Loss-of-function mutations in \textit{TNFAIP3} leading to A20 haploinsufficiency cause an early-onset autoinflammatory disease

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Systemic autoinflammatory diseases are driven by abnormal activation of innate immunity\textsuperscript{1}. Herein we describe a new disease caused by high-penetrance heterozygous germline mutations in \textit{TNFAIP3}, which encodes the NF-kB regulatory protein A20, in six unrelated families with early-onset systemic inflammation. The disorder resembles Behçet’s disease, which is typically considered a polygenic disorder with onset in early adulthood\textsuperscript{2}. A20 is a potent inhibitor of the NF-kB signaling pathway\textsuperscript{3}. Mutant, truncated A20 proteins are likely to act through haploinsufficiency because they do not exert a dominant-negative effect in overexpression experiments. Patient-derived cells show increased degradation of IkBα and nuclear translocation of the NF-κB p65 subunit together with increased expression of NF-κB–mediated proinflammatory cytokines. A20 restricts NF-κB signals via its deubiquitinase activity. In cells expressing mutant A20 protein, there is defective removal of lys63-linked ubiquitin from TRAF6, NEMO and RIP1 after stimulation with tumour necrosis factor (TNF). NF-κB–dependent proinflammatory cytokines are potential therapeutic targets for the patients with this disease.

Monogenic autoinflammatory diseases are a heterogeneous group of diseases marked by unprovoked episodic or chronic inflammatory symptoms. Mutations in more than 20 causative genes have been reported, with many of these genes encoding proteins that regulate signaling by proinflammatory cytokines, such as interleukin-1 (IL-1), type I interferon and TNF\textsuperscript{4–6}. Therapies targeting proinflammatory cytokines, especially IL-1 and TNF, have been effective in many patients\textsuperscript{7}. However, a substantial number of sporadic and familial cases remain genetically uncharacterized.

We studied a family of European ancestry (family 1) with a dominant disorder manifesting as early-onset systemic inflammation, arthralgia and/or arthritis, oral and genital ulcers, and ocular inflammation (Fig. 1a,b and Supplementary Table 1). Whole-exome sequencing on the affected mother and her two affected daughters identified 11 new candidate variants. After Sanger sequencing of maternal unaffected family members, two new variants in \textit{TNFAIP3} (tumor necrosis factor, α–induced protein 3; also known as A20) and \textit{TNFRSF9} (tumor necrosis factor receptor superfamily, member 9) remained in consideration (Supplementary Fig. 1 and Supplementary Table 2). Both genes function in the regulation of immune responses.

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 Independently, we performed whole-exome sequencing in a family of Italian ancestry (family 2) with an affected mother (P4) and daughter (P6). The family history was positive for a dominantly inherited inflammatory disease (Fig. 1a). Patients P4, P6 and P5, who is the sister of P4 and aunt of P6, had in common oral and genital ulcers and polyarthralgia. Patient P6 was initially diagnosed with an early-onset severe autoimmune condition resembling systemic lupus erythematosus (SLE), with central nervous system (CNS) vasculitis and anterior uveitis (Fig. 1b and Supplementary Table 1). We identified 30 new candidate variants that were shared by patients P4 and P6, including a new variant in TNFAIP3. Sanger sequencing of patient P5 and the three unaffected family members confirmed that the new variant, p.Phe224Serfs*4, segregated with the inflammatory phenotype (Fig. 1a). Patient P6 did not have mutations in genes associated with early-onset autoimmune diseases. We noted that family 1 and family 2 shared new variants in a single candidate gene, TNFAIP3 (NM_006290.2; Supplementary Fig. 2a). Screening of an additional 150 patients with clinically similar disease identified three new nonsense and frameshift variants in families of Turkish (family 3), European-American (family 4) and Dutch (family 5) ancestry. In total, we identified five heterozygous truncating mutations in TNFAIP3 in five families (Fig. 1a, Table 1 and Supplementary Fig. 2b). Targeted sequencing in 384 Turkish and 384 Japanese adult-onset Behçet’s disease cases from genome-wide association studies identified one patient with a new frameshift mutation in TNFAIP3, encoding p.Pro268Leufs*19 (Supplementary Fig. 2c), which was subsequently identified in her two affected daughters. The mutations identified were considered

