The CD46-Jagged1 interaction is critical for human T\textsubscript{H}1 immunity

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CD46 is a complement regulator with important roles related to the immune response. CD46 functions as a pathogen receptor and is a potent costimulator for the induction of interferon-γ (IFN-γ)-secreting effector T helper type 1 (T\textsubscript{H}1) cells and their subsequent switch into interleukin 10 (IL-10)-producing regulatory T cells. Here we identified the Notch family member Jagged1 as a physiological ligand for CD46. Furthermore, we found that CD46 regulated the expression of Notch receptors and ligands during T cell activation and that disturbance of the CD46-Notch crosstalk impeded induction of IFN-γ and switching to IL-10. Notably, CD4\textsuperscript{+} T cells from CD46-deficient patients and patients with hypomorphic mutations in the gene encoding Jagged1 (Alagille syndrome) failed to mount appropriate T\textsubscript{H}1 responses \textit{in vitro} and \textit{in vivo}, which suggested that CD46-Jagged1 crosstalk is responsible for the recurrent infections in subpopulations of these patients.

CD46 (MCP) was initially discovered as a complement-regulatory protein\textsuperscript{1}, then was identified as a cell-entry receptor ‘hijacked’ by several viruses to promote infection\textsuperscript{2} and is now emerging as an immunomodulatory molecule with vital functions in the costimulation and regulation of human T helper type 1 (T\textsubscript{H}1) cells\textsuperscript{3–5}. The ligands for CD46 and their binding sites in CD46 have been defined for the first two activities; complement-activation fragments C3b and C4b bind to the complement-control protein (CCP) domains CCP2, CCP3 and CCP4 (CCP2–CCP4)\textsuperscript{1,6,7}, whereas viral ligands such as adenosviruses knobs proteins or measles virus hemagglutinin commonly interact with domains CCP1 and CCP2 (refs. 6,7). However, not all activities of CD46 can be explained by its interaction with the ligands known at present; the egg-sperm fusion event mediated by CD46 requires CCP1 (ref. 8), and although the intrinsic generation of T cell–derived C3b is required for CD46 stimulation of CD4\textsuperscript{+} T cells\textsuperscript{4}, the molecular basis of CD46-mediated costimulatory activity is unknown. Similarly, whereas the importance of the Notch system in the induction of cells of the T\textsubscript{H}1 and T\textsubscript{H}2 lineages is undisputed and key signaling events mediated by the activation of Notch on CD4\textsuperscript{+} T cells have been identified\textsuperscript{9}, many functional aspects of this system in T cell biology remain unknown and cannot be explained solely by Notch–Notch ligand interactions. Given the similarities between these two evolutionarily old systems (complement and Notch) in T\textsubscript{H}1 biology, we investigated the possibility of a functional connection between CD46 and Notch proteins and if this potential complement–Notch system crosstalk is required for the effector function of T\textsubscript{H}1 cells.

We not only demonstrate here that the activation of CD46 on CD4\textsuperscript{+} T cells regulated the expression of Notch and its ligands but also identify Jagged1 as an additional physiological ligand for CD46. The Jagged1-binding site in CD46 was located in the amino-terminal CCP domains CCP1 and CCP2, and interference with the CD46-Jagged1 interaction resulted in substantially less induction of T\textsubscript{H}1 cells \textit{in vitro}. We obtained support for the \textit{in vivo} importance of this protein interaction by establishing that patients with mutations in the genes encoding CD46 (refs. 10,11) or Jagged1 (patients with Alagille syndrome)\textsuperscript{12} shared key features. These patients suffered recurrent infections, and although T cell proliferation and the effector function of T\textsubscript{H}1 cells was unaffected, the \textit{in vitro} induction (or regulation) of T\textsubscript{H}1 cells was absent or severely compromised. Mechanistically, the faulty induction of T\textsubscript{H}1 cells seemed to involve altered responsiveness to cytokines of the interleukin 2 (IL-2) family, as all patients had considerable deviation from normal expression of the IL-7 receptor.

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RESULTS

Jagged1 binds to CCP1 and CCP2 of CD46

Because several of the biological activities of CD46 cannot be explained by its interaction with C3b or C4b, the existence of another physiological ligand has long been suspected. Using an initial screen based on an enzyme-linked immunosorbent assay with recombinant soluble CD46 (Supplementary Fig. 1a) and available recombinant proteins of the Notch receptor and Notch ligand families (Supplementary Fig. 1b,c), we identified Jagged1 as a CD46-binding protein. CD46 bound to C3b (positive control), full-length Jagged1 and a Jagged1 construct containing the Notch1-binding site (the disulfide-rich Delta–Serrate–Lag-2 (DSL) domain and the first three epidermal growth factor (EGF)-like domains (J-1(DSL-EGF3))) but not to Notch1 or a recombinant soluble construct composed of Notch1 EGF-like domains 11–13 containing the Jagged1-binding site (N-1(11–13); Supplementary Fig. 1c) or other proteins of the Notch family (Fig. 1a and data not shown). Conversely, Jagged1 bound CD46 but not soluble recombinant complement receptor 1, which shared with CD46 the ability to bind C3b or C4b (Fig. 1b). We also did not observe binding of Jagged1 to factor H or C4bp, two fluid-phase complement regulators that contain C3b- or C4b-binding sites (data not shown). Therefore, the interaction between CD46 and Jagged1 was specific. Furthermore, our data obtained with immobilized CD46 or J-1(DSL-EGF3) indicated that the CD46- and Notch1-binding sites in Jagged1 were in that same region (Fig. 1a,b). We confirmed the binding of Jagged1 to CD46 expressed on the surface of red blood cells (RBCs) from mice with transgenic expression of human CD46 (CD46-transgenic mice). These mice express CD46 on all cells, whereas wild-type mice lack CD46 expression on somatic cells. Notably, RBCs also lack expression of Notch receptors and Notch ligands. With this system, we observed that J-1(DSL-EGF3) bound to RBCs from CD46-transgenic mice but not those from wild-type mice (Fig. 1c), which confirmed that physiological, cell-expressed CD46 was able to bind Jagged1.

