Supplementary Materials for

T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4⁺ T cells


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Materials and Methods

Antibodies, proteins, agonists and antagonists
Cell-stimulating mAbs to human CD4+ T cells were bought from BD Biosciences, San Diego, CA (anti-hCD28, CD28.2), purified from a specific hybridoma (anti-hCD3; OKT-3) or generated in-house (anti-CD46; TRA-2-10) (3). Mouse T cells were activated with anti-CD3 (145-2C11) and anti-CD28 (37.51) from Bio X Cell (West Lebanon, NH). The anti-human/mouse NLRP3 (ab4207), anti-human C5 (ab66850) and C5a (ab11878), anti-human/mouse IL1-β (ab9722) and anti-human β-actin (ab8226) antibodies were purchased from Abcam (Cambridge, UK). The anti-human C5a antibody was also biotinylated in house using the APEX™ Biotin-XX Antibody Labeling Kit (Life Technologies Ltd, Paisley, UK). Alternative antibodies to human/mouse NLRP3/NALP3 (Clone 768319) and human/mouse IL-1β (3A6, used for Western blotting and FACS experiments) were purchased from R&D Systems (Minneapolis, MN) and Cell Signalling Technology (Beverly, MA), respectively. Additional antibodies used included anti-human NLRP3/NALP3 (AG-20B-0014-C100) and ASC (AL177) from Adipogen (Liestal, Switzerland), anti-hC5aR1 (sc-53795) and anti-hNLRP3 (sc-34408) from Santa Cruz (Dallas, TX), anti-hC5aR2 (ID9-M12) and anti-hCD45RA from Biolegend, anti-hIL-1β (12-7018-81) and anti-hCD4 from eBioscience (San Diego, CA), anti-hC5aR1 (MCA2059; AbD Serotec, Oxford, UK), anti-hC5aR2 (PA1-41397; Thermo Scientific (Leicestershire, UK)), and anti-htrypsin-1 (3019-100; Biovision (Milpitas, CA)). The antibodies recognizing anti-human-CD25, CD45RA (555488), and CD45RO (559865) were purchased from BD Biosciences. The following mouse antibodies were purchased from Biolegend: Mouse Trustain (Fc-Block), anti-CD44 FITC, PE-cy7 or BV421, anti-mouse IFN-γ PE, anti-mouse CD4 (APC-cy7, BV 421 or BV605), anti-mouse CD45.1 FITC and CD45.2 PerCP, anti-mouse CD45RB FITC, anti-mouse CD25 APC. Anti-mouse/human Ki67 was purchased from BD Biosciences. The secondary antibodies anti-rabbit IgG H+L chain Alexa Fluor 594 (ab150076), anti-goat IgG H+L chain PE (ab7004) and anti-goat IgG H+L chain Alexa Fluor 488 (ab150129) were from Abcam, while anti-mouse IgG Alexa Fluor 488 (A11001), anti-rabbit IgG H+L chain Alexa Fluor 594 (A11037) and anti-rat IgG H+L chain Alexa Fluor 488 (A11006) were obtained from Molecular Probes/Life Sciences (Paisley, UK). APC streptavidin was purchased from Biolegend.

Recombinant active human IL-1β was bought from Abcam and used at 20 ng/ml in cultures, recombinant human IL18BPa (Sino Biologicals Inc.) was used at 50 μM, human IL-2 was provided by C. Pham (Washington University in Saint Louis, MO) and lipopolysaccharide (LPS) was purchased from Sigma Aldrich (Saint Louis, MO). Recombinant C5 and C5adesArg were purchased from CompTech (Tyler, TX). The specific C5aR1 antagonist (PMX53) (17) was provided by T. Woodruff (University of Queensland, AU) and used at 10 μM, the C5aR1/C5aR2 double antagonist a gift from J. Köhl (University of Lübeck, Germany) (18) and used at 7 μM, and the specific C5aR2 agonist (RHYPYWWR) was generated by T. Woodruff and P. Monk (Sheffield University, UK) (19) and used at 100 μM. The specific NLRP3 inhibitor MCC950 (36) was used at 10 μM, the specific caspase-1 inhibitor Z-YVAD-FMK (Abcam) was used at 20 μM and the reactive oxygen species (ROS) inhibitor Diphenyleneiodonium (DPI) (Sigma Aldrich) was added at 750 nM. In all experiments, cells were incubated in media for 15
minutes including the compound of choice (incubation with the corresponding vehicle buffer was used as control) before activation and culture.

