

## RESEARCH ARTICLE SUMMARY

## IMMUNE REGULATION

# T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4<sup>+</sup> T cells

Giuseppina Arbore,\* Erin E. West,\* Rosanne Spolski, Avril A. B. Robertson, Andreas Klos, Claudia Rheinheimer, Pavel Dutow, Trent M. Woodruff, Zu Xi Yu, Luke A. O'Neill, Rebecca C. Coll, Alan Sher, Warren J. Leonard, Jörg Köhl, Pete Monk, Matthew A. Cooper, Matthew Arno, Behdad Afzali, Helen J. Lachmann, Andrew P. Cope, Katrin D. Mayer-Barber, Claudia Kemper†

**INTRODUCTION:** The inflammasomes and the complement system are traditionally viewed as quintessential components of innate immunity required for the detection and elimination of pathogens. Assembly of the NLRP3 inflammasome in innate immune cells controls the maturation of interleukin (IL)-1 $\beta$ , a proinflammatory cytokine critical to host defense, whereas activation of the liver-derived complement key components C3 and C5 in serum leads to opsonization and removal of microbes and induction of the inflammatory reaction. Recent studies, however, have highlighted an unanticipated direct role for complement C3 also in human T cell immunity: The anaphylatoxin C3a receptor (C3aR)

and the complement regulator CD46 (which binds C3b) are critical checkpoints in human T cell lineage commitment, and they control initiation and resolution of T helper 1 (T<sub>H</sub>1) responses in an autocrine fashion via T cell–derived and intracellularly activated C3. We explored a novel functional cross-talk of complement with the NLRP3 inflammasome within CD4<sup>+</sup> T cells and determined how the cooperation between these two “classically” innate systems directly affects interferon- $\gamma$  (IFN- $\gamma$ ) production by adaptive immune cells.

**RATIONALE:** Given the critical role of intracellular C3 activation in human T<sub>H</sub>1 responses and

the importance of C5 activation products in inflammation, we investigated whether human CD4<sup>+</sup> T cells also harbor an “intracellular C5 activation system” and by what means this system may contribute to effector responses by using C5aR1 and C5aR2 agonists and antagonists, T cells from patients with cryopyrin-associated periodic syndromes (CAPS), and mouse models of infection and autoimmunity.

**RESULTS:** Human CD4<sup>+</sup> T cells expressed C5 and generated increased intracellular C5a upon T cell receptor activation and CD46 autocrine

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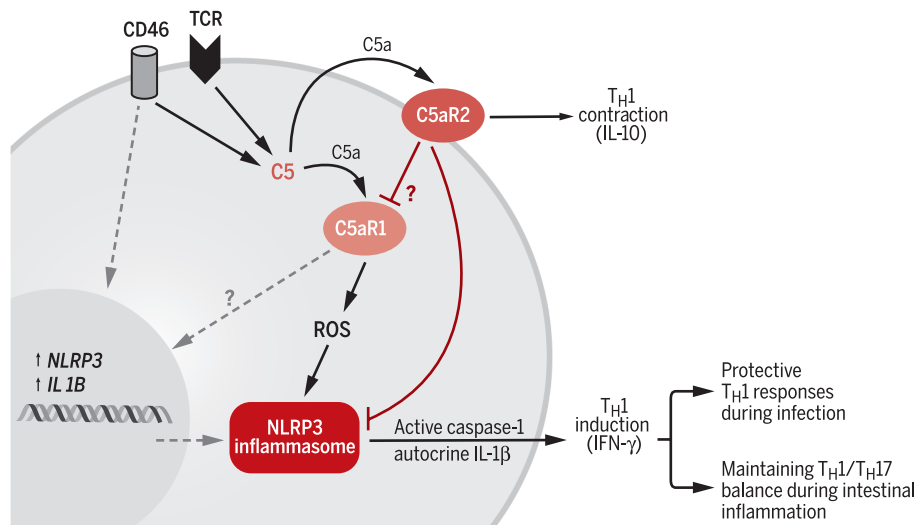
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costimulation. Subsequent engagement of the intracellular C5aR1 by C5a induced the generation of reactive oxygen species (ROS) and the unexpected assembly of a functional

NLRP3 inflammasome in CD4<sup>+</sup> T cells, whereas the surface-expressed C5aR2 negatively controlled this process.

NLRP3 inflammasome–dependent autocrine IL-1 $\beta$  secretion and activity were required for optimal IFN- $\gamma$  production by T cells; consequently, dysregulation of NLRP3 function in these cells affected their normal effector responses. For example, mutated, constitutively active NLRP3 in T cells from patients with CAPS induced hyperactive T<sub>H</sub>1 responses that could be normalized with a NLRP3 inhibitor. The in vivo importance of a T cell–intrinsic NLRP3 inflammasome was further supported by the finding that IFN- $\gamma$  production by *Nlrp3*<sup>-/-</sup> CD4<sup>+</sup> T cells was significantly reduced during viral infections in mice and that diminished T<sub>H</sub>1 induction due to lack of NLRP3 function in a CD4<sup>+</sup> T cell transfer model of colitis led to uncontrolled T<sub>H</sub>17 infiltration and/or expansion in the intestine and aggravated disease.

**CONCLUSION:** Our results demonstrate that the regulated cross-talk between intracellularly activated complement components (the “complosome”) and the NLRP3 inflammasome is fundamental to human T<sub>H</sub>1 induction and regulation. The finding that established innate immune pathways are also operative in adaptive immune cells and orchestrate immunological responses contributes to our understanding of immunobiology and immune system evolution. In addition, the results suggest that the complement–NLRP3 axis in T cells represents a novel therapeutic target for the modulation of T<sub>H</sub>1 activity in autoimmunity and infection. ■



**An intrinsic complement–NLRP3 axis regulates human T<sub>H</sub>1 responses.** T cell receptor activation and CD46 costimulation trigger *NLRP3* expression and intracellular C5a generation. Subsequent intracellular C5aR1 engagement induces ROS production (and possibly *IL1B* gene transcription) and NLRP3 assembly, which in turn mediates IL-1 $\beta$  maturation. Autocrine IL-1 $\beta$  promotes T<sub>H</sub>1 induction (IFN- $\gamma$  production) but restricts T<sub>H</sub>1 contraction (IL-10 coexpression). C5aR2 cell surface activation by secreted C5a negatively controls these events via undefined mechanisms. Dysfunction of this system contributes to impaired T<sub>H</sub>1 responses in infection or increased T<sub>H</sub>17 responses during intestinal inflammation.

The list of author affiliations is available in the full article online.

\*These authors contributed equally to this work.

†Corresponding author. Email: [claudia.kemper@kcl.ac.uk](mailto:claudia.kemper@kcl.ac.uk)  
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## RESEARCH ARTICLE

## IMMUNE REGULATION

# T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4<sup>+</sup> T cells

Giuseppina Arbore,<sup>1\*</sup> Erin E. West,<sup>2\*</sup> Rosanne Spolski,<sup>2</sup> Avril A. B. Robertson,<sup>3</sup> Andreas Klos,<sup>4</sup> Claudia Rheinheimer,<sup>4</sup> Pavel Dutow,<sup>4</sup> Trent M. Woodruff,<sup>3</sup> Zu Xi Yu,<sup>5</sup> Luke A. O'Neill,<sup>6</sup> Rebecca C. Coll,<sup>3</sup> Alan Sher,<sup>7</sup> Warren J. Leonard,<sup>2</sup> Jörg Köhl,<sup>8,9</sup> Pete Monk,<sup>10</sup> Matthew A. Cooper,<sup>3</sup> Matthew Arno,<sup>11</sup> Behdad Afzali,<sup>1,12</sup> Helen J. Lachmann,<sup>13</sup> Andrew P. Cope,<sup>14</sup> Katrin D. Mayer-Barber,<sup>15</sup> Claudia Kemper<sup>1,2,†</sup>

The NLRP3 inflammasome controls interleukin-1 $\beta$  maturation in antigen-presenting cells, but a direct role for NLRP3 in human adaptive immune cells has not been described. We found that the NLRP3 inflammasome assembles in human CD4<sup>+</sup> T cells and initiates caspase-1-dependent interleukin-1 $\beta$  secretion, thereby promoting interferon- $\gamma$  production and T helper 1 (T<sub>H</sub>1) differentiation in an autocrine fashion. NLRP3 assembly requires intracellular C5 activation and stimulation of C5a receptor 1 (C5aR1), which is negatively regulated by surface-expressed C5aR2. Aberrant NLRP3 activity in T cells affects inflammatory responses in human autoimmune disease and in mouse models of inflammation and infection. Our results demonstrate that NLRP3 inflammasome activity is not confined to “innate immune cells” but is an integral component of normal adaptive T<sub>H</sub>1 responses.

The complement system is an ancient innate immune sensor system that is essential for elimination of pathogens by the host. Processing in serum of liver-derived C3 into C3a and C3b and of C5 into C5a and C5b activation fragments leads to opsonization and removal of invading microbes, mobilization of innate im-

mune cells, and induction of inflammatory reactions (1). However, complement also profoundly regulates adaptive immunity: In addition to T cell receptor (TCR) activation, costimulation, and the presence of interleukin (IL)-12 (2), human CD4<sup>+</sup> T cells also depend on the activation of T cell-expressed complement receptors binding C3 activation fragments for normal T helper 1 (T<sub>H</sub>1) induction (3). Unexpectedly, the engagement of complement receptors on T cells is independent of systemic complement but instead is mediated in an autocrine manner by complement activation fragments produced by the T cell itself. In particular, C3a and C3b are generated intracellularly via cathepsin L-mediated cleavage of C3 in T cells upon TCR activation (4). These engage their respective receptors—a G protein-coupled receptor (GPCR) C3a receptor (C3aR) and the complement regulator CD46 (which binds C3b)—and induce autocrine interferon- $\gamma$  (IFN- $\gamma$ ) (5, 6). Mechanistically, C3aR- and CD46-mediated signals (i) regulate IL-2R assembly, (ii) up-regulate the glucose transporter GLUT1 and the amino acid transporter LAT1, and (iii) up-regulate mTORC1 activation, which is required for the metabolic programming essential for IFN- $\gamma$  induction (7).

However, CD46 costimulation is not only essential for IFN- $\gamma$  production and human T<sub>H</sub>1 induction; it also contributes to the negative control of T<sub>H</sub>1 responses. Together with IL-2, CD46-mediated signals drive the coexpression of immunosuppressive IL-10 in T<sub>H</sub>1 cells and initiate their switch into a (self-)regulatory and contracting phase (3). Accordingly, C3- and CD46-deficient patients suffer from recurrent infections and have severely reduced

T<sub>H</sub>1 responses in vitro and in vivo, whereas T<sub>H</sub>2 responses remain intact (5, 8). Conversely, uncontrolled intracellular C3 activation (or dysregulated CD46 engagement) in T cells contributes to hyperactive T<sub>H</sub>1 responses observed in autoimmunity (3, 4, 9) that can be normalized pharmacologically by targeting intracellular cathepsin L function (4). Of note, CD46 is not expressed on somatic tissue in rodents and a functional homolog has not yet been identified. This indicates the existence of substantial differences in the complement receptor-driven pathways regulating T cell responses between species [reviewed in (6)].

Given the critical role of intracellular C3 processing in human T<sub>H</sub>1 induction and contraction and the importance of C5a generation in inflammation, we investigated whether human CD4<sup>+</sup> T cells also harbor an “intracellular C5 activation” system contributing to effector responses.