To further characterize the CD46-Jagged1 interaction, we measured the binding affinity of J-1(DSL-EGF3) and truncated CD46 constructs by surface plasmon resonance and mapped the Jagged1-binding site on CD46 by nuclear magnetic resonance spectroscopy. The binding of J-1(DSL-EGF3) to CD46 constructs composed of CCP1–CCP4, CCP1–CCP3 and CCP1–CCP2 all had similar interaction affinity and kinetics, but J-1(DSL-EGF3) did not bind to the closely related mouse complement-regulatory molecule Crry (Fig. 2a–d). These experiments established that the two amino-terminal domains of CD46 were fully able to bind Jagged1. The dissociation constant (KD) for the CD46–J-1(DSL-EGF3) interaction was about 8 μM, within the less-tight affinity range of 1 μM noted for the interaction between CD46 and C3b (C. Harris, personal communication) but tighter than the assumed KD for interactions between soluble N-1(11–13) and J-1(DSL-EGF3), for which protein concentrations in excess of 50 μM have been required to observe such an interaction and the interaction is not saturated with protein concentrations up to 160 μM (ref. 13). The use of purified, soluble proteins to characterize interactions has demonstrated that most cell-surface proteins interact with each other with KD values in the micromolar range and concomitant fast on and off rates. This has often been interpreted as facilitating the fine-tuning of interactions by the avidity effects either due to the sharing of many weak interactions across two interacting cells or increases in avidity resulting from the need for cell-surface molecules on the same cell diffuse in only two dimensions rather than three dimensions to find interacting partners. This means that even apparently transient interactions will occur in vivo more frequently and for a longer duration than solution studies indicate, and it suggests that similar characteristics would be important in the interaction between CD46 and Jagged1.

We mapped the interaction site on CD46 by nuclear magnetic resonance chemical-shift perturbation. We did backbone assignment of the construct of the two amino-terminal domains of CD46 by standard triple-resonance methods1,7. The Jagged1-binding site mapped to the fine-tuning of interactions by the avidity effects either due to the sharing of many weak interactions across two interacting cells or increases in avidity resulting from the need for cell-surface molecules on the same cell diffuse in only two dimensions rather than three dimensions to find interacting partners. This means that even apparently transient interactions will occur in vivo more frequently and for a longer duration than solution studies indicate, and it suggests that similar characteristics would be important in the interaction between CD46 and Jagged1.
CD46 regulates expression of Notch and Notch ligand

Although CD46 was initially discovered as a complement-regulatory molecule, it transmits intracellular signals after engagement at the cell surface and can modulate the function of several immunologically competent cell types. For example, CD46 is a T cell costimulator and regulates the production of interferon-γ (IFN-γ) and IL-10 by human T H1 cells, as follows: coengagement of the coreceptor CD3 and CD46 in the presence of a low concentration of IL-2 induces proinflammatory T H1 cells (IFN-γ/IL-10+), whereas the presence of high environmental concentration of IL-2 initiates coexpression of IFN-γ and IL-10 with a switch to a regulatory phenotype (IFN-γ/IL-10−) and, finally, a shutdown of IFN-γ expression (IFN-γ/IL-10−)4,18,19. This CD46- and IL-2-driven (self-)regulatory pathway is defective in T cells from patients with rheumatoid arthritis, which fail to switch5. Similarly, signaling events initiated by the members of the family of human Notch receptors and ligands (Notch1–Notch4, Jagged1 and Jagged2, and Delta-like 1 (DLL1), DLL3 and DLL4) are typified by higher expression of Notch1 and Notch2, as well as DLL1 and DLL4 (Supplementary Fig. 1b,c). These results demonstrated that activation of CD46 induced a specific pattern of expression of Notch receptors and ligands on CD4+ T cells that was typified by higher expression of Notch1 and Notch2, as well as Jagged1 and Jagged2, but loss of DLL1 and CD46.

CD46-Notch crosstalk is vital for T H1 induction in vitro

Notch proteins must be processed successively by the metalloprotease ADAM10 or ADAM17 and then the presenilin-γ-secretase complex to become signaling competent, and the contributions of Notch-mediated signals have been assessed with inhibitors of ADAM and γ-secretase22. That approach cannot be used to address whether CD46-Notch crosstalk is a requisite for the regulation of T H1 cells, as activation of and signaling by CD46 on CD4+ T cells also required processing by ADAM and γ-secretase (Supplementary Fig. 2a,b). CD46 exists in four isoforms that arise through splicing of a glycosylated extracellular region and the two possible intracellular tails, CTY1 and CTY2 (ref. 1; Supplementary Fig. 1a). Human Jurkat T cells stably transfected to express the CD46-CYT1 isoform

*Figure 2* Jagged1 binds to CCP1 and CCP2 of CD46. (a) Surface plasmon resonance of the binding of J-1(DSL-EGF3) to CD46 containing CCP1–CCP4 (CD46(1–4)) coupled on the surface of the chip with a K_d of ~8 µM (normalized by subtraction of the mock-coupled channel). (b,c) Binding of CD46 containing CCP1–CCP3 (CD46(1–3)); b) or the two amino-terminal domains of CD46 (CD46(1–2); c) to immobilized J-1(DSL-EGF3), normalized as in a. Inset, equilibrium values of binding and K_d fit. (d) Binding of CD46 containing CCP1–CCP4 (21 µM), CCP1–CCP3 (25 µM) or the two amino-terminal domains (19 µM) after injection over immobilized J-1(DSL-EGF3); Crry containing CCP1–CCP4 (20 µM) serves as a negative control. Results are normalized for construct molecular weight. Below, K_d values. (e) Nuclear magnetic resonance spectroscopy overlay of the H, N-heteronuclear single-quantum coherence (HSQC) of CD46 containing the two amino-terminal domains (black), showing the chemical-shift perturbation after the addition of unlabeled J-1(DSL-EGF3) (green) at a molar ratio of 0.6. Bottom, enlargement of area outlined above. (f) Chemical-shift perturbation by residue for those unambiguously assigned and baseline-resolved residues in e. (g) Surface structure of CCP1 and CCP2 (Protein Data Bank accession code, 30BE), showing residues with unambiguous assignment (dark gray) and chemical-shift perturbation of >0.15 p.p.m. (red) after the addition of J-1(DSL-EGF3). Blue, N-linked glycosylation sites. Data are from one experiment with six replicates of two independent sets of proteins (a), twelve replicates of three independent sets of proteins (b) or nine replicates of two independent sets of proteins (c), are representative of six experiments (d); mean ± s.d. of triplicates or are from one representative of three independent experiments (e,f).
Figure 3 CD46 regulates the expression of Notch receptors and ligands on human CD4+ T cells. (a) Expression of IFN-γ and IL-10 by human CD4+ T cells activated for 36 h with anti-CD3 and anti-CD46 plus IL-2. The data were normalized to relative expression of mRNA (top left), IFN-γ/IL-10 (top right) and IL-10 (bottom right). (b,c) Expression of NOTCH1 and NOTCH2 mRNA (b) and JAG1, JAG2 and DLL1 mRNA (c) by nonactivated T cells (NA), T cells activated for 2 h with anti-CD3 (α-CD3) and anti-CD46 (plus 50 U/ml recombinant human IL-2) and T cells of the subpopulations in a. Results are presented relative to 18s mRNA expression. (d) Expression of Notch1, Jagged1 and DLL1 protein on non-subsorted bulk CD4+ T cells after stimulation for 36 h with anti-CD3 and anti-CD46. MFI, mean fluorescence intensity. NS, not significant; **P < 0.05, ***P < 0.005 and ****P < 0.001, versus nonactivated cells (Student’s one-tailed t-test and Bonferroni correction for multiple comparisons). Data are representative of four experiments (a–d; mean and s.d. in b,c).