Table 1  Ancestry and sequence alterations in TNFAIP3 mutation–positive families

| Family | Ancestry       | Inheritance | Nucleotide alteration | Exon | cDNA alterationa | Amino acid alteration | Domain      | ExACb allele frequency |
|--------|----------------|-------------|-----------------------|------|------------------|----------------------|-------------|------------------------|
| Family 1 | European Canadian | Dominant    | chr6:138197178T>A      | 5    | c.680T>A         | p.Leu227*            | OTU         | 0/122,972               |
| Family 2 | European American | Dominant    | chr6:138197169delT     | 5    | c.671delT        | p.Phe224Serfs*4     | OTU         | 0/122,972               |
| Family 3 | Turkish        | Dominant    | chr6:138198218C>T      | 6    | c.811C>T         | p.Arg271*            | OTU         | 0/122,972               |
| Family 4 | European American | De novo    | chr6:138200391delG     | 7    | c.1809delG       | p.Thr604Argfs*93    | ZnF4        | 0/122,972               |
| Family 5 | Dutch          | Dominant    | chr6:138198325C>G      | 6    | c.918C>G         | p.Tyr306*            | OTU         | 0/122,972               |
| Family 6 | Turkish        | Dominant    | chr6:138197927delG     | 5    | c.799delG        | p.Pro268Leufs*19    | OTU         | 0/122,972               |

*aDNA positions are according to the reference NM_006290.2; bThe ExAC database includes 61,486 exomes.
pathogenic on the basis of proper family segregation with the inflammatory phenotype and their absence from the Exome Aggregation Consortium (ExAC) database, dbSNP and our exome database of more than 500 exomes. Previously, frameshift mutations in TNFAIP3 have been reported only in tumor tissues in the form of biallelic somatic mutations. The pathogenic variants from our study are predicted to result in truncated proteins, suggesting haploinsufficiency or a dominant-negative effect for the mutant A20 proteins.

A20 is a 790-residue protein that consists of an N-terminal ovarian tumor (OTU) domain followed by seven zinc-finger (ZnF) domains (Fig. 1c). Five of the six disease-associated TNFAIP3 mutations map to the OTU domain, which mediates the deubiquitinase activity, and result in mutant, truncated proteins of similar length. The remaining disease-associated variant, p.Thr604Argfs*93, resides in the ZnF4 domain, which recognizes Lys63-linked ubiquitin chains and is also essential for A20 E3 ubiquitin ligase activity and dimerization.

The expression of wild-type A20 was reduced in patient-derived peripheral blood mononuclear cells (PBMCs) and fibroblasts, whereas mutant proteins were not detectable (Fig. 1d,e). We suspect that mutant proteins are not stable and undergo degradation.

We assessed the activity of each mutant A20 protein using a nuclear factor (NF)-κB luciferase assay. Overexpressed mutant proteins failed to suppress TNF-induced NF-κB activity in transfected human embryonic kidney (HEK) 293T cells (Fig. 2a) and a human T cell leukemia cell line (Jurkat cells) (Supplementary Fig. 3). Wild-type A20 suppresses NF-κB activity, and cotransfection to express mutant, truncated A20 protein with wild-type A20 did not reverse this outcome, suggesting that the mutant protein does not have a dominant-negative effect (Fig. 2a). IκB kinase (IKK)-mediated phosphorylation of IκBα, resulting in IκBα degradation and subsequent nuclear translocation of the associated p50-p65 NF-κB heterodimer, is an essential step in activation of the canonical NF-κB pathway. To define the relationship between the A20 mutants and NF-κB induction in vivo, we studied the activity of the canonical NF-κB pathway in response to stimulation with TNF. Stimulated patient-derived cells showed increased phosphorylation of IKKα/IKKβ, increased phosphorylation of the mitogen-activated protein (MAP) kinases p38 and JNK, and increased degradation of IκBα in comparison to the controls (Fig. 2b,c and Supplementary Fig. 4a,b). Consistent with these findings, we observed increased nuclear translocation of p65 in patient-derived
PBMCs (Fig. 2d and Supplementary Fig. 5a) and fibroblasts, both at rest and after stimulation with TNF (Fig. 2e and Supplementary Fig. 5b,c). These data are strong evidence for enhanced signaling in the NF-κB pathway in A20-deficient cells.