## Autocrine activation of C5a receptors regulates IFN- $\gamma$ production by human CD4<sup>+</sup> T cells

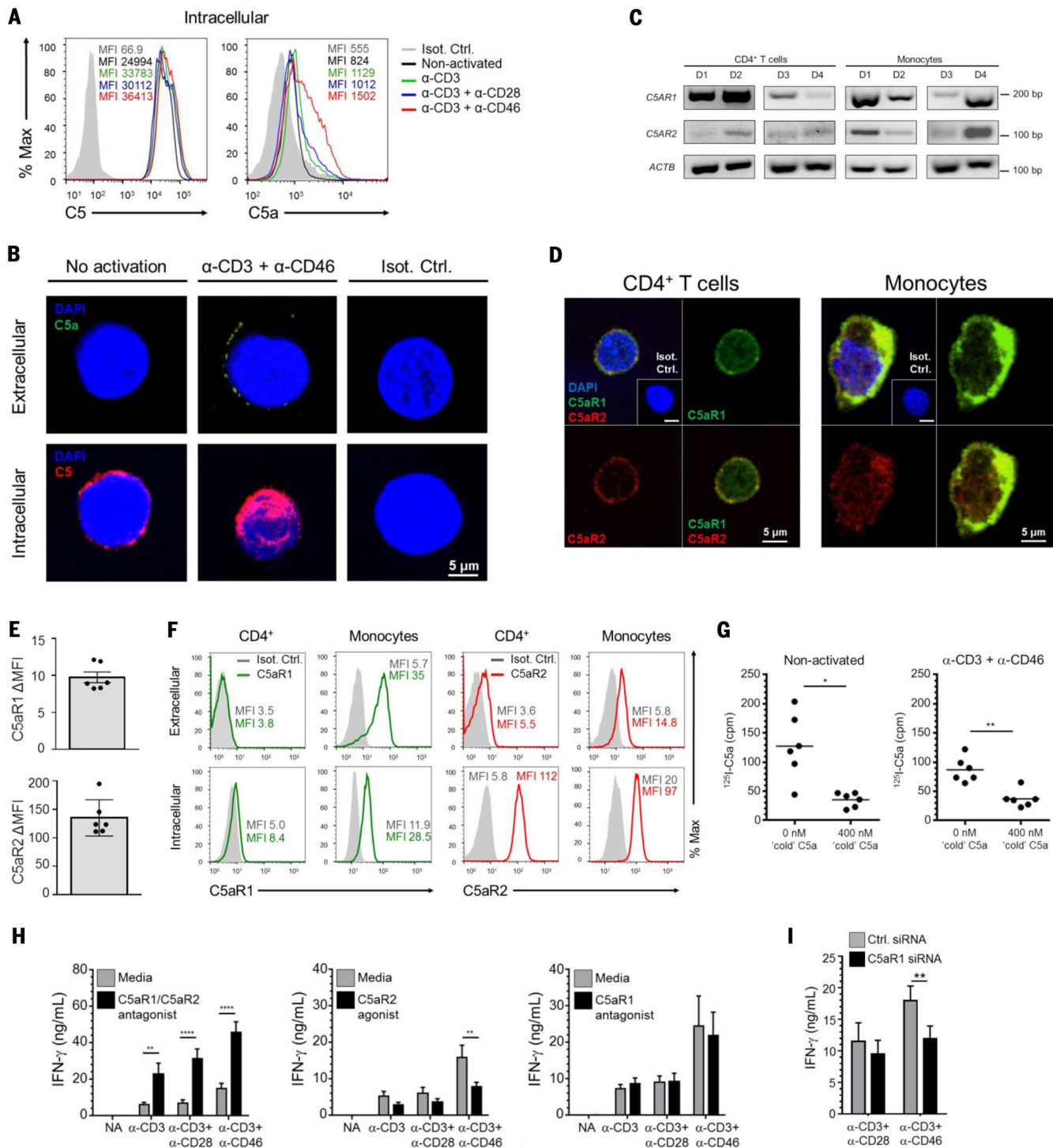
Human CD4<sup>+</sup> T lymphocytes isolated from healthy donors contained intracellular stores of C5 and produced low levels of C5a in the resting state. TCR activation, in particular TCR + CD46 costimulation, increased the amounts of intracellular C5a, and this was associated with the secretion of C5a to the cell surface (Fig. 1, A and B). C5a, as well as the C5a “des-arginized” form of C5a (C5adesArg) generated by carboxypeptidase processing, can bind two distinct GPCR receptors, C5aR1 (CD88) and C5aR2 (GPR77, C5L2) (10, 11). Binding of C5a to C5aR1 preferentially mediates proinflammatory responses. The function of C5aR2 varies with cell type; C5aR2 can act either as a nonsignaling decoy receptor antagonizing C5aR1 or as an active transducer of pro- or anti-inflammatory signals (11–14).

Both extra- and intracellular localization of C5aR1 and C5aR2 on human monocytes have been reported (14, 15), but expression patterns in human CD4<sup>+</sup> T cells have not been described in detail. We detected expression of both *C5aR1* and *C5aR2* mRNA in human CD4<sup>+</sup> T cells (Fig. 1C) and protein by immunoblotting (fig. S1A), confocal microscopy (Fig. 1D), and flow cytometry (Fig. 1, E and F). Although mRNA amounts for *C5aR1* and *C5aR2* vary in T cells (Fig. 1C) (16), the protein levels for these receptors are comparable among donors (Fig. 1E). In resting and activated CD4<sup>+</sup> T cells, C5aR1 is expressed exclusively intracellularly and in low amounts, whereas the C5aR2 receptor is abundantly present inside and to a lesser degree on the cell surface (Fig. 1F). Using human embryonic kidney (HEK) 293 cells that had been stably transfected to express C5aR1, C5aR2, or no receptor, we corroborated the specificity of reagents used for C5a receptor detection (fig. S1, B and C). Competitive binding studies of C5a labeled with radioactive <sup>125</sup>I (Fig. 1G and fig. S1D) confirmed the ability of resting and activated human CD4<sup>+</sup> T cells to bind C5a.

To determine whether autocrine engagement of the C5a receptors on T cells regulates T<sub>H</sub>1 induction, we activated human CD4<sup>+</sup> T cells with

<sup>1</sup>MRC Centre for Transplantation, Division of Transplant Immunology and Mucosal Biology, King's College London, London SE1 9RT, UK. <sup>2</sup>Laboratory of Molecular Immunology and Immunology Center, National Heart, Lung, and Blood Institute, Bethesda, MD 20892, USA. <sup>3</sup>Institute for Molecular Bioscience and School of Biomedical Sciences, University of Queensland, QLD 4072, Australia. <sup>4</sup>Institute for Medical Microbiology and Hospital Epidemiology, Medizinische Hochschule Hannover, 30625 Hannover, Germany. <sup>5</sup>Pathology Core, National Heart, Lung, and Blood Institute, Bethesda, MD 20892, USA. <sup>6</sup>School of Biochemistry and Immunology, Trinity College Dublin, Dublin, Ireland. <sup>7</sup>Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA. <sup>8</sup>Institute for Systemic Inflammation Research, University of Lübeck, Lübeck, Germany. <sup>9</sup>Division of Immunobiology, Cincinnati Children's Hospital Medical Center and University of Cincinnati College of Medicine, Cincinnati, OH, USA. <sup>10</sup>Department of Infection and Immunity, University of Sheffield, Sheffield S10 2RX, UK. <sup>11</sup>Genomics Centre, Faculty of Life Sciences and Medicine, King's College London, London SE1 9NH, UK. <sup>12</sup>Lymphocyte Cell Biology Section, Molecular Immunology and Inflammation Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, Bethesda, MD 20892, USA. <sup>13</sup>UK National Amyloidosis Centre, Division of Medicine, University College London, Royal Free Campus, London NW3 2PF, UK. <sup>14</sup>Academic Department of Rheumatology, Division of Immunology, Infection and Inflammatory Diseases, King's College London, London SE1 1UL, UK. <sup>15</sup>Laboratory of Clinical Infectious Diseases, Inflammation and Innate Immunity Unit, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20892, USA.

\*These authors contributed equally to this work. †Corresponding author. Email: claudia.kemper@kcl.ac.uk



**Fig. 1. Autocrine activation of C5a receptors regulates IFN- $\gamma$  production by human CD4<sup>+</sup> T cells.**

(A and B) Intracellular C5 and C5a generation in CD4<sup>+</sup> T lymphocytes, left nonactivated or activated (36 hours) with anti-CD3 ( $\alpha$ -CD3),  $\alpha$ -CD3 +  $\alpha$ -CD28, or  $\alpha$ -CD3 +  $\alpha$ -CD46 by flow cytometry (A) and confocal microscopy (B) (data representative of  $n = 3$ ). (C) RT-PCR analysis for *C5aR1* and *C5aR2* mRNA in resting human CD4<sup>+</sup> cells and monocytes ( $n = 4$ , donors D1 to D4, endogenous control *ACTB*). (D) Intracellular immunofluorescence on resting T cells and monocytes with antibodies to C5aR1 (green) and C5aR2 (red) (data are representative of  $n = 3$ ). (E) C5aR1 and C5aR2 protein amounts in T cells with expression normalized to respective isotype control staining for each donor (change in mean fluorescence intensity  $\Delta$ MFI  $\pm$  SEM,  $n = 6$ ). (F) Flow cytometry for C5aR1 and C5aR2 on resting T cells and monocytes, with representative histogram plots shown ( $n = 6$ ). (G) Binding of radioactively labeled <sup>125</sup>I-C5a in absence or presence of nonlabeled “cold” C5a as competitor to resting or  $\alpha$ -CD3 +  $\alpha$ -CD46 activated (4 hours) T cells ( $n = 6$ ). (H) IFN- $\gamma$  secretion in nonactivated (NA) and activated (36 hours) CD4<sup>+</sup> T cells in the absence or presence of a C5aR1/C5aR2 double receptor antagonist ( $n = 9$ ), a C5aR2 agonist ( $n = 8$ ), or a C5aR1 antagonist ( $n = 7$ ). (I) IFN- $\gamma$  production by T cells transfected with C5aR1-specific siRNA or a scrambled control siRNA (Ctrl. siRNA) 36 hours after activation ( $n = 7$ ). Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ . (G), paired *t* test; (H) and (I), two-way ANOVA with Bonferroni multiple comparison test.

(A) Flow cytometry histograms showing intracellular C5 and C5a generation. The left histogram shows C5 (% Max) with MFI values: 66.9 (Isot. Ctrl.), 24994 ( $\alpha$ -CD3), 33783 ( $\alpha$ -CD3 +  $\alpha$ -CD28), 30112 ( $\alpha$ -CD3 +  $\alpha$ -CD46), and 36413 (Non-activated). The right histogram shows C5a (% Max) with MFI values: 555 (Isot. Ctrl.), 824 ( $\alpha$ -CD3), 1129 ( $\alpha$ -CD3 +  $\alpha$ -CD28), 1012 ( $\alpha$ -CD3 +  $\alpha$ -CD46), and 1502 (Non-activated).

(B) Confocal microscopy images showing extracellular and intracellular C5 and C5a in CD4<sup>+</sup> T cells under three conditions: No activation,  $\alpha$ -CD3 +  $\alpha$ -CD46, and Isot. Ctrl. DAPI (blue) stains nuclei, C5a (green) and C5 (red) are shown in the extracellular and intracellular panels. Scale bar = 5  $\mu$ m.