(Jurkat-BC1 cells) produce IL-10 after activation with anti-CD3 and anti-CD46, whereas untransfected Jurkat cells or Jurkat cells transfected to express CD46-CYT2 (Jurkat-BC2) are unable to express IL-10 (ref. 4). To investigate whether the CD46-CYT1-mediated stimulation involves subsequent Notch1 signaling, we disrupted Notch1 signaling through the use of an inhibitory monoclonal antibody or soluble N-1(11–13) to compete with cell surface–expressed Notch1 for Notch ligands. Each treatment abrogated the CD46-mediated production of IFN-γ and diminished IL-10 secretion by >50% in Jurkat-BC1 cells (Supplementary Fig. 2c,d), which indicated that CD46 and Notch signaling pathways indeed intersected in the production of TH1 cytokines and switching to IL-10.

Consistent with this hypothesis, the addition of soluble CD46, DLL1 or Jagged1 also resulted in much less switching of purified CD4+ T cells from IFN-γ to IL-10, mediated by anti-CD3, anti-CD3 and anti-CD28, or anti-CD3 and anti-CD46, in each case in the presence of IL-2 (Fig. 4a,b). This was probably due to interference with temporally regulated changes in interactions of members of the CD46 and Notch system and, thus, signaling events during T cell activation. Super-resolution imaging experiments demonstrated that >95% of Jagged1 on resting CD4+ T cells localized together with CD46, whereas we observed only negligible interactions between Jagged1 and Notch1 (Fig. 4c). After activation with anti-CD3 and anti-CD46, which induced CD46 downregulation and the ‘release’ of Jagged1, a substantial proportion (>50%) of Notch1 molecules were bound to Jagged1 (Fig. 4c). These data suggested that our affinity measurements obtained with limited recombinant fragments of CD46, Notch1 and Jagged1 (Fig. 2) extended to intact proteins expressed on the cell surface. Further support for the possibility of regulated successive crosstalk of the CD46 and Notch system during the induction of TH1 cells (model, Supplementary Fig. 3) was provided by the observation that activation of CD46 alone, without direct antibody-mediated engagement of Notch, induced considerable transcription of the Notch target gene HES1 and transcription of the gene encoding the Notch signaling mediator RBPJk (Fig. 4d). Conversely, inadequate downregulation of CD46 hinders TH1 induction. A member of the E-cadherin network, α-E-catenin, binds to the cytoplasmic portion of CD46 in human intestinal epithelial cells123. We found that α-E-catenin also interacted with CD46 in primary human CD4+ T cells (Supplementary Fig. 4a), and knockdown of α-E-catenin protein (Supplementary Fig. 4b) impaired the downregulation of CD46 without having an effect on the expression of Notch, Jagged1 or DLL1 protein (Supplementary Fig. 4c) or that of additional molecules vital for T cell activation, such as CD3, CD25, CD28 or CD69 (Supplementary Fig. 4d). Inhibition of the downregulation of CD46 was accompanied by 50% less production of IFN-γ and IL-10 (Supplementary Fig. 4e), whereas the proliferation and viability of cells were unaffected (data not shown). Although these data suggested that α-E-catenin participated in CD46-mediated signaling events in CD4+ T cells, we cannot exclude the possibility that the changes in cytokine production were secondary to inapparent additional effects of the knockdown of α-E-catenin.

We were not able to inhibit the observed downregulation of DLL1 during T cell activation without treating CD4+ T cells with an inhibitor of ADAMs. However, we noted that the CD46-mediated downregulation of DLL1 was less efficient than on Jurkat-BC1 cells than on primary CD4+ T cells (Supplementary Fig. 4f), which might explain why Jurkat-BC1 cells produce relatively small amounts of IL-10. In agreement with that, transfection of Jurkat-BC1 cells with short hairpin RNA targeting DLL1 mRNA resulted in lower DLL1 expression in activated Jurkat-BC1 cells with proportionally greater IL-10 production (Supplementary Fig. 4f).

In sum, these data suggested that the presence of CD46 on T cell surfaces restricted interactions of Notch1 with Jagged1. They also indicated that engagement of CD46 during T cell activation led to α-E-catenin-dependent downregulation of CD46 and α-E-catenin-independent downregulation of DLL1. Disturbance in this spatially and temporally regulated crosstalk between complement and Notch proteins led to deregulated TH1 responses in vitro.