**Figure legends**

**Suppl. Fig. 1. Autocrine activation of C5a receptors regulates IFN-γ production by human CD4⁺ T cells.** (A) C5aR1 and C5aR2 Western blot analyses on cytoplasmic (Cyt.) and membrane (Mem.) fractions of resting human CD4⁺ cells (representative of n=3). (B and C) Representative flow cytometry histograms for intracellular staining (B) and immunoblot with cytoplasmic (Cyt.) and membrane (Mem.) fractions (C) on C5aR1 and C5aR2 in HEK293 cells (HEK) transfected with a vector expressing either C5aR1, or C5aR2, or an empty control vector (Ctrl. Vec.). (D) Binding of radioactively-labelled ¹²⁵I-C5a to HEK293 cells expressing either C5aR1, C5aR2 or no C5a receptor in the absence or presence of non-labelled ‘cold’ C5a as competitor (n=3). (E) IFN-γ production in CD4⁺ T cells activated for 36 hours with α-CD3 + α-CD46 in presence of increasing concentrations of exogenous C5a or C5adesArg (n=3), with significance assessed between untreated cells (0 ng/mL C5a or C5adesArg) and cells treated with indicated amounts of either C5a or C5adesArg. (F) IL-17 and IL-4 production by non-activated (NA) and activated (36 hours) CD4⁺ T cells in the absence or presence of either a C5aR1/C5aR2 double receptor antagonist (n=9), a C5aR2 agonist (n=8) or a C5aR1 antagonist (n=7). (G) Cell viability of T cells either resting or activated for 36 hours as indicated in the absence or presence of the C5aR1/C5aR2 double antagonist or C5aR2 agonist (n=2). (H) Reduction of C5AR1 mRNA levels in T cells transfected with either a C5AR1-specific siRNA or a scrambled control siRNA (Ctrl. siRNA) 48 hours post transfection. Left panel shows a representative mRNA expression sample and the right sample statistically significant reduction in C5AR1 mRNA expression in C5AR1-specific siRNA-treated CD4⁺ T cells (n=6). Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 (D to G, two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test; H, paired t-test).
Suppl. Fig. 2. NLRP3 inflammasome activation occurs in CD4⁺ T cells and enhances IFN-γ production. (A) Volcano plot showing transcripts differentially regulated in CD4⁺ T cells from 3 donors after α-CD3 + α-CD46 activation (2 hours) with or without addition of the C5aR1/C5aR2 antagonist to cultures. (B) Quantitative RT-PCR to measure NLRP3 and IL1B mRNA in non-activated (NA) or α-CD3 + α-CD46 activated human CD4⁺ T cells at 36 hours post activation (n=3; expression normalized to ACTB). (C and D) Representative NLRP3 expression assessed by flow cytometry (C) and by immunofluorescence (D) in non-activated naïve and memory human CD4⁺ T cells (n=3). (E and F) Representative caspase-1 and IL-1β immunoblot analyses (with lower arrows depicting the activated protein forms), performed on resting and α-CD3 + α-CD46 activated CD4⁺ T cells (36 hours) and resting and LPS activated monocytes (50 ng/ml, 18 hours) (representative of n=4) with densitometric analyses on activated caspase-1 and IL-1β in T cells. The corresponding quantitative data shown below the immunoblots do not depict absolute amounts of proteins in monocytes versus T cells. They depict the ratio (percentage) of non-cleaved (non-activated) versus cleaved (activated) protein in either T cells or in monocytes. (G) IL-17 and IL-4 secretion in CD4⁺ cells non-activated (NA) or activated as indicated with or without the NLRP3 inhibitor MCC950 at 36 hours post activation (n=7). (H) Cell viability of CD4⁺ cells either resting or activated for 36 hours as indicated in the absence or presence of the NLRP3-specific inhibitor MCC950 (n=2). (I) IL-1β secretion by resting (NA) and activated CD4⁺ T cells (36 hours) with or without addition of the caspase-1 inhibitor Z-YVAD-FMK (n=4). (J) IL-18 production in α-CD3 + α-CD46 activated CD4⁺ T cells (36 hours) from three HDs (left panel) and IFN-γ production in CD4⁺ T cells from HDs 1 and 2 by T cells activated with α-CD3 + α-CD46 for 36 hours in full media and for 72 hours in serum free media, in the presence of 50 μM of rIL18BP (right panel). Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01 (E and F, paired t-test; G to I, two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test).