The NF-κB signaling cascade is under tight regulation by a number of post-translational modifications, including ubiquitination1. A20 restricts cellular activation by cleaving Lys63-linked ubiquitin chains from target substrates such as IKKγ (also known as NEMO), RIP1 and TRAF6 (ref. 12). Through its E3 ubiquitin ligase activity, A20 adds Lys48-linked ubiquitin chains, restricting cellular activation by cleaving Lys63-linked ubiquitin chains (Fig. 3b). As controls for transfection efficiency, cell lysates were also blotted with antibody against each target protein (middle) or antibody against the N terminus of A20 or the GFP tag (bottom). (e,f) TNF-stimulated patient-derived PBMCs (e) or fibroblasts (f) showed increased abundance and increased molecular weight of Lys63-ubiquitinated NEMO as a result of insufficient A20 deubiquitinase activity. Primary cells from a patient and a healthy control were stimulated with TNF for the indicated times. Whole-cell lysates were subjected to immunoprecipitation with antibody against Lys63-linked ubiquitin, and the precipitates were blotted with antibodies against NEMO and ubiquitin. Cell lysates of post-translational modifications, including ubiquitination15. A20 truncation-causing mutations, patient-derived PBMCs displayed reduced recruitment of A20 to the TNFR complex in comparison to cells from healthy donors (Fig. 3a). To investigate whether the A20 mutants still retained their deubiquitinase function, wild-type and mutant A20 constructs were cotransfected into 293T cells with plasmids encoding Lys63-linked ubiquitin and the A20 ubiquitination targets RIP1, NEMO and TRAF6 (Fig. 3b–d).

Figure 3 Impaired TNFR signaling and deubiquitinase function of mutant A20.
(a) Reduced recruitment of mutant A20 to the TNFR signaling complex. PBMCs from patient P4 and a healthy control were stimulated with TNF for the indicated times. Whole-cell lysates were subjected to immunoprecipitation with antibody against TNFR1, TRAF2 or RIP1, and the precipitates were blotted with antibody against the N terminus of A20 and reblotted with antibodies against TNFR1, TRAF2, RIP1 and Ubc13. (b–d) A20 mutants lose their ability to deubiquitinate Lys63-linked ubiquitin chains but do not antagonize the deubiquitinase function of wild-type A20. 293T cells were transiently transfected with expression plasmids for the A20 target proteins RIP1 with a Myc tag (b), NEMO (c) and TRAF6 (d), together with plasmids for HA-Lys63-ubiquitin (Ub) and GFP-tagged wild-type or mutant A20. Cells were collected 48 h later, and equal amounts of the whole-cell lysates were subjected to immunoprecipitation with antibodies against the respective target proteins. High-molecular-weight (HMW) ubiquitin aggregates (top) were detected by immunoblotting the precipitates with an HA-specific antibody. As controls for transfection efficiency, cell lysates were also blotted with antibody against each target protein (middle) or antibody against the N terminus of A20 or the GFP tag (bottom). (e,f) TNF-stimulated patient-derived PBMCs (e) or fibroblasts (f) showed increased abundance and increased molecular weight of Lys63-ubiquitinated NEMO as a result of insufficient A20 deubiquitinase activity. Primary cells from a patient and a healthy control were stimulated with TNF for the indicated times. Whole-cell lysates were subjected to immunoprecipitation with antibody against Lys63-linked ubiquitin, and the precipitates were blotted with antibodies against NEMO and ubiquitin. Cell lysates were also blotted with antibodies against the target protein and β-actin as internal controls. Red arrows point to the differences in lines used for comparison.
Figure 4: Patient-derived immune cells have a strong inflammatory signature. Cytokine profiles are compared for A20-deficient patients and healthy controls. (a) Serum cytokine levels from six patients and eight controls. (b) Cytokine levels in the supernatants of PBMCs derived from patients (n = 5) or controls (n = 6) stimulated either with bacterial LPS or staphylococcal enterotoxin B (SEB). Each sample was assessed in duplicate or triplicate. Values are represented as means ± s.e.m. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001. (c) Flow cytometry analysis of CD3+CD4+CD45RO+ single live cells. Top, representative flow cytometry plots of IL-9 expression by intracellular cytokine (ICC) staining. A20-deficient patients (n = 4) expressed significantly more IL-9 than paired controls (n = 5), and ex vivo transcript levels of SPI1 (encoding PU.1) were likewise significantly increased in patient-derived PBMCs. Bottom, representative flow cytometry plots of IL-17A and IL-4 expression in an A20-deficient patient as compared to a paired control. A significant increase in intracellular IL-17A expression was observed in patients (n = 5) as compared to controls (n = 8). Consistent with an absence of clinical allergic disease, no significant difference in intracellular IL-4 expression was seen. *P < 0.05, Mann-Whitney test; NS, not significant. Values are represented as means ± s.e.m. (d) Increased prevalence of inflammatory monocyte subsets in patients. Top, representative flow cytometry analysis of monocyte subsets in one control and one A20-deficient patient. Three monocyte subsets are identified by CD14highCD16− (classical; P1), CD14highCD16+ (inflammatory; P2) and CD14highCD16+ (non-classical; P3) profiles. The prevalence (percentage) of the different subsets is shown in the different gates. Bottom, quantification of CD14highCD16+ (inflammatory) monocyte subsets in four A20-deficient patients and four controls. Data are shown as means ± s.d. (P = 0.00013).