CD46 deficiency causes defective TH1 function in vivo

Rodents (mice, rats and guinea pigs) lack CD46 expression on somatic tissues, and this restricted expression pattern impedes direct evaluation of the in vivo importance of the CD46-Jagged1 interaction with a small animal model15. Although the mouse Crry protein compensates for
Figure 4 Undisturbed crosstalk by the CD46 and Notch system is required for normal switching of human T<sub>H1</sub> cells from IFN-γ to IL-10. (a) Expression of IFN-γ and IL-10 by CD4<sup>+</</sup> T cells activated with anti-CD3 alone (top), anti-CD3 and anti-CD28 (middle) or anti-CD3 and anti-CD46 (bottom) in the presence of recombinant human IL-2 (25 U/ml) and treated with medium alone (Med) or soluble (s) DLL1, J-1 (DSL-EGF3) or CD46. Numbers in plots indicate subpopulations (as in Fig. 3a). (b) Ratio of IFN-γ to IL-10 secreted into the media of cells treated as in a, bottom. (c) Super-resolution confocal microscopy and three-dimensional analysis of nonactivated T cells and T cells activated with anti-CD3 and anti-CD46, stained with anti-CD46, anti-Notch1 or anti-Jagged1 to assess molecular colocalization (white areas, far right). Outlined area (bottom left), staining for CD46. Original magnification, ×600. (d) Quantitative PCR analysis of the transcription of HES1 and RBPJ mRNA in purified CD4<sup>+</</sup> T cells (as in Fig. 3b,c). *P < 0.05, **P < 0.005 and ***P < 0.001 (Student’s one-tailed t-test and Bonferroni correction for multiple comparisons), versus medium alone (b) or nonactivated cells (d). Data are representative of six experiments (a,b,d) and mean and s.d. (c) to two independent experiments (c).

The complement-regulatory function of CD46, it does not regulate T<sub>H1</sub> responses<sup>10</sup>. For that reason, we obtained CD4<sup>+</</sup> T cells from patients with mutations in either CD46 or JAG1 and assessed their ability to mount T<sub>H1</sub> responses in vitro and in vivo. Mutations in CD46 that affect protein expression or complement-regulatory function cause atypical hemolytic uremic syndrome<sup>24</sup>. At present, fewer than ten patients worldwide have been identified as having homozygous mutations in CD46 but, notably, over 50% of those patients have common variable immunodeficiency and recurrent chest infections, which indicates that CD46 mutations can indeed induce immunological defects<sup>10,11</sup>. The following three patients with homozygous CD46 mutations participated in this study (Fig. 5a): patient CD46-1, who has a splice-site alteration between exons 1 and 2 that leads to only 10% of normal CD46 expression on peripheral blood mononuclear cells<sup>10</sup>; patient CD46-2, who has a similar splice-site alteration that causes aberrant mRNA transcripts and loss of CD46 expression on >90% of peripheral blood mononuclear cells<sup>11</sup> and normal expression on the remaining 10% of those cells; and patient CD46-3, with two mutations in exon 2 and absence of detectable cell-surface expression of CD46 (Supplementary Table 1 and Supplementary Fig. 5a). Although hospital records for patient CD46-1 are unavailable, patients CD46-2 and CD46-3 have suffered confirmed recurrent infections, have been diagnosed with common variable immunodeficiency and are being treated with intravenous immunoglobulin infusion. All three patients have normal numbers of B cells and CD4<sup>+</</sup> and CD8<sup>+</</sup> T cells<sup>10,11</sup> (data not shown), and activation of their purified CD4<sup>+</</sup> T cells showed that they proliferated at normal rates (data not shown) and mounted strong T<sub>H1</sub> responses (Fig. 5b): this suggested that an intrinsic thymus-derived defect in induction of the T cell lineage was unlikely. However, activation with anti-CD3, with anti-CD3 plus anti-CD28 or with anti-CD3 and anti-CD46 did not induce IFN-γ secretion in cells from patients CD46-1 and CD46-3; consequently, the cells also failed to switch to an IFN-γ- and IL-10-coexpressing phenotype, and these patients thus lacked a major T cell population key to the prevention and clearance of infections caused by intracellular pathogens<sup>25</sup> (Fig. 5b). In contrast, T cells from patient CD46-2 produced normal amounts of IFN-γ and IL-10 after activation with anti-CD3 or with anti-CD3 plus anti-CD28 (although they lacked the usual CD46-mediated increase in IFN-γ production and switch to IL-10). The reason for this is unclear. Notably, B cells from patient CD46-1 are fully functional<sup>26</sup>, but that has not been confirmed for patients CD46-2 and CD46-3.

As predicted, lack of CD46 resulted in altered regulation of the expression of Notch1 and Jagged1, as nonactivated T cells from patients CD46-2 and CD46-3 had higher expression of Jagged1 than those from healthy donors (Table 1 and Supplementary Fig. 5a) but then failed to upregulate the expression of Jagged1 protein after activation with anti-CD3 and anti-CD46. Similarly, T cells from patient CD46-3 were defective in activation-induced upregulation of Notch1, whereas T cells from patient CD46-2 overexpressed Notch1 after activation (data on Notch1 and Jagged1 expression not available for patient CD46-1; Table 1 and Supplementary Fig. 5a). We also assessed the expression of additional key cell-surface markers required for normal T<sub>H1</sub> responses on resting and activated T cells from healthy donors and the patients with CD46 mutations. We found no substantial differences among those cells in the expression and regulation of CD3, CD11a (α<sub>x</sub>-chain of LFA-1), CD28, CD69, CD122 and CCR7 (data not shown). Although all three patients showed a trend toward less upregulation of CD25 and downregulation of CD62L after activation with anti-CD3 or with anti-CD3 plus anti-CD28,
Figure 5 T cells from CD46-deficient patients have defective in vitro T_{H}1 induction. (a) Location of CD46 mutations (‘†’; bottom) in CD46-deficient patients CD46-1, CD46-2 and CD46-3 (including exon structure); above, corresponding protein domains of CD46; SP, signal peptide; (STA, STB, STC), serine-threonine-proline–rich regions; ? , region of unknown function; TM, transmembrane; CYT1 and CYT2, cytoplasmic tail.

