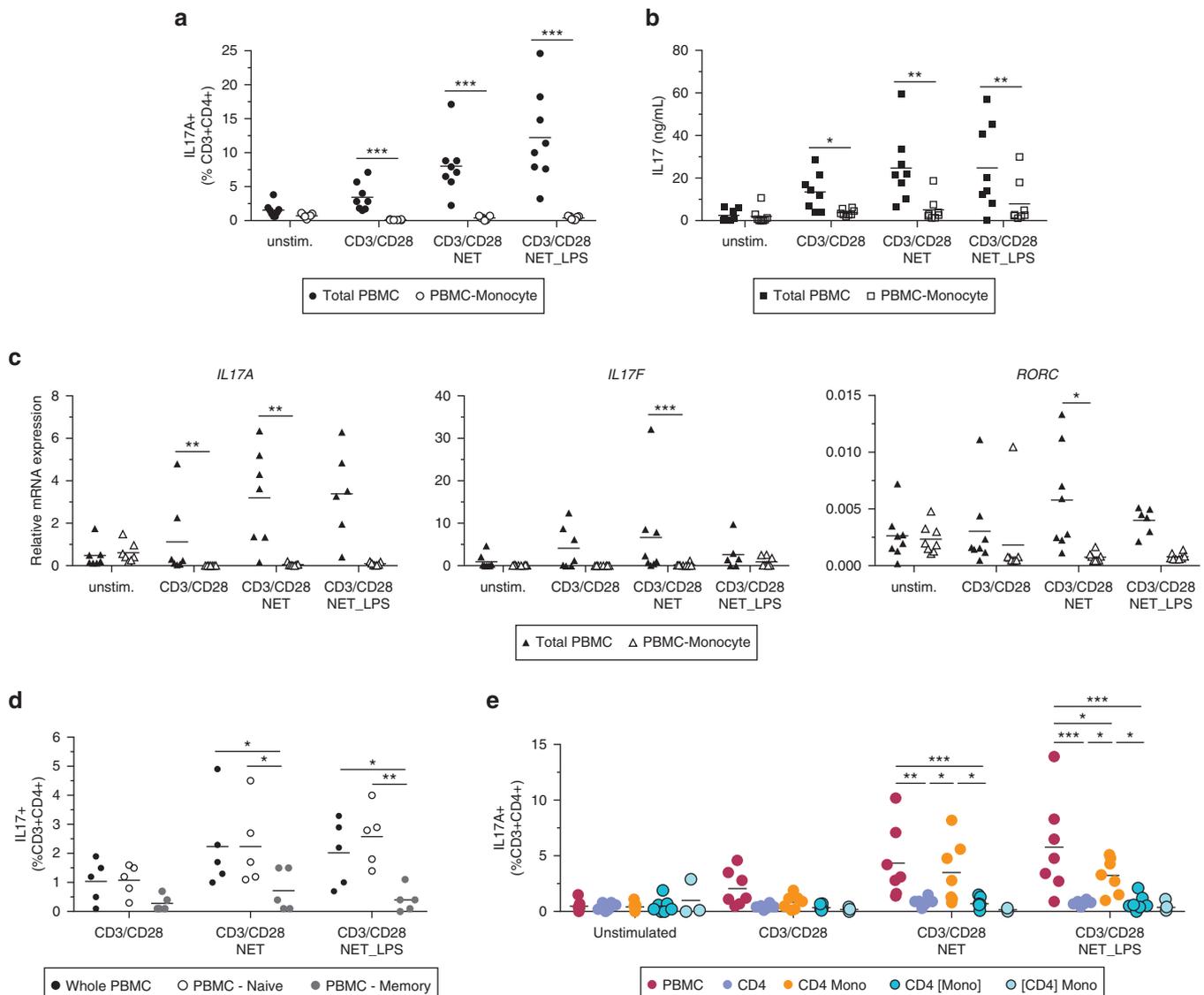
Figure 3. Monocytes, memory CD4$^+$ T cells, and cell-to-cell contact are required for neutrophil extracellular trap -induced Th17 induction. (a–c) Monocyte depletion experiment. (a) Percentage of IL-17$^+$ T cells in whole PBMC (filled squares) or monocyte-depleted PBMC (open squares), as determined by flow cytometry. (b) Secreted IL-17 protein, as determined by ELISA. (c) *IL-17A, IL-17F,* and *RORC* mRNA levels, as determined by quantitative reverse transcription PCR. Statistics: two-way repeated measures analysis of variance of transformed data (a, logit, b, log (ln), c, log$_2$) with all *P* values corrected to maintain experiment-wise type I error rate for the tested factor. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. (d) T-cell depletion experiment. Memory or naïve T cells were removed from PBMC prior to 7 days of CD3/CD28 stimulation. Closed circles represent whole PBMC, open squares indicate depletion of naïve T cells, and gray squares indicate depletion of memory T-cells. Statistics: analysis of non-transformed data by two-way analysis of variance with Tukey's test for multiple comparisons, *$P < 0.05$, **$P < 0.01$. (e) Cell-to-cell contact experiment. Percentage of IL-17$^+$ T cells in whole PBMC, CD4 T cells, CD4 T cells with monocytes, or CD4 T cells or monocytes seeded in Transwell determined by flow cytometry. Brackets indicate the cell type confined to the Transwell. Statistics: analysis of non-transformed data by two-way analysis of variance with Tukey's test for multiple comparisons, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. LPS, lipopolysaccharide; NET, neutrophil extracellular trap; PBMC, peripheral blood mononuclear cell.