Suppl. Fig. 3. T cells from CAPS patients have increased caspase-1 activity and IL-1β secretion upon in vitro stimulation. (A) Percentages of naïve and memory CD4⁺ T cell subpopulations in the blood of a second cohort of CAPS patients P8 to P14 and of
three sex- and age-matched healthy donors (HD5-HD7). (B) Correlation between active caspase-1 and IL-1β production in T cells from patients P8-P14 upon CD3 + CD46 activation (Spearman’s correlation analysis).

**Suppl. Fig. 4. C5a receptors regulate NLRP3 activation to modulate IFN-γ responses.** (A and B) Quantitative RT-PCR to measure *IL1B* (A) and *NLRP3* mRNA (B) in resting or α-CD3 + α-CD46 activated (2 hours) human CD4+ T cells in the absence or presence of the C5aR1/C5aR2 antagonist with the respective corresponding bar graphs (panel below) showing relative expression in activated versus non-activated cells with or without C5aR1/C5aR2 antagonist-treated T cells (n=3, expression normalized on *ACTB*). (C) IL-17 and IL-4 production in resting or activated T cells in presence or absence of MCC950 and/or the C5aR1/C5aR2 double antagonist at 36 hours (n=4). (D) NLRP3 expression in CD4+ T lymphocytes either left non-activated or activated with α-CD3, α-CD3 + α-CD28 or α-CD3 + α-CD46 for 36 hours with or without addition of the C5aR1/C5aR2 antagonist (upper row) or the C5aR2 agonist (lower row) to cultures (data representative of n=3). (E) NLRP3 expression after α-CD3 + α-CD46 activation (36 hours) in T cells transfected with either C5aR1-specific siRNA or scrambled control (Ctrl.) siRNA (data representative of n=3). Error bar graphs represent mean ± SEM. *p <0.05 (A and B, paired *t*-test; C, two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test).

**Suppl. Fig. 5. Effect of ROS inhibition on IL-17 and IL-4 production by CD4+ T cells.** IL-17 and IL-4 secretion (shown as % normalized to respective untreated conditions) from CD4+ T cells left non-activated (NA) or activated as indicated with or without a specific ROS inhibitor and/or the C5aR1/C5aR2 antagonist at 36 hours post activation (n=3). Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 (two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test).