(b) Secretion of cytokines by CD4+ T cells purified from freshly drawn blood samples from two healthy donors (HD3 and HD4; representative of 12 age- and sex-matched donors) and patients CD46-1, CD46-2 and CD46-3 (above plots), then left nonactivated or activated for 36 h with various combinations of immobilized anti-CD3, anti-CD28 and anti-CD46 (horizontal axes) in the presence of recombinant human IL-2 (25 U/ml). TNF, tumor-necrosis factor, ND, not detectable. Data are representative of three experiments with duplicate samples (mean).

Table 1 Expression of surface markers on CD4+ T cells from CD46-deficient patients

| Jagged1 | Notch1 | CD46 | CD127 | CD132 |
|---------|--------|------|-------|-------|
| HD3     | +      | ↑    | +     | +     |
| HD4     | +      | ↑    | +     | +     |
| CD46-1  | NDA    | NDA  | NDA   | NDA   |
| CD46-2  | + (†)  | ↔    | +     | +     |
| CD46-3  | + (†)  | ↔    | +     | +     |

Expression of surface markers on CD4+ T cells from healthy donors and CD46-deficient patients, left nonactivated or activated with anti-CD3 and anti-CD28 (CD3,CD28) or with anti-CD3 and anti-CD46 (CD3,CD46); +, present on resting T cells; −, not present on resting (or activated) T cells; ↑, higher expression; ↓, lower expression after activation; ↔, no change in expression; †, higher baseline expression (>250% and >250%, respectively) than that of cells from healthy donors; ††, more upregulation (≥250% and ≥250%, respectively) on activated cells than that on cells from healthy donors; †††, no change in expression relative to that of nonactivated cells. NDA, no data available. Data are representative of three experiments.

Expression remains higher than that of cells from healthy donors. *Expression similar to that of activated T cells from healthy donors. †Expression remains higher than that of cells from healthy donors. Raw data, Supplementary Figure 5.

**Articles**

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Figure 6  T cells from patients with Alagille syndrome have defective in vitro Th1 induction. (a) Location of JAG1 mutations in patients AP1–AP4 with Alagille syndrome (presented as in Fig. 5a). CR, cysteine-rich region; PDZ, conserved scaffolding structural domain. (b) Secretion of cytokines by CD4+ T cells purified from freshly drawn blood samples from two healthy donors (HD1 and HD2) and patients AP1–AP4 (above plots), then left nonactivated or activated for 36 h with various combinations of immobilized anti-CD3, anti-CD28 and anti-CD46 (horizontal axes) in the presence of recombinant human IL-2 (25 U/ml). Data are representative of four experiments with duplicate samples (mean).

We studied four patients with Alagille syndrome (AP1–AP4) who had mutations in exon 3, 4, 18 or 19 of JAG1 and suffered recurrent and persistent otitis media and chest infections (Fig. 6a and Supplementary Table 2). Because Notch–Notch ligand interactions participate in development of the CD4+ and CD8+ T cell lineage in the thymus, we first assessed the composition of peripheral blood mononuclear cell populations and found no deviation in the frequency of key lymphocyte subpopulations in samples from patients AP1–AP4 relative to those from healthy donors (Supplementary Table 2). However, when we compared the expression of T111 and T112 cytokines by activated purified CD4+ T cells from patients AP1–AP4 and their counterparts from healthy donors, we observed a profile reminiscent of that of CD46-deficient patients (Fig. 6b). Cell proliferation and secretion of tumor-necrosis factor was normal for all patients, but T cells from patients AP1 and AP2 produced no IFN-γ (and did not switch to IL-10 production) after activation with anti-CD3 alone or with anti-CD3 plus anti-CD48 (data not shown) or activation with anti-CD3 and anti-CD46, whereas patients AP3 and AP4 had much less T111 induction (≤50%). T cells from patients AP3 and AP4 also had notably enhanced T112 responses (Fig. 6b).

Although basal Notch1 expression and upregulation of Notch1 expression after activation was unaffected, patients AP1–AP4 had unexpectedly higher expression of Jagged1 protein on resting T cells (Table 2 and Supplementary Fig. 6a). In contrast to results obtained for the CD46-deficient patients, however, Jagged1 was further upregulated after activation (Table 2 and Supplementary Fig. 6a). Whereas the expression and regulation of CD3, CD11a, CD25, CD28, CD69, CD12 and CCR7 was also normal in patients AP1–AP4 (data not shown), we found substantial deviation (similar to that observed for the CD46-deficient patients) in the regulation of CD127 and CD132 by T cells from the patients with Alagille syndrome. Cells from patients AP1 and AP4 completely lacked downregulation of CD127 after activation with anti-CD3 and anti-CD46, and whereas all patients had more CD132 on resting T cells than did healthy donors, after activation with anti-CD3 and anti-CD46, T cells from patients AP1, AP3 and AP4 upregulated CD132 expression well beyond the normal expression achieved by such activation. Furthermore, we observed that T cells from patients AP2 and AP3 were unable to efficiently downregulate CD46 after activation with anti-CD3 and anti-CD46 (Table 2 and Supplementary Fig. 6a).

Similar to T cells from patient CD46-1, T cells from patients AP1 and AP3 were also unable to induce T111 responses in vivo or cause graft-versus-host disease when injected into IL-2 receptor (IL-2R) γ-chain–deficient mice of the nonobese diabetic–severe combined immunodeficiency strain (Supplementary Fig. 6b–d). However, this failure to cause graft-versus-host disease might have been the result of poor engraftment, because in contrast to T cells from patient CD46-1, which engrafted at a ‘reasonable’ rate (Supplementary Fig. 5b), T cells from patients with Alagille syndrome failed to engraft in the mice. Notch1 signaling on human CD4+ T cells has been shown to regulate the adhesion, migration and chemotaxis of these cells via modulation of GTPases of the Rho family. Thus, defects in Notch system signaling may affect not only T111 cytokine production in patients with Alagille syndrome but also the homing ability of their T cells. In summary, patients with Alagille syndrome with recurrent infections had a T effector cell phenotype similar to that of CD46-deficient patients, characterized by defective induction of T111 cells and deregulation of the expression of CD127 and CD132 but unaffected function of T112 cells.