Cells transfected to express various disease-associated A20 mutants showed a marked defect in the deubiquitination of each of these target molecules, and cells cotransfected to express wild-type and mutant A20 molecules, mimicking the situation in patients, had intermediate levels of deubiquitination.

Consistent with the transfection data, TNFR1 signaling complexes from patients with TNFAIP3 mutations accumulated sustained levels of Lys63-ubiquitinated NEMO and RIP1 and accumulated high-molecular-weight ubiquitin aggregates (Fig. 3f, top, lanes 5–8 and Supplementary Fig. 6a, lanes 5–8). In comparison, in PBMCs and fibroblasts from healthy controls, the abundance of high-molecular-weight ubiquitin aggregates gradually decreased over time after stimulation with TNF (Fig. 3f, top, lanes 1–4 and Supplementary Fig. 6a, lanes 1–4). Taken together, these results indicate that inefficient deubiquitination of A20 target proteins might explain higher NF-kB signaling activity in A20-mutant cells.

Active NF-kB subunits promote the transcription of genes encoding proinflammatory cytokines, such as IL-1β, IL-6 and TNF, and facilitate the differentiation and activation of a variety of lymphocyte lineages. Multiple experiments showed evidence of increased expression of NF-kB target genes in patient-derived immune cells (Supplementary Figs. 7 and 8). Levels of several proinflammatory cytokines were substantially higher in serum from patients and in the supernatants of stimulated patient-derived PBMCs relative to cells from healthy controls (Fig. 4a,b). Intracellular cytokine staining also showed increased polarization toward T helper type 9 (Th9) and type 17 (Th17) CD4+ effector cell lineages, a finding that is consistent with the participation of IL-1β signaling in the differentiation of these
Figure 5 Spontaneous NLRP3 inflammasome activity in PBMCs from patients with TNFAIP3 mutations. (a) Immunoblots of total cell lysates from LPS-stimulated patient cells and control cells show increased expression of pro-IL-1β, NLRP3 and activated caspase-1 (casp-1; p10) and increased production of mature IL-1β in patient-derived cells. (b) The secretion of IL-1β in supernatants of LPS-stimulated patient-derived cells is regulated by the activity of the NLRP3 inflammasome. Activation of adenosine cyclase (via NKH477, an adenosyl cyclase activator), inhibition of PLC (via U73122) and addition of a small molecule, MCC950, attenuate NLRP3 inflammasome activation. U73343 is an inactive analog of U73122. (c) Treatment with an IL-1 inhibitor, anakinra, normalized markers of systemic inflammation in patient P6. Anakinra was initially given at a dose of 200 mg daily (May 2014), with the dose later increased to 400 mg daily (June 2014–September 2014) and then to 500 mg daily (October 2014–August 2015). The patient was also on 10 mg of prednisone and 200 mg of azathioprine daily. Besides the addition of anakinra, she had no other medication changes from 2012–2014. ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

lineages, and these lineages may further contribute to tissue inflammation in A20-deficient patients (Fig. 4c). Finally, the frequency of CD14+ inflammatory monocytes was significantly higher in patients than in healthy controls (Fig. 4d).

Recent studies in mouse macrophages lacking A20 suggest that A20 functions as a negative regulator of the Nlrp3 inflammasome independently of its role in NF-kB regulation. Consistent with these data, patient-derived cells showed constitutive activation of the NLRP3 inflammasome (Fig. 5a), which resulted in activation of caspase-1 and increased secretion of active IL-1β and IL-18 (Fig. 4b). Patient-derived PBMCs exhibited enhanced NLRP3 inflammasome–mediated caspase-1 activation and secreted IL-1β in response to lipopolysaccharide (LPS) priming alone, and this effect was attenuated by use of a phospholipase C (PLC) inhibitor, an adenosyl cyclase activator or MCC950, all of which are known NLRP3 inflammasome inhibitors (Fig. 5b). Furthermore, initial experience in a patient with an agent targeting IL-1β has been positive (Fig. 5c).