than *differentiation* or *expansion*, to describe our findings regarding Th17 cells.

We confirmed previous observations (Evans et al., 2007) that monocytes are required for CD3/CD28-mediated Th17 induction from PBMC (Figure 3). These findings suggest that myeloid cells comprise a "bridge" between NETs on the one hand, and Th17 cells on the other. This would be consonant with the in vivo environment of psoriatic skin, in which NETs are abundantly present (Hu et al., 2016; Lin et al., 2011) in an environment rich in myeloid cells (Kim and Krueger, 2015) and memory T cells (Clark, 2015).

We utilized LPS to enhance NET formation and found that this significantly increased the percentage of Th17 cells (Figure 1). However, LPS has also been reported to enhance the ability of monocytes to induce Th17 from memory T cells (Evans et al., 2007), raising the possibility that our results might be due to effects of LPS on monocytes, rather than its effects upon NETosis. We consider this to be unlikely for several reasons. First, we found that LPS levels in our cultures were below the limit of sensitivity of the limulus amebocyte lysate assay (<0.01 ng/ml), whether measured immediately after NET washing or at the end of the culture period on day
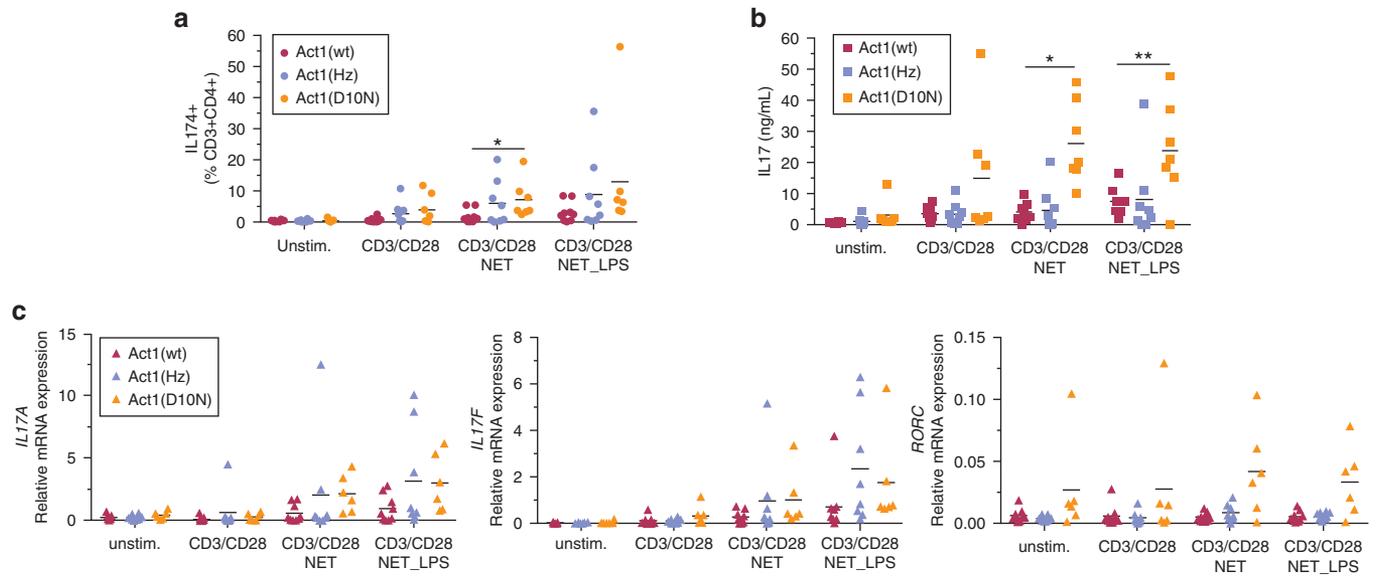
Figure 4. The Act1 D10N variant promotes Th17 induction. Unstimulated peripheral blood mononuclear cells, peripheral blood mononuclear cells activated with anti-CD3/CD28 beads in presence of neutrophil extracellular traps or lipopolysaccharide-induced neutrophil extracellular traps were incubated for 7 days followed by (**a**) flow cytometry to determine the percentage of IL-17$^+$T cells; (**b**) ELISA to determine secreted IL-17 protein; and (**c**) quantitative reverse transcription PCR to determine *IL17A, IL17F*, and *RORC* mRNA levels. Means are indicated by horizontal lines. Statistics: two-way repeated measures analysis of variance of transformed data (**a**, logit, **b**, log, **c**, log$_2$) with all *P* values corrected to maintain experiment-wise type I error rate for the tested factor. *$P < 0.05$, **$P < 0.01$. Graphical results for the transformed data and a summary of statistically significant outcomes are presented in Supplementary Figure S4. LPS, lipopolysaccharide; NET, neutrophil extracellular trap.