(C) RT-PCR analysis of *C5aR1*, *C5aR2*, and *ACTB* mRNA in CD4<sup>+</sup> T cells and monocytes at days 1, 2, 3, and 4. Molecular weight markers are indicated on the right (200 bp for *C5aR1*, 100 bp for *C5aR2* and *ACTB*).

(D) Immunofluorescence images of CD4<sup>+</sup> T cells and monocytes stained for C5aR1 (green) and C5aR2 (red). DAPI (blue) stains nuclei. Scale bar = 5  $\mu$ m.

(E) Bar graphs showing the change in mean fluorescence intensity ( $\Delta$ MFI) for C5aR1 and C5aR2 in CD4<sup>+</sup> T cells. C5aR1  $\Delta$ MFI is approximately 10, and C5aR2  $\Delta$ MFI is approximately 140.

(F) Flow cytometry histograms for C5aR1 and C5aR2 expression in CD4<sup>+</sup> T cells and monocytes. Extracellular and intracellular expression are shown. MFI values are provided for each cell type and receptor.

(G) Scatter plot showing the binding of <sup>125</sup>I-C5a to CD4<sup>+</sup> T cells. The left plot shows non-activated cells, and the right plot shows  $\alpha$ -CD3 +  $\alpha$ -CD46 activated cells. Data points represent individual donors, and horizontal lines indicate mean  $\pm$  SEM.

(H) Bar graph showing IFN- $\gamma$  secretion (ng/mL) in CD4<sup>+</sup> T cells. Media, C5aR1/C5aR2 antagonist, and C5aR2 agonist are shown for NA,  $\alpha$ -CD3,  $\alpha$ -CD3 +  $\alpha$ -CD28, and  $\alpha$ -CD3 +  $\alpha$ -CD46 conditions.

(I) Bar graph showing IFN- $\gamma$  production (ng/mL) in CD4<sup>+</sup> T cells transfected with Ctrl. siRNA or C5aR1-specific siRNA under the same activation conditions as in (H).

immobilized antibodies to CD3, CD3 and CD28, or CD3 and CD46 in the presence or absence of (i) a specific antagonist to C5aR1 [PMX53 (17)]; (ii) the C5aR1/C5aR2 receptor double antagonist A8<sup>A71-73</sup> [dRA (18)], targeting only C5aR2 (as the C5aR1 is expressed intracellularly); or (iii) a specific C5aR2 agonist (19). All reagents were cell-impermeable. Blocking C5aR2 activity significantly increased T<sub>H</sub>1 induction (Fig. 1H, left), and activating C5aR2 with the agonist or with C5a or C5adesArg reduced T<sub>H</sub>1 responses (Fig. 1H, middle, and fig. S1E). Blockade of C5aR2 also led to increased T<sub>H</sub>17 (IL-17) but not T<sub>H</sub>2 (IL-4) responses (fig. S1F) without altering cell viability (fig. S1G). Consistent with the solely intracellular localization of C5aR1, the C5aR1-specific antagonist had no effect on IFN- $\gamma$  production because it could not “reach” and block intracellular C5aR1 (Fig.

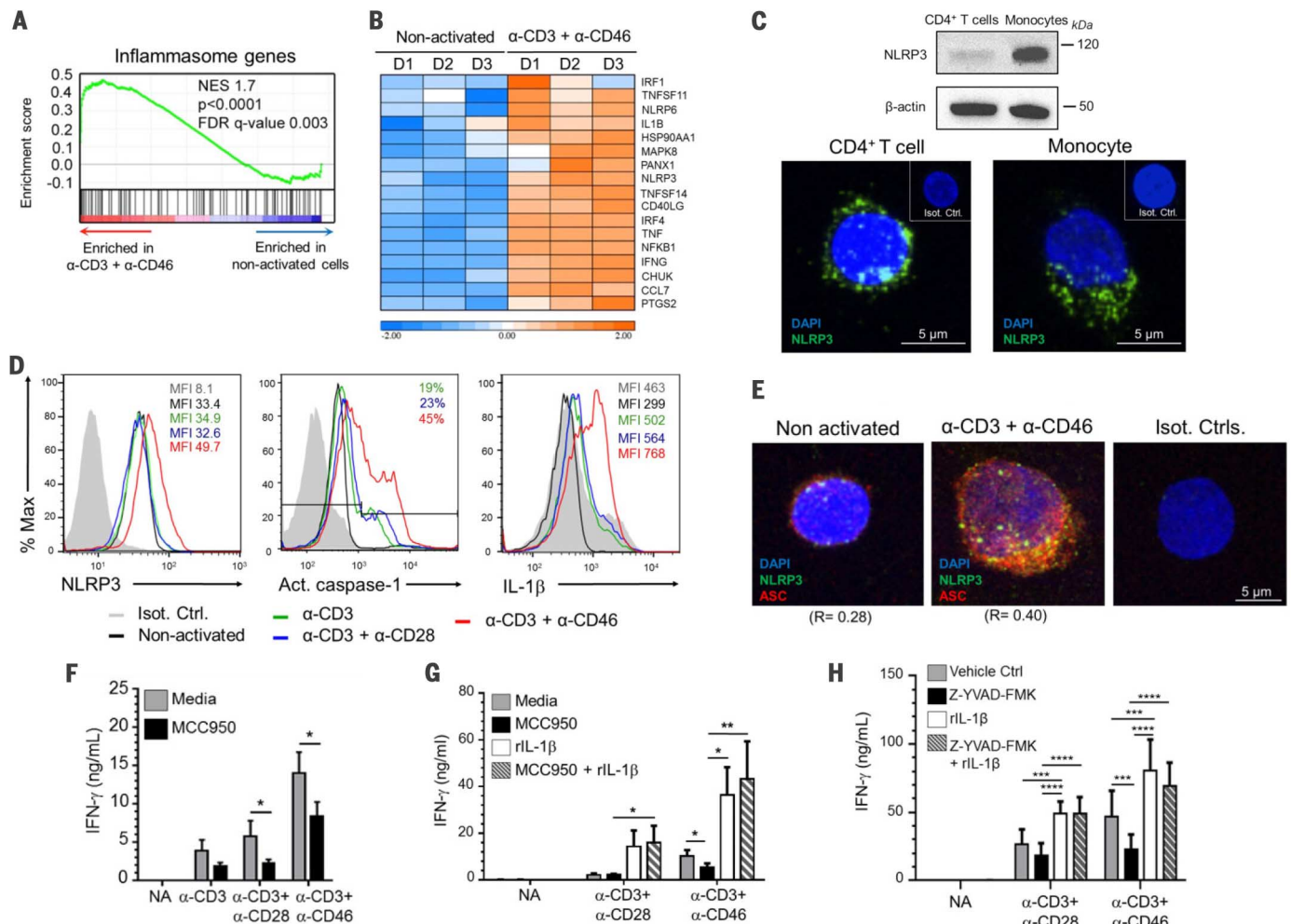
1H, right). However, reduction of intracellular C5aR1 by small interfering RNA (siRNA) gene targeting led to a commensurate decrease in IFN- $\gamma$  production (Fig. 1I and fig. S1H). Together, these data show that intracellular C5 activation contributes to induction of IFN- $\gamma$  production in CD4<sup>+</sup> T cells via intracellular C5aR1 engagement, and that the surface-expressed C5aR2 exerts negative control of IFN- $\gamma$ , possibly via suppression of intracellular C5aR1 signals.

### Canonical NLRP3 inflammasome activation in CD4<sup>+</sup> T cells enhances IFN- $\gamma$ production

To delineate the autocrine C5-driven pathways contributing to regulation of IFN- $\gamma$  in CD4<sup>+</sup> T cells, we performed a transcriptome analysis using T cells from three healthy donors activated, or not,

with anti-CD3 and anti-CD46 in the presence or absence of the C5aR1/C5aR2 antagonist. Surprisingly, we observed enrichment of transcripts associated with inflammasome activation, including *NLRP3* and *IL1B* (Fig. 2, A and B, and table S1), in cells activated with anti-CD3 and anti-CD46. Inhibition of C5aR2 during these activation conditions further increased some of these transcripts, notably *IL1A* and *IL1B* (fig. S2A and table S2); this finding offers further support for the idea that blocking C5aR2 leads to unrestrained or increased engagement of intracellular C5aR1 driven by the anti-CD3- and anti-CD46-induced increase in intracellular C5a generation.

IL-1 $\alpha$  and IL-1 $\beta$  are prototypical proinflammatory cytokines involved in innate immune responses and contributing to the development of several pathogenic autoimmune diseases, including



**Fig. 2. NLRP3 inflammasome activation occurs in CD4<sup>+</sup> T cells and enhances IFN- $\gamma$  production.** (A) Gene set enrichment analysis (GSEA) for inflammasome-related genes in CD4<sup>+</sup> T cells after  $\alpha$ -CD3 +  $\alpha$ -CD46 activation (2 hours) compared to resting cells (donors D1 to D3). (B) Heat map depicting leading edge analysis (the core enriched genes) of the data in (A). (C) NLRP3 immunoblot (upper panel) and immunofluorescence (lower panel) on CD4<sup>+</sup> lymphocytes and monocytes (data representative of  $n = 3$ ). (D) NLRP3, activated caspase-1 and total IL-1 $\beta$  protein expression in activated CD4<sup>+</sup> cells (data rep-

resentative of  $n = 3$ ). (E) Representative immunofluorescence costaining for NLRP3 (green) and ASC (red) on resting and  $\alpha$ -CD3 +  $\alpha$ -CD46 activated T cells ( $r =$  Pearson correlation coefficient between NLRP3 and ASC fluorescence,  $n = 3$ ). (F and G) IFN- $\gamma$  production by resting (NA) and activated CD4<sup>+</sup> T cells with or without MCC950 addition ( $n = 7$ ) (F) and with or without rIL-1 $\beta$  supplementation ( $n = 3$ ) (G). (H) IFN- $\gamma$  production in presence of the specific caspase-1 inhibitor Z-YVAD-FMK with or without rIL-1 $\beta$  addition. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ . (F) to (H), two-way ANOVA with Bonferroni multiple comparison test.

type 1 diabetes and arthritis (20–22). Both IL-1 $\alpha$  and IL-1 $\beta$  bind to IL-1 receptor 1 (IL-1R1). Antigen-presenting cell (APC)-derived IL-1 $\beta$  supports T cell priming and imprinting of T helper effector function (23), including enhancement of IFN- $\gamma$  and IL-17 production from CD4 $^+$  T cells (24–26). Further, mice with deletion of the IL-1 $\beta$  signal transducer MyD88 in T lymphocytes cannot generate memory T cells (27). Pro-IL-1 $\beta$  is synthesized as a 31-kDa precursor and converted to mature 17-kDa IL-1 $\beta$  via caspase-1 cleavage (28). Caspase-1 is regulated by proteolytic activation during oligomerization with NLRP3 and the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), which is triggered in response to danger signals (29, 30). NLRP3 inflammasome function requires a priming signal 1 (which induces NLRP3 and *IL1B* gene transcription) and a signal 2 that induces functional inflammasome assembly (30) and has been described in myeloid innate immune cells, with monocytes as the main source of IL-1 $\beta$  (25, 31), and in several nonimmune cell types (such as microglia, endothelial cells, and retinal pigment epithelial cells) (32–34). However, canonical NLRP3 inflammasome activity has not been demonstrated in lymphoid adaptive immune cells.