In summary, this is the first report, to our knowledge, of a human disease that we are calling haploinsufficiency of A20 (HA20), caused by high-penetrance loss-of-function germline mutations in TNFAIP3. The findings of multiple nonsense and frameshift variants associated with impaired regulatory function of A20 provide strong evidence that these mutations are pathogenic. Although patients with HA20 and Behçet’s disease have similar symptoms, the underlying genetics are distinct. Most sporadic late-onset Behçet’s disease cases are not caused by highly penetrant germline mutations in TNFAIP3. A20-deficient mice (Tnfaip3−/−) display persistent NF-kB activation, spontaneous multiorgan inflammation and early lethality. Aging heterozygous mice (Tnfaip3+/−) develop autoantibodies resembling those in human autoimmune conditions. Targeted ablation of A20 in specific hematopoietic cells in murine models leads to phenotypes reminiscent of autoimmunity. Although five patients in our study developed autoantibodies, an overt autoimmune disease was only diagnosed in patient P6. Notably, none of our patients have developed lymphomas or other malignancies.

TNFAIP3 variants have been linked to many human diseases. Somatic inactivating mutations in TNFAIP3 have been described in B cell lymphomas, suggesting that A20 acts as a tumor suppressor. Common low-penetrance coding and noncoding variants in TNFAIP3 have been associated with multiple autoimmune diseases and susceptibility to allergy and asthma. The current study, however, is the first, to our knowledge, to delineate the consequences of germline inactivating TNFAIP3 mutations in human biology.

Next-generation sequencing will likely identify other rare monogenic inflammatory diseases, possibly involving genes in the same pathway, which might further inform genetic studies of common polygenic immune diseases. There are also potential therapeutic implications from studying patients with monogenic diseases such as HA20 that may apply to more common inflammatory conditions such as Behçet’s disease.

URLs. Exome Aggregation Consortium (ExAC) database, http://exac.broadinstitute.org/; National Heart, Lung, and Blood Institute (NHLBI) GO Exome Sequencing Project (ESP), http://evs.gs.washington.edu/EVS/; 1000 Genomes Project, http://www.1000genomes.org/; dbSNP, http://www.ncbi.nlm.nih.gov/projects/SNP/; Picard, http://broadinstitute.github.io/picard/; Genome Analysis Toolkit (GATK), http://www.broadinstitute.org/gatk/; ANNOVAR, http://www.openbioinformatics.org/annovar.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Whole-exome sequencing data have been deposited in the Sequence Read Archive (SRA) under accession SRP062234.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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LETTERS

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with adult-onset disease. Pooled DNA was prepared as previously described. Dominant inheritance. With over 500 exomes, and variants were selected on the basis of autosomal

Exome Sequencing Project and ClinSeq databases and an in-house database (STR) markers across chromosome 6 were genotyped for all family members. NEMO (sc-56919 and sc-8330), ubiquitin (sc-271289), caspase-1 (sc-515 and sc-622), GFP (sc-8334), HA probe (sc-7392) and c-Myc (sc-40) were from Santa Cruz Biotechnology; antibodies to the N terminus of A20 (5630), NF-κBα (11930), IκBα (2859), NF-κBβ (4814 and 9242), phosphorylated p44/42 MAPK (ERK1/2) (4370), phosphorylated SAPK/JNK (4686), p44/42 MAPK (ERK1/2) (4695), SAPK/JNK (9252), p38 MAPK (8690), horseradish peroxidase (HRP)-linked anti-rabbit IgG (7074) and HRP-linked anti-mouse IgG (7076) were from Cell Signaling Technology; antibody to IL-1β (AF-201-NA) was from R&D Systems; and antibody to NLRP3 (ALX-804-819-C100) was from Enzo Life Sciences. pRKS-HA-ubiquitin-K63 (Addgene plasmid 17606) was a gift from T. Dawson (Johns Hopkins University). PEGFP-C1-NTFAP3 (Addgene plasmid 22141) was a gift from Y. Ye (Wuhan University). Constructs for Myc-ΔDKT–tagged human RIPK1 (RC126024), Myc-ΔDKT–tagged human NFKBIA (RC200711) and untagged human TRAF6 (sc109845) were from Origene. 3×Flag-NTFAP3 (Ex-K6040-M120) was from Genecopoeia. pEF-NEMO was a gift from C. Ma (National Institute of Allergy and Infectious Diseases, USA). The plasmids encoding GFP- or 3×FLAG-tagged mutant A20 were constructed by PCR-mediated mutagenesis.