7. Thus, LPS levels in our cultures were at least 10,000 times lower than those used by Evans et al. (2007) (100 ng/ml). Moreover, addition of LPS did not stimulate the induction of Th17 cells in our system (Supplementary Figure S3a), possibly because we used a considerably lower concentration of LPS (5 vs. 100 ng/ml).

We utilized 25 nM PMA as an alternative stimulus for NETosis and were interested to find that PMA-induced NETs were completely ineffective for induction of Th17 cells (Supplementary Figure S2c), despite PMA being a strong stimulus for NETosis (Supplementary Figures S2a, S2b). Recent studies (Khan et al., 2017) have shown that LPS and PMA utilize different signaling pathways to induce NETosis, with LPS triggering a Toll-like receptor 4—dependent activation of nicotinamide adenine dinucleotide phosphate oxidase to effect NETosis (Khan et al., 2017). While both sets of experiments utilized *E. coli* 0111:B4 LPS, we used a much lower concentration (5 ng/ml vs. 100—25,000 ng/ml). Consistent with our findings (Supplementary Figure S2b), Khan et al. (2017) observed very little enhancement of DNA release at 100 ng/ml LPS (<10% over control, not significant). These results suggest that LPS may be enhancing Th17 via one or more Toll-like receptor 4—dependent effects on neutrophils other than, or in addition to, the stimulation of NETosis per se.

A major puzzle in psoriasis genetics is why the rs33980500T variant in *TRAF3IP2*, encoding Act1 D10N, is associated with increased risk for psoriasis and psoriatic arthritis (Ellinghaus et al., 2010; Huffmeier et al., 2010; Strange et al., 2010; Stuart et al., 2015), given that the Act1 D10N variant is hypofunctional in response to IL-17 in mouse embryo fibroblasts (Wang et al., 2013) and Act1 silencing leads to lower responses to IL-17 in human keratinocytes

(Lambert et al., 2017). To ask whether the Act1 D10N variant might promote Th17 induction in human cells, we used our model to study individuals of varying Act1 D10N genotype. We observed a significant stimulatory effect of Act1 D10N genotype on induction of Th17 cells and IL-17A secretion, which was potentiated in the presence of NETs (Figures 4a, 4b).

We next utilized RNA sequencing to more globally assess gene expression changes influenced by NETs in the context of CD3/CD28 activation. Volcano plot analysis confirmed a significant effect of NETs in this model, with upregulated genes exceeding downregulated genes (Figure 5a). Functional analysis of DEGs revealed enrichment for genes associated with the Gene Ontology term *cytokine activity* and related terms (Supplementary Table S2). On average, DEGs associated with cytokine activity were increased by NET exposure, with a further increase in Act1 D10N homozygotes versus nullizygotes (Figure 5b). Moreover, Act1D10N-dependent upregulation was observed for *IL17A, IL17F*, and *RORC* (Figure 5b). By measuring the gene expression ratios for Act1 D10N versus wild-type homozygotes, we observed a highly significant ($P < 1 \times 10^{-16}$) global effect of Act1 D10N genotype on gene expression, which was enhanced by NETs (Figure 5c). Together, these results corroborate those of our Th17-focused experiments (Figures 1—4).