We confirmed the presence of an “NLRP3 signature” in T cells by demonstrating NLRP3 and *IL1B* gene (fig. S2B) and protein expression, as well as generation of activated caspase-1 and mature IL-1 $\beta$ , in activated human CD4 $^+$  T cells (Fig. 2, C and D, and fig. S2, C to F). Consistent with our gene array data, anti-CD3 and anti-CD46 activation led to robust NLRP3 activation and IL-1 $\beta$  generation (Fig. 2D) and increased colocalization of NLRP3 and ASC (Fig. 2E). Notably, both resting naïve

and memory CD4 $^+$  T cells expressed NLRP3 protein (fig. S2, C and D).

Because IL-1 $\beta$  supports T<sub>H</sub>1 induction (35) and is most strongly induced by the T<sub>H</sub>1 driver CD46, we next assessed whether inhibition of NLRP3 activity in CD4 $^+$  T cells perturbs IFN- $\gamma$  production. To this end, CD4 $^+$  T cells were activated in the presence of MCC950, a specific NLRP3 inhibitor (36), and T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 cytokine production was measured 36 hours after activation. NLRP3 inhibition during T cell activation specifically attenuated IFN- $\gamma$  (Fig. 2F), whereas differences in IL-4 and IL-17 production did not reach significance (fig. S2G) and cell viability was unaffected (fig. S2H). The effects of the NLRP3 inhibitor could be fully reversed by the addition of recombinant human IL-1 $\beta$  (rhIL-1 $\beta$ ) to cultures (Fig. 2G). Similarly, reduction of active caspase-1 activity by the specific inhibitor Z-YV AD-FMK repressed IL-1 $\beta$  and IFN- $\gamma$  secretion (Fig. 2H and fig. S2I), and rhIL-1 $\beta$  provision normalized T<sub>H</sub>1 induction in these cultures. The role for IL-1 $\beta$  as critical autocrine “T<sub>H</sub>1 supporter” is reinforced by our observation that no IL-18 [which also depends on NLRP3 activation and can support T<sub>H</sub>1 responses (37)] was measurable in our cultures and that addition of IL-18 binding protein had no effect on cytokine production (fig. S2J).

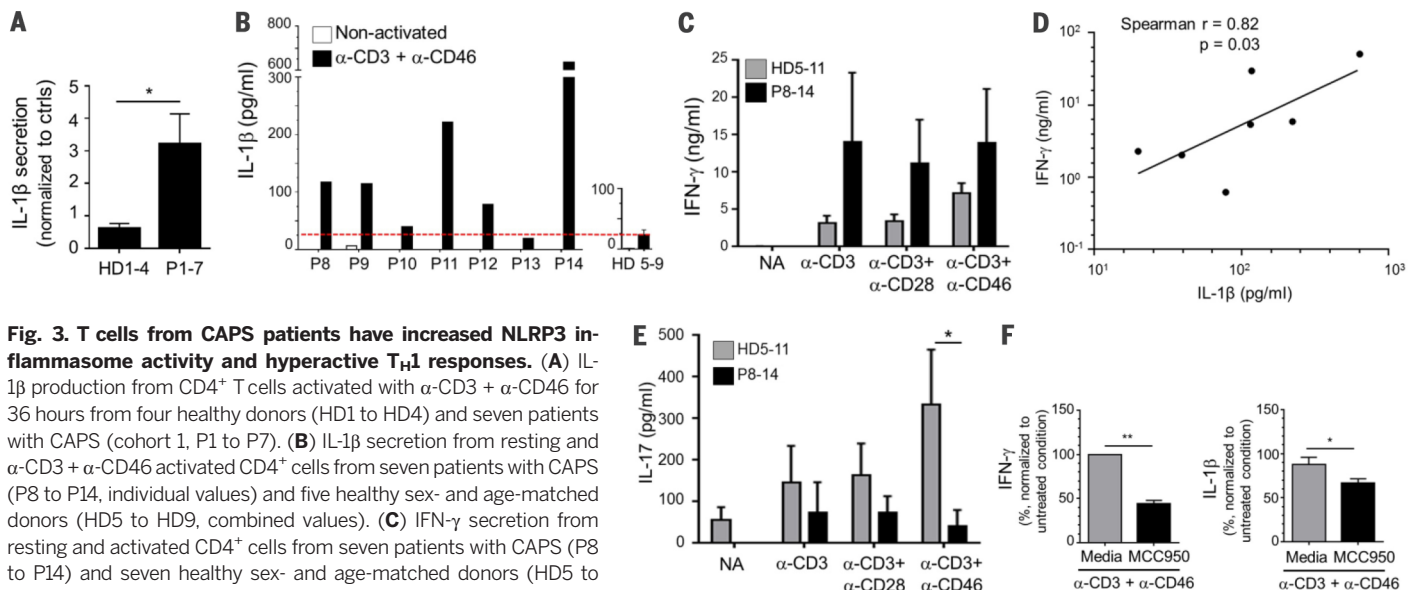
### The hyperactive in vitro T<sub>H</sub>1 response in CAPS patients is normalized by NLRP3 inhibition

To further explore this pathway, we measured the effects of NLRP3 hyperactivity in CD4 $^+$  T cells isolated from the blood of patients with distinct gain-of-function mutations in NLRP3 (patient char-

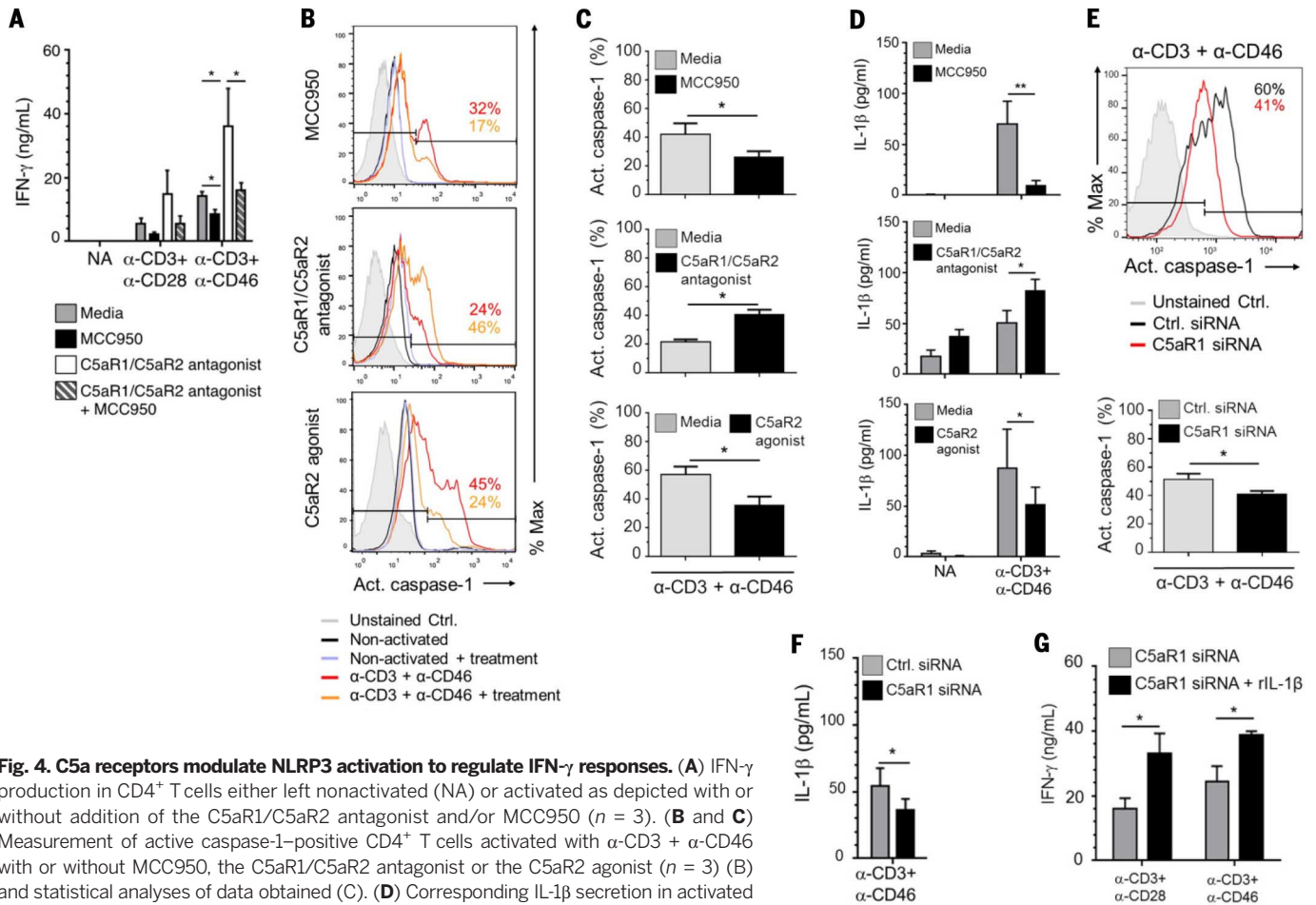
acteristics are summarized in table S3). This class of NLRP3 mutations is associated with a group of heritable monogenic syndromes known as cryopyrin-associated periodic syndromes (CAPS), characterized by excessive production of IL-1 $\beta$  from APCs with recurrent fevers, skin rashes, joint and ocular inflammation, and amyloidosis (38). Therapeutic suppression of the inflammatory responses can be achieved by IL-1R blockade with anakinra, an IL-1R antagonist, or canakinumab, a monoclonal antibody (mAb) targeting IL-1 $\beta$  (38, 39). Despite their medication regimen and the fact that cytokine production by immune cells from CAPS patients can vary with their respective “flare status” (40), T cells from a first cohort of CAPS patients that we assessed had significantly increased IL-1 $\beta$  secretion relative to sex- and age-matched healthy donors (Fig. 3A), indicating that increased NLRP3 activity in CD4 $^+$  T cells indeed induces heightened IL-1 $\beta$  secretion.

We next performed a more in-depth analysis of T cell in vitro responses from another cohort of seven CAPS patients (table S4). All patients had a naïve versus memory T cell distribution comparable to those of healthy donors (fig. S3A), and T cells from five patients of this second cohort also showed significantly increased IL-1 $\beta$  secretion upon activation (Fig. 3B). Furthermore, CD4 $^+$  T cells from these patients trended toward substantially increased IFN- $\gamma$  relative to T cells from sex- and age-matched healthy donors, and we observed a statistically significant correlation between increased IL-1 $\beta$  and IFN- $\gamma$  secretion (Fig. 3, C and D).

T cells from CAPS patients displayed significantly reduced in vitro IL-17 responses (Fig. 3E).