Cell cultures. 293T cells (human embryonic kidney cells expressing SV40 large T antigen; negative for mycoplasma; originally obtained from the American Type Culture Collection) and skin fibroblast cells derived from A20-deficient patients or normal donors were grown in DMEM (Life Technologies) supplemented with 10% FCS (Gemini Bio-Products) and 1× antibiotics (Life Technologies). Jurkat T cells (human leukemic T lymphoma cell line; negative for mycoplasma; originally obtained from the American Type Culture Collection) and human PBMCs were grown in RPMI-1640 medium (Life Technologies) supplemented with 10% FCS and antibiotics. For intracellular TNF staining, pan-T cells purified from PBMCs by negative selection (Miltenyi Biotech) were cultured in complete RPMI-1640 in the presence of plate-bound antibody to CD3 and soluble antibody to CD28 (both at 1 µg/ml) for 5 d. For IL-9 staining, recombinant human IL-4 (30 ng/ml), transforming growth factor (TGF)-β (5 ng/ml), IL-1β (10 ng/ml) and IL-2 (10 U/ml) together with antibody to interferon-γ (IFNγ, 10 µg/ml), were added to total PBMCs, and cells were cultured for 48 h under the same conditions.
of interest in ImageJ software and used to measure nuclear p65 intensity. Data were analyzed in GraphPad Prism 6 and are shown as means ± s.e.m.

**Immune cell cytokine production and serum cytokine detection.** PBMCs (2 × 10^6 cells/ml) were stimulated with bacterial LPS (Sigma), flagellin (Sigma) or SEB (Sigma) for 24 h at 37 °C. The concentrations of cytokines in the supernatants of stimulated and non-stimulated PBMCs and in serum were determined using Bio-Plex Pro Human Cytokine 27-plex and 21-plex immunoassay kits (Bio-Rad). Bio-Plex Pro Human Cytokine Standard Group I and Group II were used as standards for the assays. Differences in cytokine concentrations were analyzed statistically using the Mann-Whitney test and were plotted with the GraphPad Prism software package.

**Primary human monocyte isolation, differentiation and stimulation.** Monocytes were purified from PBMCs by negative selection (Monocyte Isolation Kit II, Miltenyi Biotec). Monocytes were suspended in monocyte attachment medium (PromoCell) and seeded at a density of 150,000 cells/cm² for 2 h. Monocytes were co-stained with APC-Cy7–conjugated antibody to CD11b, PE-Cy7–conjugated antibody to CD11c, FITC-conjugated antibody to CD14, APC-conjugated antibody to CD16 or relevant isotype control and were then analyzed using a FACS Canton II instrument (BD Biosciences).

Primary human monocytes were differentiated into M1 macrophages using 20 ng/ml human granulocyte-macrophage colony-stimulating factor (GM-CSF, Sigma) or into M2 macrophages using 100 ng/ml human macrophage colony-stimulating factor (M-CSF, Sigma) for 10 d. Human GM-CSF–differentiated M1 macrophages were stimulated with LPS (Sigma) at 100 ng/ml for 6 h or left unstimulated.

**RT-PCR.** RNA was isolated from M1 macrophages using the RNeasy kit (Qiagen) and from unstimulated PBMCs using TRIzol. Reverse transcription was performed using the TaqMan kit (Applied Biosystems). RT-PCR was performed using the iQ SYBR Green kit (Bio-Rad) or TaqMan Gene Expression Master Mix and TaqMan gene expression assay probes. 18S rRNA served as a control. The reactions were run on an Applied Biosystems 7500 Real-Time PCR System (Life Technologies).

**Intracellular cytokine staining in T cells.** Intracellular cytokine staining for IL-17A, TNF, IL-4 and IL-9 following stimulation with either phorbol 12-myristate 13-acetate (PMA) and ionomycin (IL-17A, IL-4 and IL-9) or SEB (TNF) was performed as previously described. All events were collected on an LSRFortessa instrument (BD Biosciences) and analyzed with FlowJo software.

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