Table 2 | Expression of surface markers on CD4+ T cells from patients with Alagille syndrome

|           | Jagged1 | Notch1 | CD3, CD46 | CD3, CD46 | CD3, CD46 | CD3, CD46 | CD3, CD46 |
|-----------|---------|--------|-----------|-----------|-----------|-----------|-----------|
| HD1       | +       | +      | +         | +         | +         | +         | +         |
| HD2       | +       | +      | +         | +         | +         | +         | +         |
| AP1       | + (†)   | + (†)  | +         | +         | +         | +         | + (†)     |
| AP2       | + (†)   | + (†)  | +         | +         | + (50%)   | +         | + (†)     |
| AP3       | + (†)   | + (†)  | + (‡)     | +         | +         | +         | + (†)     |
| AP4       | + (†)   | + (†)  | + (‡)     | +         | +         | + (†)     | + (‡)     |

Expression of surface markers on CD4+ T cells from healthy donors and patients with Alagille syndrome, left nonactivated or activated with anti-CD3 and anti-CD46: symbols as in Table 1; †, lower baseline expression than that of cells from healthy donors. Data are representative of four experiments.

**DISCUSSION**

Here we have identified Jagged1 as a physiological ligand for CD46 and have demonstrated that coordinated CD46-Jagged1 crosstalk was required for T111 responses. Activation of the Notch system is controlled by spatial and temporal restriction of the availability of receptors and ligands during cell-cell interactions, and our study has suggested that CD46 participates in this process. We propose a model in which CD46 sequesters Jagged1 on resting T cells,
in terms of cell-surface receptors involved in TH1 biology was the CD46 or a combination thereof. However, the most notable phenotype studied here unexpectedly had higher Jagged1 expression on rest-

In addition, expression studies of additional Alagille syndrome–associated mis-

tion34. Thus, similar to DLL1 expression, in the absence of antigen or danger signals, CD46 expression on T cells may function as the ‘brake.’ After engagement of the T cell antigen receptor, the CD46 ligand C3b (ref. 34) is generated locally. Binding of C3b to CD46 initi-

ates CD46-mediated signaling events, including the migration and cluster formation of T cells35, downregulation of CD46 and DLL1 and maintenance of the surface availability of Notch1 and Jagged1. This change in surface expression of CD46 and Notch proteins releases the brake and allows orchestrated Notch1 and DLL1 interactions in trans (that generate IFN-γ), as well as binding of Notch1 and Jagged1 in cis or trans (necessary for IL-10 induction36). The role of IL-2 and potential functions of generated soluble CD46 and members of the Notch family remain to be integrated into this model. In support of this model is our observation that both CD46-deficient patients and patients with JAG1 mutations who suffer recurrent infections did not generate normal TH1 responses. Both patient groups also shared additional key features in their T cell phenotype as additional evidence that an overlapping CD46 and Notch pathway is affected. First, in line with published observations that CD46-mediated sig-

daurs are specifically needed for TH1 induction19, TH2 responses and tumor-necrosis factor factor were induced. Moreover, both patient groups showed a trend toward exaggerated TH2 responses, which may explain why patients with Alagille syndrome also suffer a greater prevalence of TH2-driven conditions, including otitis media, asthma and eczema12,25. Furthermore, and consistent with our model, CD4 + T cells from C3-deficient patients (which cannot produce the CD46 ligand C3b locally) were also unable to assume a TH1 phenotype and had deregulated IL-2R expression but produced large amounts of TH2 cytokines (data not shown).

The mutations in patients AP2 and AP3 are predicted to lead to nonsense-mediated decay of JAG1 mRNA that results in the expres-

sion of only wild-type Jagged1 on the cell surface. Furthermore, expression studies of additional Alagille syndrome–associated mis-

sense mutations in cell lines have shown that they led to retention of the mutant protein in the endoplasmic reticulum (data not shown), which suggests that haploinsufficiency is the pathogenic mechanism that operates in most cases. The patients with Alagille syndrome studied here unexpectedly had higher Jagged1 expression on rest-

ing T cells; we have no explanation for this observation at present. Nonetheless, each patient with Alagille syndrome and CD46-deficient patient had distinct deviations in the expression of Jagged1, Notch1 or CD46 or a combination thereof. However, the most notable phenotype in terms of cell-surface receptors involved in TH1 biology was the considerable deregulation of CD127 and CD132 (which together form the receptor for IL-7) on T cells from each patient group. Notably, not only is IL-7 required for T cell homeostasis and the enhancement of TH1 and TH17 responses46 but the gene encoding CD127 has also been identified as a strong risk locus not linked to the major histo-

compatibility complex for the T cell–driven disease multiple sclero-

sis37,38. Similarly, deregulation of the expression of CD46 isoforms has been connected with the progression of multiple sclerosis39. Future studies should assess whether T cells from patients with CD46 mutations have an altered responses to IL-7. However, CD132 is also an essential component of the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 (the IL-2 receptor family)37. Most members of this family are involved in the normal function of T cells and B cells as well as natural killer cells, and CD132 deficiency is linked to X-linked severe combined immunodeficiency27. Deregulation of CD132 would therefore also affect the responsiveness of T cells to members of the IL-2

cytokine family other than IL-7. IL-2–mediated signaling through the high-affinity receptor for IL-2 (CD25, CD122 and CD132) is needed for cell-activation induction of TH1 responses27, and chemical inhibi-

tion of Notch1 signaling impedes normal IL-2R expression and TH1 induction40. IL-2 is also linked to CD46 as follows: CD46 negatively regulates IL-2 expression but also integrates IL-2R signals for IL-10 and IFN-γ coexpression in TH1 cells4,5. We therefore speculate that T cells from CD46-deficient patients and patients with Alagille syn-

drome may be unable to induce TH1 responses, at least in part because of aberrant IL-2R signaling.

The immunomodulatory function of CD46 is probably one reason for the use of CD46 as a receptor by several human pathogens. CD46-interacting viruses target CCP1 and CCP2 of CD46 (refs. 2,6,7), which contain the Jagged1-binding site. Structures of CCP1 and CCP2 in complex with viral proteins that bind CD46 have demonstrated sub-

stantial reorientation of these two CCPs relative to each other, which suggests that their arrangement is highly ligand specific. The interaction surfaces identified here for the binding of Jagged1 to CD46 sug-

gested that a Jagged1-specific conformation of CCP1–2 was required for binding and indicated that Jagged1-bound CD46 would not be able to bind viral ligands simultaneously without displacement of Jagged1 from CD46. Hence, the observation that the binding of adenovirus serotype 35 to CD46 on human CD4+ T cells induced downregulation of CD46 but less production of IL-2 and IFN-γ could have been due to interference with the coordinated CD46-Notch system signaling events during T cells activation41,42.