**Fig. 3. T cells from CAPS patients have increased NLRP3 inflammasome activity and hyperactive T<sub>H</sub>1 responses.** (A) IL-1 $\beta$  production from CD4 $^+$  T cells activated with  $\alpha$ -CD3 +  $\alpha$ -CD46 for 36 hours from four healthy donors (HD1 to HD4) and seven patients with CAPS (cohort 1, P1 to P7). (B) IL-1 $\beta$  secretion from resting and  $\alpha$ -CD3 +  $\alpha$ -CD46 activated CD4 $^+$  cells from seven patients with CAPS (P8 to P14, individual values) and five healthy sex- and age-matched donors (HD5 to HD9, combined values). (C) IFN- $\gamma$  secretion from resting and activated CD4 $^+$  cells from seven patients with CAPS (P8 to P14) and seven healthy sex- and age-matched donors (HD5 to HD11). (D) Correlation between IL-1 $\beta$  and IFN- $\gamma$  production in T cells from patients P8 to P14 upon  $\alpha$ -CD3 +  $\alpha$ -CD46 activation (Spearman correlation analysis). (E) IL-17 production by resting and activated T cells from CAPS patients P8 to P14 and healthy donors H5 to H11. (F) IFN- $\gamma$  and IL-1 $\beta$  secretion by CD4 $^+$  T cells from P8, P11, and P14 after  $\alpha$ -CD3 +  $\alpha$ -CD46 activation with or without MCC950 treatment (% normalized to nontreated). Analyses on (A) to (F) were performed at 36 hours after activation. Values correspond to two technical replicates for every patient and healthy control sample in each experiment. Data are means  $\pm$  SEM. \* $P$  < 0.05, \*\* $P$  < 0.01. (A), unpaired  $t$  test; (C) and (E), two-way ANOVA with Bonferroni multiple comparison test; (D), Spearman correlation test; (F), paired  $t$  test.



**Fig. 4. C5a receptors modulate NLRP3 activation to regulate IFN- $\gamma$  responses.** (A) IFN- $\gamma$  production in CD4<sup>+</sup> T cells either left nonactivated (NA) or activated as depicted with or without addition of the C5aR1/C5aR2 antagonist and/or MCC950 ( $n = 3$ ). (B and C) Measurement of active caspase-1-positive CD4<sup>+</sup> T cells activated with  $\alpha$ -CD3 +  $\alpha$ -CD46 with or without MCC950, the C5aR1/C5aR2 antagonist or the C5aR2 agonist ( $n = 3$ ) (B) and statistical analyses of data obtained (C). (D) Corresponding IL-1 $\beta$  secretion in activated CD4<sup>+</sup> cells treated as in (B) ( $n = 5$ ). (E and F) Active caspase-1 levels [(E),  $n = 4$ ] and IL-1 $\beta$  secretion [(F),  $n = 7$ ] in T cells after transfection with either C5aR1-specific siRNA or scrambled control (Ctrl.) siRNA. (G) IFN- $\gamma$  production in activated CD4<sup>+</sup> T cells after transfection with C5aR1-specific siRNA or a scrambled control siRNA (Ctrl. siRNA) with or without addition of rhIL-1 $\beta$  ( $n = 3$ ). Analyses were performed at 36 hours after activation. Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ . (A), (D), and (G), two-way ANOVA with Bonferroni multiple comparison test; (C), (E), and (F), paired  $t$  test.

Although caspase-1 activity was not significantly increased in the patients' T cells at the time point assessed (36 hours), the patients with highest IL-1 $\beta$  secretion also had the highest active caspase-1 levels (fig. S3B). Activation of CD4<sup>+</sup> T cells from CAPS patients in the presence of the NLRP3 inhibitor MCC950 led to a reduction of both IL-1 $\beta$  and IFN- $\gamma$  secretion (Fig. 3F). Together, these data demonstrate that human CD4<sup>+</sup> T cells produce IL-1 $\beta$  in an NLRP3-dependent manner, that autocrine IL-1 $\beta$  generation supports IFN- $\gamma$  secretion, and that dysregulation of this pathway occurs in human autoinflammatory disease.

### C5a receptors modulate NLRP3 inflammasome activity to regulate IFN- $\gamma$ production

We next asked whether C5aR signaling could directly regulate NLRP3 activity in human CD4<sup>+</sup> T cells. C5aR2 blockade in CD3 + CD46-activated T cells further increased *IL1B* but not *NLRP3* mRNA (fig. S4, A and B). Enhanced IFN- $\gamma$  secretion driven by C5aR2 blockade could be reversed by inhibition of NLRP3 with MCC950 (Fig. 4A) without affect-

ing IL-17 or IL-4 production (fig. S4C). Pharmacological targeting of C5aR2 via either dRA (blockage of C5aR2 signaling) or a C5aR2 agonist (activation of C5aR2 signaling) revealed that C5aR2 negatively regulates active caspase-1 and mature IL-1 $\beta$  expression in T cells (Fig. 4, B to D) but does not affect NLRP3 protein levels per se (fig. S4D). Silencing of *C5AR1* expression had also no effect on NLRP3 protein levels (fig. S4E) but reduced active caspase-1 (Fig. 4E) and IL-1 $\beta$  expression (Fig. 4F). Moreover, the reduction of IFN- $\gamma$  secretion after *C5AR1* gene silencing was "rescued" by addition of rhIL-1 $\beta$  (Fig. 4G). Together, these data suggest that CD46-mediated signals increase *NLRP3* mRNA expression in T cells, whereas C5aR1 supports subsequent NLRP3 assembly and C5aR2 is a negative regulator of this process.

Reactive oxygen species (ROS) are "classical" upstream stimulators (signal 2) of NLRP3 assembly (41) and are strongly induced by C5aR1 in monocytes and neutrophils (42). Furthermore, generation of ROS within CD4<sup>+</sup> T cells is required for T cell activation and induction of IL-2, a key cytokine for T<sub>H</sub>1 biology (43). We therefore as-

essed whether autocrine C5aR1 engagement by intracellular C5a generation in T cells induces NLRP3 inflammasome assembly via ROS generation. We observed potent generation of ROS in anti-CD3- and anti-CD46-induced T<sub>H</sub>1 cells (Fig. 5A) and poor T<sub>H</sub>1 induction in the presence of a ROS inhibitor (Fig. 5B). Further, reduction of C5aR1 protein expression by gene silencing decreased ROS production (Fig. 5C, left), whereas inhibition of C5aR2 surface activation significantly increased ROS generation in T cells (Fig. 4C, right).

Enhanced IFN- $\gamma$  production by T cells induced by C5aR2 blockade could be entirely reversed by the presence of a ROS inhibitor (Fig. 5B and fig. S5). This finding suggests that NLRP3 activation in human T cells involves intracellular C5-driven ROS production.

### NLRP3 activity in CD4<sup>+</sup> T cells is required for optimal IFN- $\gamma$ responses during viral infection

To address the biological importance of NLRP3-driven autocrine IL-1 $\beta$  production by CD4<sup>+</sup> T cells, we analyzed CD4<sup>+</sup> T cell responses of *Nlrp3*<sup>-/-</sup>,

*Il1a*<sup>-/-</sup>/*Il1b*<sup>-/-</sup>, and *Il1r1*<sup>-/-</sup> mice initially in vitro and subsequently in an established in vivo viral infection model. Similar to human CD4<sup>+</sup> lymphocytes, CD4<sup>+</sup> cells from wild-type mice expressed NLRP3 and IL-1 $\beta$ ; neither NLRP3 nor IL-1 $\beta$  mRNA (Fig. 6A) and protein (fig. S6A) were detectable in T cells from respective gene-deficient animals. We observed no difference in the proportion of naïve versus memory T cells or in T cell survival between wild-type and knockout strains (fig. S6, B and C). However, upon in vitro CD3 + CD28 activation, CD4<sup>+</sup> T cells from *Nlrp3*<sup>-/-</sup>, *Il1a*<sup>-/-</sup>/*Il1b*<sup>-/-</sup>, and *Il1r1*<sup>-/-</sup> mice had a reduction of ~75% in IFN- $\gamma$  production when compared to T cells from wild-type animals (Fig. 6B); in contrast, IL-10, IL-4, and IL-17 production were unaffected in all three mouse mutant lines (fig. S6D). Further, although activation of T cells from wild-type mice in the presence of the NLRP3 inhibitor MCC950 had no effect on cell viability (fig. S6E), only IFN- $\gamma$  production was significantly reduced (Fig. 6C and fig. S6F). These results indicate that diminished IFN- $\gamma$  secretion in the “knockout T cells” was not due to a developmental defect, whereas NLRP3 activity is required for normal IFN- $\gamma$  induction. Moreover, both naïve and memory mouse CD4<sup>+</sup> T cells displayed a requirement for NLRP3-driven IL-1 $\beta$  activity for optimal IFN- $\gamma$  secretion (fig. S6, G and H).

Using a lymphocytic choriomeningitis virus (LCMV) model (Fig. 6D), we next demonstrated an in vivo role for NLRP3-driven IL-1 $\beta$  generation in T<sub>H1</sub> responses during infection. Irradiated mice were reconstituted with equal parts bone marrow cells isolated from wild-type mice mixed with bone marrow cells from *Nlrp3*<sup>-/-</sup>, *Il1a*<sup>-/-</sup>/*Il1b*<sup>-/-</sup>, or *Il1r1*<sup>-/-</sup> mice before infection with LCMV. Analysis of splenic CD4<sup>+</sup> T cells 12 days after infection revealed comparable numbers of GP66-77<sup>+</sup> Ki67<sup>+</sup> LCMV tetramer<sup>+</sup> cells generated by all animals (Fig. 6, E and F), indicating that *Nlrp3*<sup>-/-</sup>, *Il1a*<sup>-/-</sup>/*Il1b*<sup>-/-</sup>, and *Il1r1*<sup>-/-</sup> CD4<sup>+</sup> T cells survived normally. However, T cells deficient in any of these components displayed substantially reduced ability to generate IFN- $\gamma$ <sup>+</sup> virus-specific cells in vivo (with an average decrease of ~50%) (Fig. 6, G and H). Together, these data demonstrate that autocrine canonical NLRP3 inflammasome activity is required for optimal protective IFN- $\gamma$  production by CD4<sup>+</sup> T cells during viral infection.