**Suppl. Fig. 6. NLRP3 function in CD4+ T cells is required for optimal IFN-γ response *in vivo.*** (A) Representative immunofluorescence analysis for NLRP3 and IL-1β
protein expression on CD4+ T cells isolated from wild type (WT), Nlrp3−/− and combined Il1a−/− and Il1b−/− (Il1a/b−/−) mice. (B) Percentages of naïve and memory CD4+ T cells isolated from the spleen of unchallenged wild type (WT) and Nlrp3−/−, combined Il1a−/− and Il1b−/− (Il1a/b−/−), and Il1r1−/− mice (n=3). (C) Cell viability of sorted CD4+ T cells from WT and knock out mice 96 hours post CD3 + CD28 activation (n=3). (D) IL-10, IL-4 and IL-17 secretion from CD4+ T cells isolated from WT and knock out mice activated 96 hours with antibodies to CD3 and CD28 (n=3). (E) Cell viability of sorted CD4+ T cells from WT and Nlrp3−/− mice assessed with or without MCC950 addition (right panel) during activation (96 hours post α-CD3 + α-CD28 activation, n=4). (F) IL-10, IL-4 and IL-17 secretion from CD4+ T cells activated 96 hours with antibodies to CD3 and CD28 from WT and Nlrp3−/− mice with or without addition of MCC950 (n=4). (G and H) IFN-γ, IL-10, IL-4 and IL-17 secretion from sorted naïve (G) and memory (H) CD4+ T cells from WT, Nlrp3−/−, Il1a/b−/−, and Il1r1−/− mice 96 hours post α-CD3 + α-CD28 activation (n=3). Error bar graphs represent mean ± SEM. *p <0.05 (B, E and F, two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test; C, D, G and H, one-way analysis of variance (ANOVA) with Tukey’s multiple comparison test).

Suppl. Fig. 7. Lack of intrinsic NLRP3 inflammasome function in CD4+ T cells impacts on GvHD disease. (A-D) Disease scores and Th1 and Th17 T cell populations. T cell-depleted C57BL/6 bone marrow was transferred into lethally irradiated BALB/c mice alone (control group), or with the addition of 1 x 10^6 naïve CD4+ T cell from either C57BL/6 or Nlpr3−/− mice. (A) Colon length at study endpoint (12 days post-cell transfer). (B-D) Intracellular IFN-γ and IL-17A staining of mesenteric lymph node CD4+ T cells at the study endpoint after overnight α-CD3 and α-CD28 stimulation and brefeldin A and monensin addition for 5 hours (Gated on live CD4+ Thy1.2+ C57BL/6 (H-2K^dD^d+) T cells). Percent (B) and mean fluorescence intensity (MFI) of IFN-γ+ cells (C) and percent IL-17A+ cells (D). For (A-D) n=4 WT, 5 KO, 2 controls. Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01, ***p <0.001 (B to D, one-way ANOVA with Sidak’s multiple comparisons test, A; unpaired t-test).
Suppl. Fig. 8. The C5aR2-NLRP3-IL-1β axis may regulate ‘IL-10 switching’ in human Th1 cells. (A) IFN-γ to IL-10 ratio in α-CD3 + α-CD28 or α-CD3 + α-CD46 activated (36 hours) CD4+ T cells in the absence or presence of either a C5aR1/C5aR2 double receptor antagonist (n=9), a C5aR2 agonist (n=8) or a C5aR1 antagonist (n=7). (B) IL-10 production by resting and activated CD4+ T cells, in presence or absence of MCC950 and/or rhIL-1β measured at 36 hours post activation (n=3). (C) IL-10 secretion at 36 hours post indicated activation by CD4+ cells from the patients with CAPS P8-P14 and seven sex- and age-matched healthy donors (HD5-11) with data represented as mean ± SEM. *p <0.05, **p <0.01 (two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test).

Suppl. Fig. 9. Cytokine production of T cells from C5ar2−/− mice in the presence and absence of NLRP3 inhibition. In vitro cytokine production of CD4+ T cells from wild type and C5ar2−/− mice with or without addition of MCC950 at 48 hours post α-CD3 + α-CD28 activation. Error bar graphs represent mean ± SEM. *p <0.05, **p <0.01 (two-way analysis of variance (ANOVA) with Bonferroni’s multiple comparison test).
Table S1.