Our observations may provide a platform from which to advance understanding of the complex signaling networks that underlie the biology of TH1 cells as well as differences in the human and mouse systems in the induction and regulation of TH1 responses. Future analy-

sis of the effect of the binding of virus to CD46 and its effect on the interaction of Jagged1 with CD46 may also provide new insights into how CD46-binding pathogens may interfere with the CD46-Jagged1–

mediated normal TH1 induction to foster infection. Furthermore, the identification of the surface expression of CD46 as a ‘stop signal’ offers the following hypothesis to explain the counterintuitive downregula-

tion of CD46 on most activated cell types: it provides a ‘go’ signal when immune activation is apparent5,23. Finally, as the Notch system also has fundamental roles in tissue morphogenesis and renewal, we anticipate that the CD46-Jagged1 interaction may be important in these biological processes as well.

METHODS

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

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Note: Supplementary information is available in the online version of the paper.

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ONLINE METHODS

Healthy donors and patients. Purified T cells were obtained from buffy coats (National Blood Service) or blood samples from healthy volunteers. Informed consent was obtained from all subjects, and blood was collected and processed with the approval of and in accordance with the King’s College Ethics Committee guidelines (06/Q0705/20). Adult patients with CD46 deficiencies were recruited in France under appropriate institutional guidelines; two cases have been described\(^{10,11}\). Six Caucasian children between 2 and 12 years of age were recruited, of whom four were diagnosed with Alagille syndrome and two were healthy (Review Board of National Research Ethics Committee London: 09/H0711/38). Patients with Alagille syndrome all had repeated infections and/or allergies and food intolerances. None of the patients were taking immunosuppressants or had undergone liver transplantation. Blood samples were processed within a maximum of 3 h from the time of collection.

Mice and graft-versus-host disease model. CD46-transgenic mice were generated by backcrossing an established CD46-transgenic line onto outbred MF1 mice\(^{14}\). Mice were handled and samples were obtained and processed under UK Home Office license 70/6906. Mice of the nonobese diabetic–severe combined immunodeficiency strain that were deficient in β₂-microglobulin (Taconic Farms) or IL-2R common γ chain (Charles River) were used for the injection of T cells from patient CD46-1 or from patients with Alagille syndrome, respectively, and were maintained under pathogen-specific sterile conditions. Graft-versus-host disease was induced as described\(^{19}\). Peripheral blood mononuclear cells from healthy donors, CD46-deficient patients or patients with Alagille syndrome were activated for 72 h with immobilized monoclonal antibody (mAb) to CD3 and mAb to CD28 before adoptive transfer via injection into the tail vein (1 × 10⁷ T cells: 80–85% CD⁴⁺ and 15–20% CD⁸⁺). Engraftment of human cells was monitored by counting of human CD⁴⁵⁺ cells to mouse CD⁴⁵⁺ cells and measurement of human IFN-γ in mouse blood at various time points. Body weight was monitored and mice were culled when they reached the human end point of a decrease of 15% in body weight. Disease was further confirmed by immunohistochemical analysis of intestinal tissue.

T cell isolation and activation. T cells were isolated and activated as described\(^{5}\). The human embryonic kidney HEK293T and Jurkat cell lines were cultured according to the manufacturer’s protocol (American Type Culture Collection). Jurkat cells (including those stably transfected to express either CD46-CYT1 (Jurkat-BC1) or CD46-CYT2 (Jurkat-BC2))\(^{4}\) were activated as described for purified CD⁴⁺ T cells but for 5 d with IL-2 supplementation every 2 d.

Recombinant proteins. Serum-purified C3b was from Complement Technologies, and recombinant complement receptor 1 (CR1) was produced as published\(^{43}\). Recombinant human DLL1 and Jagged1 and recombinant mouse Jagged1 were from R&D Systems. N-1(11–13) and (J-1(DSL-EGF3)) with carboxy-terminal biotinylation were produced as described\(^{13}\). CD46 constructs (Adprotech) were subcloned into the PETib vector and were transformed into B834 cells. Labeled proteins were produced as described\(^{44}\) and proteins were refolded by an established protocol\(^{45}\). Recombinant Crry (containing CCP1–CCP4) was generated as described\(^{46}\).

Antibodies and inhibitors. The following cell-stimulating monoclonal antibodies were used: anti–human CD28 (CD28.2), anti–mouse CD28 (145-2C11), anti–mouse CD28 (37.51), anti–CD3 purified from a specific hydridoma line (OKT-3; all from American Biosciences); and anti–CD46 (TRA-2-10; generated by Bioproducts for Bioassay). N-1(11–13) and J-1(DSL-EGF3) were obtained from R&D Systems. Biotinylated J-1(DSL-EGF3) and N-1(11–13) were produced as described\(^{45}\). Recombinant Crry (containing CCP1–CCP4) was generated as described\(^{46}\).

Surface plasmon resonance. All data were collected with a Biacore T100 (GE Healthcare) with Jagged1 or CD46 immobilized through primary amine-coupling to the surface of the Biacore CM5 Chip. CD46 or J-1(DSL-EGF3) constructs were passed over the chip in a solution of 10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.005% surfactant P20. Multiple titrations were done over a concentration range of 0.1 µM to 40 µM, with flow rates from 25 µl/min to 40 µl/min at 25 °C. Data were processed with the manufacturer’s BIAevaluation software and were fit with a Langmuir 1:1 equilibrium model or, where possible, kinetic analysis with simultaneous fits of the on and off rates (SigmaPlot).