### Autocrine NLRP3 activity in T cells controls the T<sub>H1</sub>-T<sub>H17</sub> balance during intestinal inflammation

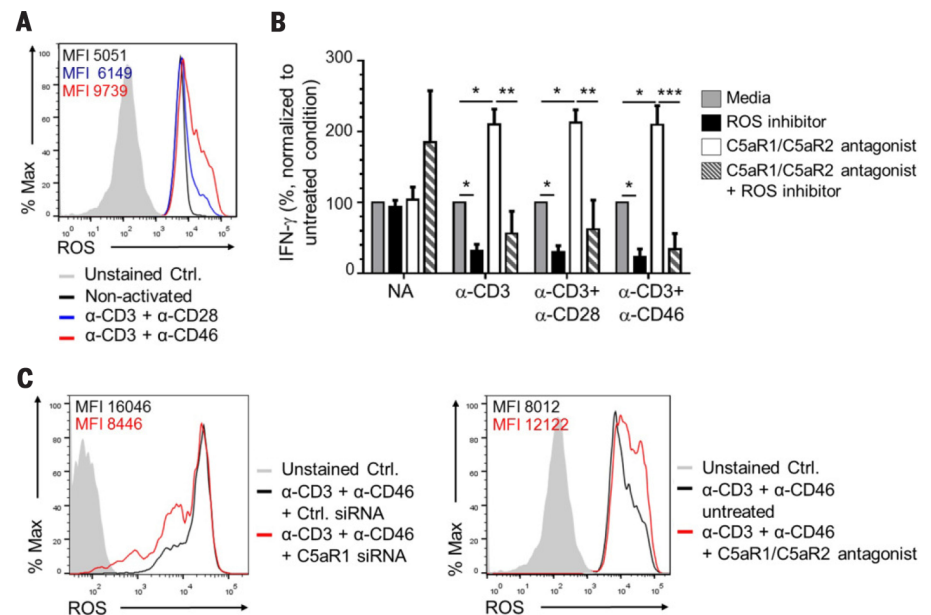
To further substantiate the in vivo importance of NLRP3 inflammasome activity in CD4<sup>+</sup> T cells, we also measured the effects of NLRP3 deficiency in an autoimmune disease setting by assessing its influence on disease outcome in a CD4<sup>+</sup> T cell transfer model of colitis, where IL-1 $\beta$  and both T<sub>H1</sub> and T<sub>H17</sub> responses in the intestine have been shown to be involved (44, 45). To this end, sorted CD4<sup>+</sup>CD25<sup>+</sup>CD45RB<sup>hi</sup> T cells isolated from C57BL/6 wild-type or *Nlrp3*<sup>-/-</sup> mice were injected intraperitoneally (i.p.) into age- and sex-matched C57BL/6 *Rag2*<sup>-/-</sup> mice. Body weight and disease score were monitored and cytokine production by lam-

ina propria CD4<sup>+</sup> T cells measured after animals displayed disease symptoms and were killed. Unexpectedly, relative to mice injected with wild-type CD4<sup>+</sup> T cells, mice that had received *Nlrp3*<sup>-/-</sup> CD4<sup>+</sup> T cells developed more severe disease with significantly increased weight loss, reduction in colon length, and higher disease scores (Fig. 7, A to C). Similar to our observation in the LCMV model, colonic *Nlrp3*<sup>-/-</sup> T cells displayed a substantial reduction in IFN- $\gamma$  production (average decrease ~45%); however, we also observed a concurrent significant increase in T<sub>H17</sub> responses in these animals (Fig. 7, D and E). These observations were confirmed using a CD4<sup>+</sup> T cell-driven model of graft-versus-host disease (GvHD) where mice receiving *Nlrp3*<sup>-/-</sup> T cells also displayed more severe illness with reduced T<sub>H1</sub> but concurrently increased T<sub>H17</sub> induction (fig. S7, A to D). Together, these data demonstrate that the NLRP3 inflammasome mediates functionally important CD4<sup>+</sup> T cell intrinsic effects that not only are required for normal IFN- $\gamma$  production but also control the T<sub>H1</sub>-T<sub>H17</sub> balance during (at minimum) intestinal inflammation. These latter findings align with our observation that T cells from CAPS patients indeed have increased T<sub>H1</sub> but also decreased in vitro T<sub>H17</sub> responses (Fig. 3, C and E).

### Discussion

Our results show that canonical NLRP3 inflammasome function is not confined to innate immune cells but is operative in adaptive CD4<sup>+</sup> T cells and, via autocrine IL-1 $\beta$  activity, is required for the optimal production of the key host defense

factor IFN- $\gamma$ . Further, and unexpectedly, NLRP3 assembly in human T cells requires TCR-induced intracellular C5 activation and stimulation of intracellular C5aR1, which initiates the generation of ROS and thereby provides a critical signal 2 for inflammasome assembly (30). Secretion of intracellularly generated C5a/C5ades-Arg engages the surface-expressed “alternative” C5aR2, which negatively controls NLRP3 activation either through inhibition of the C5aR1 or via a yet undefined mechanism. Given that APCs provide generally ample amounts of IL-1 $\beta$  during the cognate APC/T cell interaction, our observation that normal IFN- $\gamma$  production requires also T cell autocrine IL-1 $\beta$  production is initially somewhat surprising. However, we envisage that, whereas APC-derived NLRP3-activated IL-1 $\beta$  supports initial T<sub>H1</sub> priming, proper “imprinting” or maintenance of the T<sub>H1</sub> phenotype during differentiation and migration into the periphery may rely on autocrine NLRP3 activity. IL-1 $\beta$  production by T cells, relative to myeloid cells, is comparatively low and, as we have shown, tightly regulated by an autocrine C5aR1 versus C5aR2 activation balance. The likely reason for this is that rapid control of local IL-1 $\beta$  is critical to normal termination of T<sub>H1</sub> responses: Human T<sub>H1</sub> cells co-induce IL-10 secretion in a CD46-dependent fashion during their contraction phase, and failure of this “IL-10 switch” underlies hyperactive T<sub>H1</sub> responses observed in rheumatoid arthritis and multiple sclerosis (3, 9). IL-1 $\beta$  is a strong suppressor of IL-10 production (23) and, accordingly, we found that blockade of C5aR2 increased the IFN- $\gamma$ /IL-10 ratio in CD4<sup>+</sup> T cells (fig. S8A), whereas



**Fig. 5. Intracellular C5aR1 activation induces ROS generation in CD4<sup>+</sup> T cells.** (A) ROS production in CD4<sup>+</sup> T cells activated under the depicted conditions (data shown are representative of  $n = 3$ ). (B) IFN- $\gamma$  production from CD4<sup>+</sup> T cells left nonactivated or activated as indicated with and without a specific ROS inhibitor and/or the C5aR1/C5aR2 antagonist ( $n = 3$ ). Data are from a two-way ANOVA with Bonferroni multiple comparison test. (C) ROS production in  $\alpha$ -CD3 +  $\alpha$ -CD46 activated CD4<sup>+</sup> cells after transfection with C5aR1-specific siRNA (left panel) or with or without the C5aR1/C5aR2 double antagonist (right panel) (data shown are representative of  $n = 3$ ). Analyses were performed 36 hours after activation. Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

IL-1 $\beta$  addition to cultures increased IFN- $\gamma$  (Fig. 2G) but blocked proportional IL-10 secretion (fig. S8B). Moreover, IFN- $\gamma$  to IL-10 switching was significantly reduced in T cells from CAPS patients (fig. S8C).

Further supporting the notion that autocrine T cell cytokine production needs to be carefully controlled in the microenvironment is our observation that the reduction in IFN- $\gamma$  secretion by T cells from *Nlrp3*<sup>-/-</sup> mice led to a concurrent increase in colonic lamina propria T<sub>H</sub>17 cells and increased intestinal inflammation. These findings demonstrate that T<sub>H</sub>1 cells negatively control the influx and/or expansion of T<sub>H</sub>17 cells during colitis and that T<sub>H</sub>17 induction at this location (and in this model) is independent of intrinsic T cell NLRP3 activity. These observations may also help to explain why some groups observed protection of *Nlrp3*<sup>-/-</sup> animals in models of inflammatory bowel disease while others observed aggravated disease (46, 47); these earlier studies had not controlled for a T cell-intrinsic function of the

NLRP3 inflammasome. Also, because IL-1 $\beta$  also boosts the production of other cytokines including IL-4 and IL-17 (35), the activation of the NLRP3 inflammasome in T cells and its functional outcome could be context-dependent. For example, Bruchard *et al.* recently observed a noncanonical function for NLRP3 in mouse CD4<sup>+</sup> T cells (they did not assess human T cells) independent of inflammasome formation and IL-1 $\beta$  secretion, during T<sub>H</sub>2 induction and tumor growth (48).

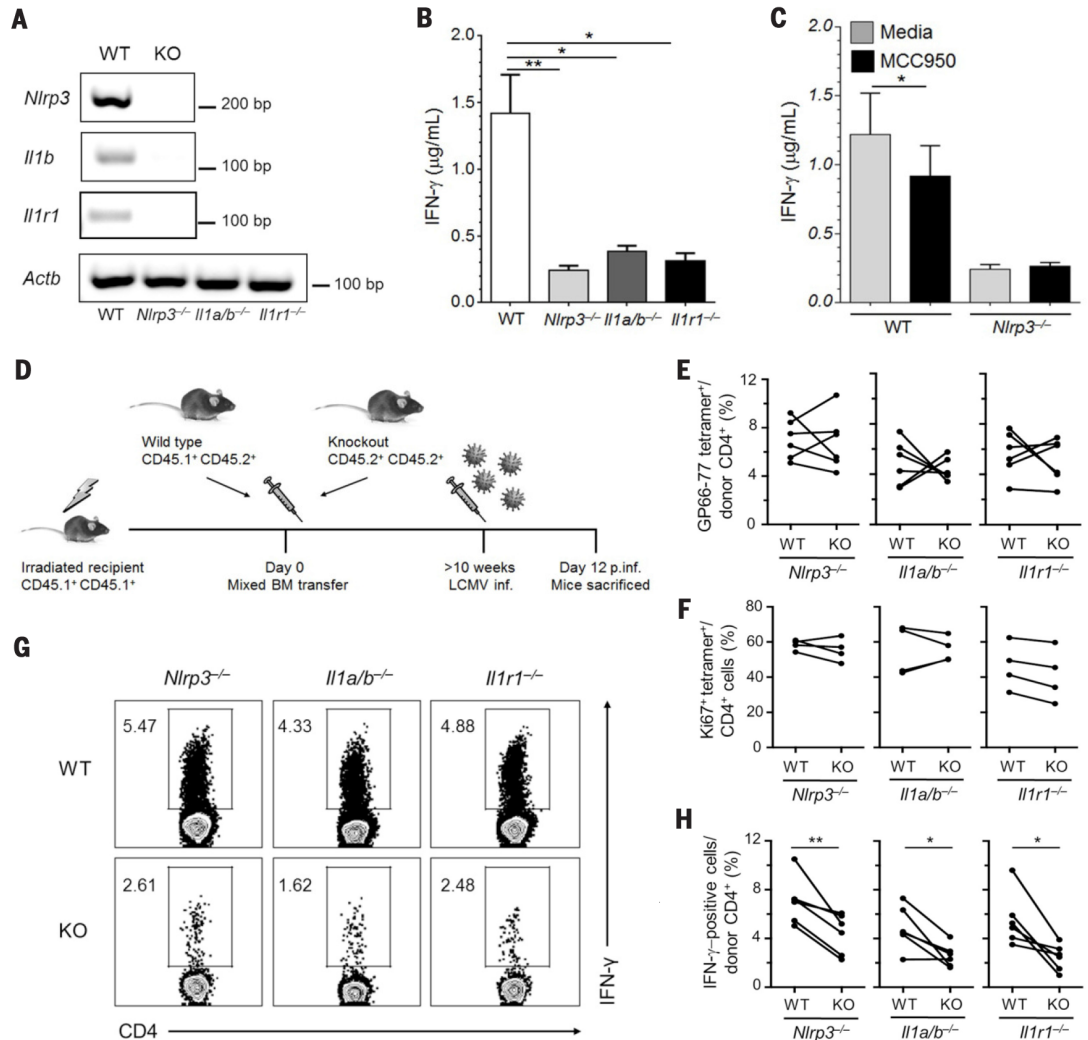
In addition, there are clear species-specific differences in the relative contributions of complement receptor activities to IL-1 $\beta$  and/or IFN- $\gamma$  induction in CD4<sup>+</sup> T cells. Although we found that CD4<sup>+</sup> T cells from *C5ar2*<sup>-/-</sup> mice have increased in vitro IFN- $\gamma$  production, which was reduced to normal levels by MCC950 treatment (fig. S9), and *C5ar1*<sup>-/-</sup> mice have impaired in vitro and in vivo T<sub>H</sub>1 responses (49), the role and expression of anaphylatoxin receptors on mouse T cells remains a matter of controversy (49–51). But more important, mice lack expression of CD46 on all immune

cells (6), whereas in humans, CD46 costimulation is required for IL-2R assembly and the metabolic reprogramming in CD4<sup>+</sup> T cells that drives IFN- $\gamma$  secretion (5, 7, 52). Further, we show here that CD46 engagement, aside from amplifying intracellular C5a generation and ROS production (signal 2), also delivers an important signal 1 for NLRP3 inflammasome activation by mediating increased transcription of the *NLRP3* gene [this likely occurs via CD46-induced increased NF- $\kappa$ B nuclear translocation during T cell activation (6)]. Because a functional homolog for CD46 has not been identified in rodents, the exact upstream signals controlling NLRP3 inflammasome activation and IL-1 $\beta$  production in murine T cells remain to be defined.