Overall, these results are in agreement with studies in mice indicating that the Act1 D10N variant results in a hyperactive Th17 response and increased skin inflammation, despite the fact that it behaves like a loss of function variant in response to IL-17 stimulation (Wang et al., 2013; Wu et al., 2014). While the mechanism underlying this effect requires further investigation, our observations would be consistent with a recent report (Zhang et al., 2018) indicating that Act1 inhibits STAT3,
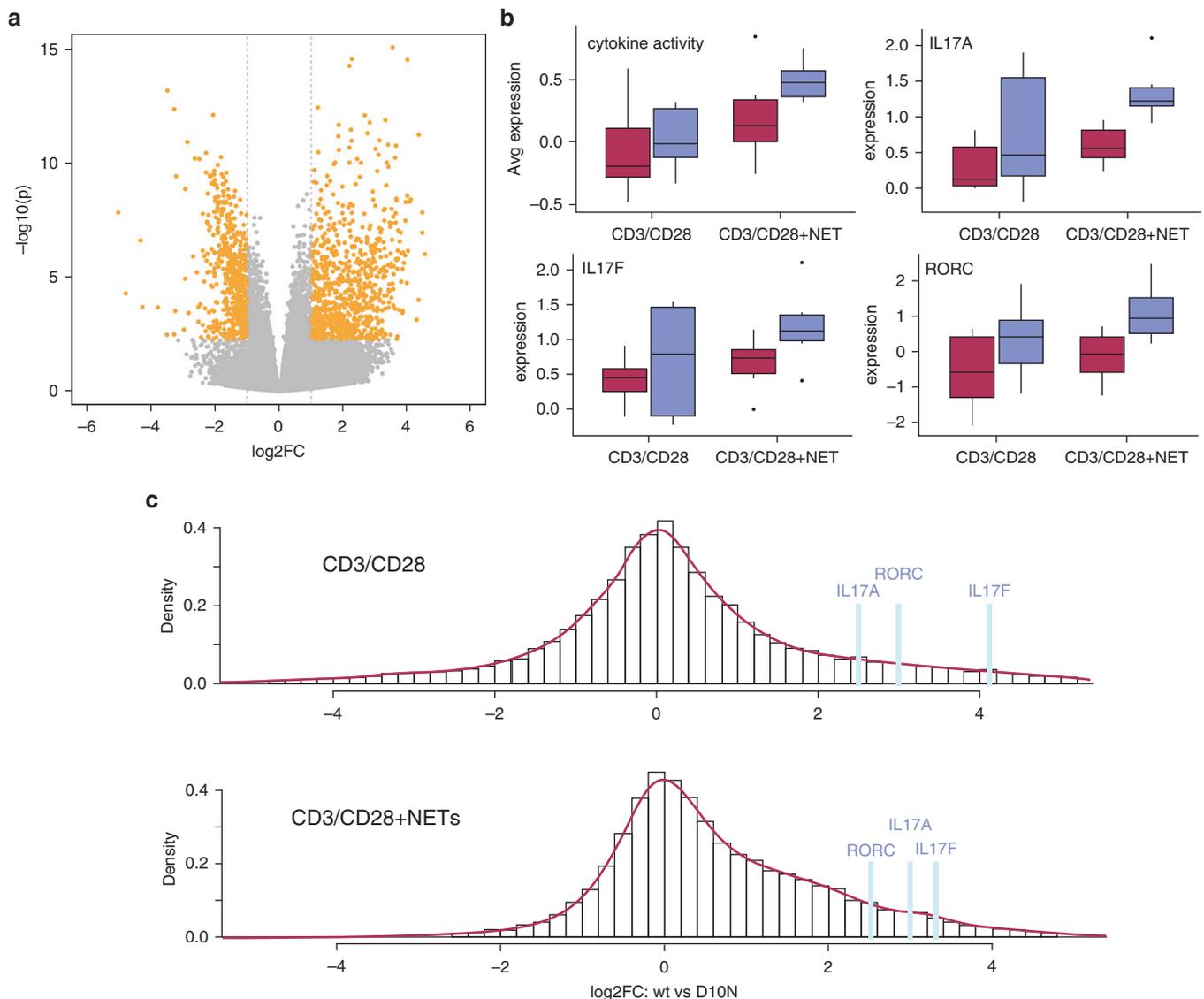
Figure 5. NETs enhance the effect of the Act1 D10N variant on global gene expression in CD3/CD28-stimulated peripheral blood mononuclear cells.
(**a**) Volcano plot showing log_2 fold-change values for global gene expression determined by RNA sequencing for 7-day CD3/CD28-stimulated peripheral blood mononuclear cells in the presence or the absence of NETs. Significant differentially expressed genes ($|log_2$ fold-change $| \geq 1$, $P_{adj} < 0.05$) are highlighted in orange. Eight hundred and forty genes were significantly upregulated and 503 genes were significantly downregulated. (**b**) Box and whisker plots showing average gene expression across all expressed genes belonging to the Gene Ontology term *cytokine activity*, as well as individual gene expression values for *IL17A*, *IL17F*, and *RORC*. Red bars indicate values obtained for Act1 wild-type homozygotes and blue bars indicate values obtained for Act1 D10N homozygotes. The top and the bottom of the box represent 25th and 75th percentiles, respectively, and the centerline is the 50th percentile; upper and lower whiskers extend to the 1.5-fold interquartile range of the 25th and 75th percentiles, respectively; and dots represent outliers beyond the 1.5-fold interquartile range. (**c**) Histograms summarizing the distribution of fold-change values (Act1 D10N/WT) after CD3/CD28 stimulation in the absence of NETs (upper panel) versus the presence of NETs (lower panel). Individual gene expression ratio values for *IL17A*, *IL17F*, and *RORC* are indicated above the histograms. Note the extended "tail" of positive gene expression ratios in the presence of NETs. FC, fold-change; NET, neutrophil extracellular trap.