Nuclear magnetic resonance spectroscopy. A sample containing 65 µM of the construct of the two amino-terminal domains of CD46 uniformly enriched in 15N in 25 mM sodium acetate (pH 5.5) and 5% D₂O was used for collection of sensitivity-enhanced 1H, 15N–HSQC\(^{48}\) on a 500-MHz Bruker Avance (Bruker U) equipped with a cryoprobe. Another 1H, 15N–HSQC was collected with the addition of 38 µM unlabeled Jagged1 BirA as (described\(^{13}\)). In addition, the 1H, 15N–HSQC was repeated with a sample of unlabeled Jagged1 without the BirA tag (data not shown). For the 1H, 15N–HSQC, acquisition times for t₁ were 42 ms with 256 complex data points. Data were processed and analyzed with NMRPipe NMR data-processing software and Sparky NMR display software. Chemical shift perturbation values were calculated with the following equation:

\[
\Delta d = \sqrt{\left(\Delta d_H \right)^2 + \left(\Delta \delta_{t} / \delta_R \right)^2}
\]

Assignments were done on [U-15N, 13C, 1H] the construct of the two amino-terminal domains of CD46 with standard triple resonance correlation experiments. HNCA/HN(CO)CA, HNCO/HN(CA)CO and HNCA/CB/HNCA(CO)CB experiments were done as described\(^{49}\).

High-resolution microscopy and co-localization analysis. T cells were treated and then were stained for 25 min at 4 °C with anti-CD46, anti-Jagged1 or anti-Notch1. Cells were then mounted with Fluoromount-G (SouthernBiotech). Images were obtained by confocal fluorescence microscopy with a laser-scanning microscope (Fluorview 1000; Olympus) with a 60x oil objective with a numerical aperture of 1.35. For three-dimensional image analysis, z-stacks were obtained with an interval of 0.1 µm with the confocal microscope with 20–30 slices per stack to visualize cells in their full extension. Stacks were then used for image analysis with IMARIS software (version 7.4.2; Bitplane), operating with IMARIS Surpass (volume and isosurface rendering analysis), to visualize and locate points of interest (expression of Jagged1, CD46 and Notch1). For colocalization studies, data sets were analyzed with IMARIS software. Data of colocalization events were determined with the statistical modules of the colocalization software of the IMARIS package.

Cytokine measurements. Cytokines from cell cultures or mouse serum were measured with human TH1/TH2 Cytometric Bead Arrays (BD Biosciences) or anti-CD26L (599772), anti-CD69 (555350), anti-CD127 (557938), anti-CD132 (555900) and anti-mouse CD45RB (16A) were all from BD Biosciences. The broad-spectrum matrix metalloproteinase inhibitor TAPI-2 was from Merck Chemicals; marimastat was from Tocris Biosciences; and the presenilin and γ-secretase inhibitor L-685,458 was from Sigma-Aldrich.

Enzyme-linked immunosorbent assay. After 96-well microplates were coated overnight at 4 °C with 5 µg/ml of protein (soluble CD46, human C3b, J-1(DSL-EGF3) or recombinant CR1), they were blocked with 1% BSA and then incubated for 1.5 h at 37 °C with protein samples diluted to a concentration of 0.5 µg/ml (and roughly equimolar amounts) in 4% BSA, 0.005% Tween20, 0.25% NP-40, 20 mM HEPES and 10 mM CaCl₂, pH 7.4. Because recombinant mouse C3b and C4b are commercially unavailable, 5% mouse serum was used as source for mouse C3b and C4b. Bound proteins were detected with the appropriate primary mAbs, followed by horseradish peroxidase–linked secondary antibodies or streptavidin and subsequent visualization with OPD substrate (O-phenylene diamine dihydrochloride; Sigma-Aldrich).

Images were obtained by confocal fluorescence microscopy with a laser-scanning microscope (Fluorview 1000; Olympus) with a 60x oil objective with a numerical aperture of 1.35. Three-dimensional image analysis, z-stacks were obtained with an interval of 0.1 µm with the confocal microscope with 20–30 slices per stack to visualize cells in their full extension. Stacks were then used for image analysis with IMARIS software (version 7.4.2; Bitplane), operating with IMARIS Surpass (volume and isosurface rendering analysis), to visualize and locate points of interest (expression of Jagged1, CD46 and Notch1). For co-localization studies, data sets were analyzed with IMARIS software. Data of co-localization events were determined with the statistical modules of the co-localization software of the IMARIS package.
the human IFN-γ and IL-10 Cytokine Secretion Assay Kits (Miltenyi Biotec) in combination according to the manufacturer's protocol.

Quantitative real-time RT-PCR. Primers used to quantify mRNA transcription in CD4+ T cells were as follows: NOTCH1 forward, 5′-CGCACAAGGTTCTTCCAG-3′, and reverse, 5′-AGGATCAGTGGCGTCTGTG-3′; NOTCH2 forward, 5′-TTGAGAGTTATACCTTGTGTC-3′, and reverse, 5′-GATACACCTGTCAATGCTAAAGG-3′; JAG1 forward, 5′-ACGCTTTGTCCGAAATACG-3′, and reverse, 5′-AGGCTTTGTCGGCAA-3′; NOTCH2 forward, 5′-GGAGGCTATTACACCCTTGTTTA-3′; DLL1 forward, 5′-CTGAACTCCGGAACCT-3′; HES1 forward, 5′-GACGGACATCGTTCATGCACTC-3′, and reverse, 5′-TTTCGACATCGTTCATGCACTC-3′.

RNA silencing. Indocarbocyanine-labeled small interfering RNA targeting human α-E-catenin (s3718) and negative control siRNA were from Ambion; these experiments done as described4. Transfection efficiency and cell viability was consistently above 80% and 75%, respectively, and protein knockdown peaked at 24–36 h after transfection. For short hairpin RNA-mediated silencing of DLL1 in Jurkat T cells, the appropriate lentivirus was generated by cotransfection of HEK293T cells with the packaging plasmid psPAX2 (Addgene), envelope plasmid pMD2.G (Addgene) and pLKO.1 vector containing short hairpin RNA targeting DLL1 (Abgene) through the use of FuGENE 6 Transfection Reagent (Roche Diagnostics). After 48 h, medium was collected and filtered and was added to Jurkat cell cultures. Virus-infected cells were selected by puromycin. Knockdown of DLL1 protein was consistently above 50%.

Statistical analysis. Statistical analyses were done with the Student's one-tailed t-test and Bonferroni correction for multiple comparisons (Excel software; Microsoft).

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