Normalized read values from microarrays for Fig. 2A and B
Table is provided in Other Supplementary Material as an Excel file.
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Table S2.

**Genes differentially regulated by the C5aR1/C5aR2 double antagonist**

Reported the p-value and the fold change ($\alpha$-CD3 + $\alpha$-CD46 + C5aR1/C5aR2 double antagonist vs $\alpha$-CD3 + $\alpha$-CD46)
### Table S3.
Details of seven patients (1 to 7) with cryopyrin-associated periodic syndrome (CAPS)

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<th>Age/gender</th>
<th>NLRP3 Mutation</th>
<th>Treatment</th>
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<td>4</td>
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### Table S4.
Details of seven patients (8 to 14) with cryopyrin-associated periodic syndrome (CAPS) and A439V mutation

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<td>14</td>
<td>67 y./female</td>
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### Table S5.
Listed primers sequences

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Fig. S2

A

B

C

D

E

F

G

H

I

J
Fig. S3

A

![Bar chart showing cell populations (%).]

B

![Scatter plot showing IL-1β (pg/ml) against Active caspase-1 (%). Spearman r = 0.74, p = 0.03.]

Cell populations (%)

H55 H56 H57 P8 P9 P10 P11 P12 P13 P14

IL-1β (pg/ml)

Active caspase-1 (%)
Fig. S4

A

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C

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D

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<td><img src="image7.png" alt="Image" /></td>
<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
<td><img src="image10.png" alt="Image" /></td>
<td><img src="image11.png" alt="Image" /></td>
</tr>
</tbody>
</table>

E

<table>
<thead>
<tr>
<th>NLRP3</th>
<th>α-CD3 + α-CD346</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image12.png" alt="Image" /></td>
<td><img src="image13.png" alt="Image" /></td>
</tr>
</tbody>
</table>
Fig. S5
Fig. S6

A

Wild type  Nlrp3<sup>–/–</sup>  Il1αβ<sup>–/–</sup>

B

Isot. Crls.

Cell populations (%)

WT  Nlrp3<sup>–/–</sup>  Il1αβ<sup>–/–</sup>  Il1β<sup>–/–</sup>

C

Viable cells (%)

WT  Nlrp3<sup>–/–</sup>  Il1αβ<sup>–/–</sup>  Il1β<sup>–/–</sup>

D

IL-10 (ng/ml)

WT  Nlrp3<sup>–/–</sup>  Il1αβ<sup>–/–</sup>  Il1β<sup>–/–</sup>

E

Viable cells (%)

WT  Nlrp3<sup>–/–</sup>

F

IL-10 (ng/ml)

WT  Nlrp3<sup>–/–</sup>

G

IFN-γ (ng/ml)

WT  Nlrp3<sup>–/–</sup>  Il1αβ<sup>–/–</sup>

H

IFN-γ (μg/ml)

WT  Nlrp3<sup>–/–</sup>  Il1αβ<sup>–/–</sup>

E

Media  MCO950

F

Media  MCO950

G

NS

H

ND  ND  ND

17
Fig. S7

A

Colon length (cm)

WT  Nlrp3\textsuperscript{\textasciitilde}  no tx

B

IFN-\gamma-\textsuperscript{positive cells/donor CD4\textsuperscript{+} cell} (%)  

WT  Nlrp3\textsuperscript{\textasciitilde}

C

IFN-\gamma MFI

WT  Nlrp3\textsuperscript{\textasciitilde}

D

IL-17A-\textsuperscript{positive cells/donor CD4\textsuperscript{+} cell} (%)  

WT  Nlrp3\textsuperscript{\textasciitilde}
Fig. S8

A

![Graph showing the ratio of IFN-γ/IL-10 in different conditions.](image)

B

![Graph showing the concentration of IL-10 in different conditions.](image)

C

![Graph showing the concentration of IL-10 in different conditions.](image)
Fig. S9