which in turn exerts multifaceted effects on Th17 biology as a downstream mediator of IL-23 signaling (Mathur et al., 2007). Future studies dissecting the constituent cell populations of the PBMC assay using single-cell techniques should prove fruitful in understanding both the effects of the Act1 D10N variant and the role of NETs in Th17 induction in psoriasis.

MATERIALS AND METHODS
This study was approved by the University of Michigan's Institutional Review Board, and all patients provided

written informed consent in adherence with the Declaration of Helsinki principles.

PBMC and neutrophils were isolated from blood by centrifugation over Ficoll 1.077D (Sigma-Aldrich, St. Louis, MO). NETs were prepared by adding 5×10^5 neutrophils in 0.5 ml RPMI 1640 to 24-well poly-L-lysine−coated tissue culture plates (Corning BioCoat, Corning, NY), with or without 5 ng/ml *E. coli*-derived LPS (Serotype O111:B4; Sigma Aldrich) or 25 nM PMA and incubated for 4 hours to allow for NETosis, then washed twice with fresh RMPI 1640 lacking phenol red (Thermo Fisher Scientific, Waltham, MA). Th17

cells were induced by cultivating 5×10^5 PBMC in 0.5 ml RPMI 1640 containing 10% fetal bovine serum with Human T-Activator CD3/CD28 Dynabeads (Thermo Fisher) in 24-well plates. PBMC were suspended in empty wells or wells in which NETs from the same donor had just been generated, with or without LPS or PMA. In other experiments, we added 5 ng/ml LPS for the duration of the culture, or we depleted monocytes, memory T cells, or naïve T cells from PBMC using immunomagnetic bead kits (Miltenyi Biotec, Auburn, CA). Cultures were fed fresh medium after 4 days. After 7 days, cells were harvested for analysis by flow cytometry or quantitative reverse transcriptase PCR, and conditioned medium was assayed for IL-17A by ELISA (DuoSet assay, R&D Systems, Minneapolis, MN). For flow cytometry experiments cells were stimulated for 6 hours with PMA (5 ng/ml) and ionomycin (1 μg/ml) in presence of Brefeldin A (BioLegend, San Diego, CA). The gating strategy for flow cytometry is shown in Supplementary Figure S5 online. We activated cultures with PMA and ionomycin (without Brefeldin A) for 6 hours to assess mRNA expression (including RNA sequencing) and for 24 hours to assess protein secretion, as these time points revealed the best induction of IL-17 mRNA or protein in preliminary experiments (Supplementary Figure S6 online). Details of flow cytometry, RNA sequencing, statistical analysis, and other procedures are provided in the Supplementary Material online.

RNA-sequencing data have been submitted to Gene Expression Omnibus, accession number GSE121315.

CONFLICTS OF INTEREST
The authors state no conflicts of interest.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.11.021.

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