In summary, the regulated cross-talk between intracellularly activated complement components (“complosome”) and the NLRP3 inflammasome emerges as fundamental to human T<sub>H</sub>1 induction and regulation. That established innate immune pathways previously not thought to be operative

### Fig. 6. NLRP3 function in CD4<sup>+</sup> T cells drives optimal IFN- $\gamma$ production during viral infection.

(A) RT-PCR analysis on CD4<sup>+</sup> T cells isolated from wild type (WT), *Nlrp3*<sup>-/-</sup>, combined *Il1a*<sup>-/-</sup> and *Il1b*<sup>-/-</sup> (*Il1a/b*<sup>-/-</sup>) and *Il1r1*<sup>-/-</sup> mice for corresponding gene mRNA expression. (B) Cytokine secretion from CD4<sup>+</sup> T cells isolated from wild-type and knockout mice at 96 hours after  $\alpha$ -CD3 +  $\alpha$ -CD28 activation ( $n = 3$ ). (C) Cytokine production from CD4<sup>+</sup> T cells from wild-type and *Nlrp3*<sup>-/-</sup> mice after  $\alpha$ -CD3 +  $\alpha$ -CD28 activation (96 hours) with or without addition of MCC950 ( $n = 4$ ). (D) Schematic of the acute lymphocytic choriomeningitis virus (LCMV) infection model used in this study. (E and F) Percentage of LCMV tetramer-positive CD4<sup>+</sup> T cells isolated from the spleens of the three bone marrow chimeric mice groups used 12 days after infection (E) and percentages of Ki67<sup>+</sup>GP66-77<sup>+</sup>/tetramer-positive cells (F). (G and H) Representative intracellular IFN- $\gamma$  staining in splenic CD4<sup>+</sup> T cells of one mouse from each group after LCMV peptide restimulation (5 hours) [(G),  $n = 6$ ] with corresponding statistical analyses [(H),  $n = 6$ ]. Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ . (B), one-way ANOVA with Tukey multiple comparison test; (C), two-way ANOVA with Bonferroni multiple comparison test; (E) to (H), paired  $t$  test. Data in (G) are representative of two independent experiments; data in (E), (F), and (H) are pooled from two independent experiments.





in adaptive immune cells are not only present but also are key in directing immunological responses is of substantial importance to our understanding of immunobiology and immune system evolution. Further, the possibility that this normal functional cross-talk may also be target of viral immune evasion strategies (53) suggests that the complement-NLRP3 axis in T cells could represent a novel therapeutic target for the modulation of IFN- $\gamma$  responses in autoimmunity and infection. In this regard, it will be valuable to explore whether optimal production of IFN- $\gamma$  by CD8 $^+$  T cells (54), natural killer T (NKT) cells, and/or innate lymphoid type 1 (ILC1) cells also relies on autocrine complement-NLRP3 inflammasome activity.

## Materials and methods

### Healthy donors and patients

Blood samples were obtained with ethical and institutional approvals (Wandsworth Research Ethics Committee, REC number 09/H0803/154). T cells were purified blood samples from healthy volunteers after informed consent. Fourteen adult patients with CAPS were recruited at the National Amyloidosis Centre, University College London (ethical approval REC reference number 06/Q0501/42) with key information on the patients summarized in tables S3 and S4. In all experiments that involved T cells from CAPS patients, T cells from age- and sex-matched healthy volunteers were used as controls.

### Mice used in the study

All mice used in this study are on a C57BL/6 background (with the exception of the GvHD exper-

iment, where Balb/c mice were used). Wild-type and *Il1r1* $^{-/-}$  mice were purchased from Jackson Laboratories and subsequently backcrossed to B6 for 10 generations at NIH (Bar Harbor, ME). The *C5ar2* $^{-/-}$  (human gene symbol GPR77) mice were previously described (12), *Nlrp3* $^{-/-}$  animals were provided by V. Dixit of Genentech, and mice deficient in *Il1a* and *Il1b* (*Il1a/Il1b* $^{-/-}$  animals) were kindly provided by Y. Iwakura (Tokyo University) (55). The C57BL/10 RAG2 $^{-/-}$  mice were obtained from Taconic. All animals were maintained in AALAC-accredited BSL2 or BSL3 facilities at the NIH or FDA and experiments performed in compliance with an animal study proposal approved by the NIAID or FDA Animal Care and Use Committee.

### Cell isolation and activation

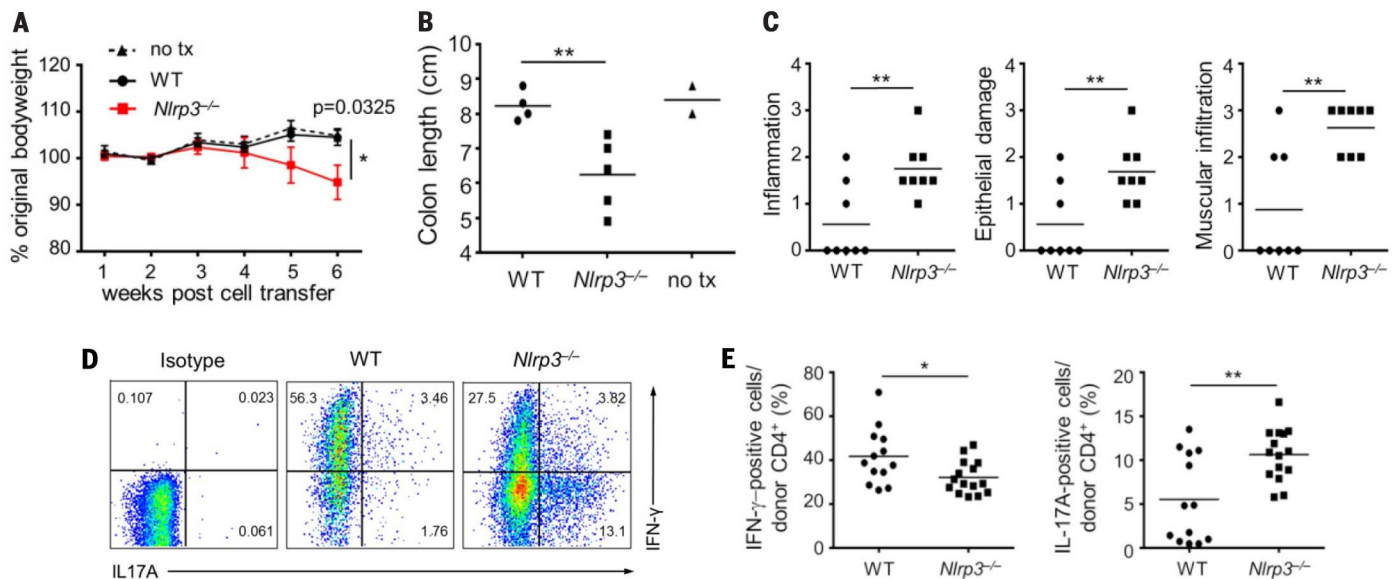
**Human cells:** CD4 $^+$  T cells and monocytes were isolated from blood as previously published using the MACS Human CD4 $^+$  Positive T cell Isolation Kit or the MACS Human CD14 $^+$  Cell Positive Isolation Kit (both Miltenyi Biotech, Bisley, UK), respectively (7). Purity of bead-isolated T lymphocyte fractions was typically >98% and for monocytes >95%. For naïve and memory CD4 $^+$  T cell sorting, cells were stained with appropriate antibodies (naïve cells, CD4 $^+$ , CD45RA $^+$ , CD45RO $^-$ , and CD25 $^-$ ; memory cells, CD4 $^+$ , CD45RA $^+$ , CD45RO $^+$ , and CD25 $^-$ ) and sorted with a BD FACSAria II Cell Sorter (KCL Flow Core facility). CD4 $^+$  T cells were activated in 48-well culture plates ( $2.5 \times 10^5$  to  $3.0 \times 10^5$  cells per well) coated with mAbs to CD3, CD28, or CD46 (2.0  $\mu$ g/ml PBS each) and addi-

tion of rhIL-2 (25 U/ml), thus, under nonskewing conditions. Monocytes were activated in 24-well plates ( $2.5 \times 10^5$  to  $5.0 \times 10^5$  cells per well) by addition of LPS (50 ng/ml). Cell viability was monitored by either propidium iodide (BD Biosciences) or the LIVE/DEAD Cell Viability Assay (Life Technologies).

**Mouse cells:** Single cell suspensions of spleen cells were generated and red blood cells lysed using ACK lysis buffer (Life Technologies). CD4 $^+$  T cells were isolated by negative selection using the Stem Cell Technologies EasySep Mouse CD4 $^+$  T Cell Isolation Kit (Tukwila, WA). To obtain pure CD4 $^+$  T cell populations, CD4 $^+$  cells were sorted using a FACS Aria (BD Biosciences) based on CD4 $^+$  CD45.2 $^+$  staining and to separate naïve versus memory CD4 $^+$  T lymphocytes. T cells were sort-separated based on CD4 $^+$  CD44 $^+$  (memory) and CD4 $^+$  CD44 $^-$  (naïve) stainings. For in vitro T cell activation, 48- or 96-well plates were coated with anti-CD3 (2  $\mu$ g/ml) overnight at 4°C, and CD4 $^+$  T cells ( $0.5 \times 10^6$  to  $1.0 \times 10^6$  per well of 48-well plates or  $0.2 \times 10^6$  per well of 96-well plates) were added to the appropriate wells. Anti-CD28 (1  $\mu$ g/ml) was added to the media to provide costimulation.

### Lymphocytic choriomeningitis virus (LCMV) infection in mice

**Preparation of mixed bone marrow (BM) chimeric mice:** B6.SJL (CD45.1,1) mice were lethally irradiated (950 rad) and reconstituted with a total of  $10^7$  donor BM cells from C57BL/6 CD45.1,2 wild-type mice mixed at equal parts with BM cells from CD45.2,2 mice deficient (KO) in *Nlrp3* $^{-/-}$ , *Il1r1* $^{-/-}$ ,



**Fig. 7. T cell intrinsic NLRP3 activity regulates the TH1-TH17 balance in intestinal inflammation.** (A to E) Naïve splenic CD25 $^-$ CD45RB $^{\text{hi}}$  CD4 $^+$  T cells from wild-type or *Nlrp3* $^{-/-}$  mice were transferred into C57BL/10 RAG2 $^{-/-}$  mice. (A) Weight change over the course of colitis induction. (B) Colon length at the study endpoint. (C) Inflammation score of the colons according to blinded histological analysis with assessment of inflammation (left panel), epithelial damage (middle panel) and muscular immune cell infiltration (right panel). (D and E) Intracellular IFN- $\gamma$  and IL-17A staining of colonic CD4 $^+$  T cells at the

study endpoint after overnight  $\alpha$ -CD3 +  $\alpha$ -CD28 stimulation and brefeldin A and monensin addition for 5 hours (gated on live CD4 $^+$  Thy1.2 $^+$  T cells). Representative flow cytometric plots (D) with corresponding statistical analysis shown from two combined independent experiments [(E),  $n = 13$  wild-type,  $n = 15$  KO]. Data are means  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ . (A) and (B), one-way ANOVA with Sidak multiple-comparisons test; (C) and (E), unpaired  $t$  test. Data are representative of two experiments [(A) to (C)] or are combined from two experiments (E).

or *Ila/Iib*<sup>-/-</sup>. Mice were allowed to reconstitute for 10 weeks before infection with LCMV.

**LCMV infection and assessment of antigen-specific CD4<sup>+</sup> T cell response:** Ten weeks after reconstitution, the mice were infected i.p. with 10<sup>5</sup> pfu of LCMV-Armstrong. On day 12 after infection, the mice were killed and the spleens removed for processing. For ex vivo cytokine staining of mouse cells after LCMV infection, cells were incubated with LCMV GP61-80 peptide (1 µg/ml) in the presence of monensin and brefeldin for 5 hours at 37°C. Staining for LCMV-specific CD4<sup>+</sup> T cells was performed using an APC-labeled 1A<sup>b</sup> LCMV GP66-77 tetramer (NIH tetramer core facility) as described (56). Data were acquired with a FACS Calibur, Fortessa LSRIII, or FACS Aria cytometer (BD Biosciences) and analyzed with FlowJo 10.0.8 software (Ashland, OR).

### Induction of colitis and colon cell isolation

Splenic CD4<sup>+</sup> T cells were isolated from C57BL/6 or *Nlpr3*<sup>-/-</sup> mice using a negative selection CD4 T cell enrichment kit (Stemcell tech), were stained with anti-CD45RB FITC, anti-CD25 APC, and anti-CD4 BV421, and sorted on a FACS Aria (BD biosciences) for CD4<sup>+</sup> CD25<sup>-</sup> CD45RB<sup>hi</sup> (brightest 35%) cells. Wild-type or *Nlpr3*<sup>-/-</sup> cells (2 × 10<sup>5</sup> each) were injected i.p. into age- and sex-matched C57BL/10 RAG2<sup>-/-</sup> mice. The mice were killed when symptoms of clinical disease (5 to 10% weight loss of original body weight and/or diarrhea) were observed in at least one group, approximately 6 to 11 weeks after adoptive transfer. Colon lamina propria cells were isolated as described (57), with the additional step of further purifying the cells over a 44 and 67% Percoll gradient to enrich for the mononuclear cells.

### Scoring of intestinal inflammation

Samples of the proximal, mid-, and distal colon were excised after feces were flushed from the colons, placed into 3.7% formaldehyde solution, and then paraffin-embedded. Cross-sectional sections were cut and stained with hematoxylin and eosin (H&E). Colon pathology scores were based on severity of mononuclear cell inflammation, intestinal wall thickening, including infiltration to the muscularis, and epithelial damage, including edema, degeneration, and necrosis on a graded scale where 0 = normal, 0.5 = very mild, 1 = mild, 2 = moderate, 3 = severe. Samples were scored blinded by a pathologist from the NIH Pathology Score.

### Induction of graft versus host disease (GVHD)

Balb/c mice were lethally irradiated with 900 cGy (two doses of 450 cGy 3 hours apart) on day -1. C57BL/6 wild-type bone marrow was depleted of T cells with the use of a CD90.2 Positive Selection Kit (Stemcell tech), and 5 × 10<sup>6</sup> cells were transferred on the following day (day 0) alone (control), or in addition to 1 × 10<sup>6</sup> wild-type B6 or *Nlpr3*<sup>-/-</sup> naïve CD4<sup>+</sup> T cells isolated with the Negative Selection Naïve CD4 T Cell Kit (Stemcell tech). Mice were killed upon clinical symp-

toms of disease (diarrhea and weight loss) on day 12 after cell transfer.

### Detection of active caspase-1 and reactive oxygen species (ROS)

Generation of cleaved and active caspase-1 in cells was monitored by Western blotting for appropriate active fragment generation and by using the Green FLICA Caspase-1 Assay Kit (ImmunoChemistry Technologies, Bloomington, MN) according to the manufacturer's protocol with subsequent FACS analysis. ROS staining was performed by incubating cells to be assayed in dihydrorhodamine 123 (17 µg/ml) diluted in Hank's balanced salt solution with 10 mM HEPES (all from Sigma Aldrich) for 15 min at 37°C. Data were acquired on a FACS Calibur or Fortessa LSRIII cytometer (BD Biosciences) and analyzed with FlowJo software.

### Confocal microscopy

Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD Biosciences) and stained with the indicated primary antibodies overnight and with secondary antibodies for 30 min at 4°C. Cells were mounted using VECTASHIELD media with DAPI (Vector Laboratories, Burlingame, CA) and images were acquired with a Nikon AIR confocal microscope (Nikon Imaging Centre, King's College London) and analyzed using NIS Elements (Nikon) and ImageJ software (National Institutes of Health).

### Binding studies with recombinant human <sup>125</sup>I-labeled C5a

CD4<sup>+</sup> T cells from healthy donors were left non-activated or activated for 4 hours with immobilized antibodies to CD3 and CD46 and then incubated for 2 hours at 4°C (1 × 10<sup>7</sup> cells/ml) with 10 µl of 0.1 nM <sup>125</sup>I-rhC5a (Perkin Elmer) and either 400 nM nonlabeled rhC5a in HAG-CM buffer (1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 0.25% bovine serum albumin, 0.5 mM glucose, pH 7.4) or buffer without rhC5a addition. Cells were vacuum-transferred onto 96-well MultiScreen-HV filter plates (MAHVN4510; Millipore/Merck), nonbound <sup>125</sup>I-rhC5a removed by washing and cell-bound <sup>125</sup>I-rhC5a detected on the filter membranes by <sup>125</sup>I using a Packard Cobra II Gamma Counter (Perkin Elmer). For binding controls, HEK 293 cells (ATCC CRL 1573) were stably transfected with the pQCXIN vector expressing hC5aR1 or hC5aR2 (leading to expression of >1 Mio. of the respective C5aR/cell) or with the "empty" vector as control (58) (these cell lines also served as specificity controls for the anti-C5a receptor antibodies used in this study). In order to get comparable low CPM values as observed with purified T cells, only 5 × 10<sup>4</sup> cells/ml of C5aR1- or C5aR2-expressing HEK cells were applied. They were diluted in buffer containing "no-C5aR-expressing control" cells. The constant higher number of cells (5 × 10<sup>5</sup> HEK cells/ml in the 30-µl volume later used in the binding assay) permitted repetitive washing without cell loss and ensured identical nonspecific binding in all samples containing the same cell type. C5aR1-, C5aR2-expressing or control HEK 293 cells were incubated for 1 hour with or without 100 nM of nonlabeled rhC5a,

washed thoroughly and then incubated for an additional 2 hours with 10 µl of 0.1 nM <sup>125</sup>I-rhC5a. After removal of nonbound rhC5a, binding of <sup>125</sup>I-C5a to the respective HEK 293 cell lines was determined by measuring gamma radioactivity. To exclude C5a-induced C5aR-internalization during all binding studies all steps in the binding experiments were performed at 4°C and HEK 293 cells were additionally preincubated 15 min at 37°C with 0.1% NaAcid and Cytochalasin B (21 µg/ml) and then cooled on ice for 5 min before their incubation with rhC5a.

### Cytokine measurements

Cytokine production by cells in culture was quantified from cell supernatants using either the human or mouse T<sub>H</sub>1/T<sub>H</sub>2/T<sub>H</sub>17 Cytometric Bead Array (BD Bioscience) or via intracellular cytokine staining after treated for 4 hours with PMA (50 ng/ml), ionomycin (1 µg/ml) (both Sigma Aldrich), and 1× Golgi Plug (BD Biosciences). Secreted human IL-1β and IL-18 were measured using the Human IL-1β/IL-1F2 DuoSet Kit or the Human IL-18 Platinum ELISA kit (R&D Systems and eBiosciences, respectively) in combination with SIGMAFAST OPD tablets (Sigma Aldrich) as substrate for detection.

### RNA extraction, reverse transcription polymerase chain reaction (RT-PCR), and quantitative RT-PCR

RNA was extracted with the RNeasy Mini Kit including DNase digestion and DNA cleanup (Qiagen), and reverse transcription was performed with the One Step RT-PCR (Qiagen). For quantitative PCR, RNA was reverse-transcribed with 2.5 µM random hexamers, 1 mM dNTPs, 40 U of RiboLock RNase inhibitor, and 400 U of RevertAid H Minus Reverse Transcriptase (Thermo Scientific). Quantitative PCR was performed using KI-Q Hot Start Sybr Green Mix (Sigma Aldrich), with 150 pmol of forward and reverse primers and data acquired on the CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Primer sequences are listed in table S5.

### RNA silencing

siRNA targeting human C5aR1 mRNA and control scrambled siRNA were purchased from Origene (Rockville, MD) and delivered at a final concentration of 15 nM (mixture of three different C5aR1 siRNA used at 5 nM each or scramble control at 15 nM) into primary human CD4<sup>+</sup> T cells by transfection with Lipofectamine RNAiMAX (Life Technologies) following the manufacturer's instructions. C5aR1 mRNA level reduction was consistently about 30%.

### Microarray data generation and analysis

Transcriptome profiling was performed by the KCL Genomic Centre (London) using human exon 1.0 ST arrays (Affymetrix) on CD4<sup>+</sup> T cells isolated from three different healthy donors that were left either nonactivated or were activated with antibodies to CD3 and CD46 for 2 hours in the absence or presence of the C5aR1/C5aR2 antagonist. Expression data were analyzed using



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#### SUPPLEMENTARY MATERIALS

[www.sciencemag.org/content/352/6292/aad1210/suppl/DC1](http://www.sciencemag.org/content/352/6292/aad1210/suppl/DC1)  
Materials and Methods  
Figs. S1 to S9  
Tables S1 to S5

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**T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4<sup>+</sup> T cells**

Giuseppina Arbore, Erin E. West, Rosanne Spolski, Avril A. B. Robertson, Andreas Klos, Claudia Rheinheimer, Pavel Dutow, Trent M. Woodruff, Zu Xi Yu, Luke A. O'Neill, Rebecca C. Coll, Alan Sher, Warren J. Leonard, Jörg Köhl, Pete Monk, Matthew A. Cooper, Matthew Arno, Behdad Afzali, Helen J. Lachmann, Andrew P. Cope, Katrin D. Mayer-Barber and Claudia Kemper (June 16, 2016)

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Editor's Summary

**Innate immune crosstalk in T cells**

The classical view of immune activation is that innate immune cells, such as macrophages and dendritic cells, recognize invading microbes and then alert adaptive immune cells, such as T cells, to respond. Arbore *et al.* now show that innate and adaptive immunity converge in human and mouse T cells. Activated T cells express components of the complement cascade, which in turn leads to the assembly of NLRP3 inflammasomes—both critical components of innate immunity that help hosts detect and eliminate microbes. In T cells, complement and inflammasomes work together to push T cells to differentiate into a specialized subset of T cells important for eliminating intracellular bacteria.

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