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Junctional adhesion molecule-A on dendritic cells regulates Th1 differentiation

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ABSTRACT

The junctional adhesion molecule-A (JAM-A) is an adhesion molecule present in the surface of several cell types, such as endothelial cells and leukocytes as well as Dendritic Cells (DC). Given the potential relevance of JAM-A in diverse pathological conditions such as inflammatory diseases and cancer, we investigated the role of JAM-A in $CD4^+$ T cell priming. We demonstrate that JAM-A is present in the immunological synapse formed between T cells and DC during priming. Furthermore, an antagonistic anti-JAM-A mAb could disrupt the interaction between $CD4^+$ T cell and DC. Antagonism of JAM-A also attenuated T cell activation and proliferation with a decrease in T-bet expression and increased IL-6 and IL-17 secretion. These findings demonstrate a functional role for JAM-A in interactions between $CD4^+$ T cells and DCs during T cell priming as a positive regulator of Th1 differentiation.

1. Introduction

The junctional adhesion molecule-A (JAM-A/JAM-1/F11R) is a transmembrane glycoprotein expressed by platelets, epithelial cells, endothelial cells and leukocytes, such as dendritic cells (DC) [1–3]. The JAM-A extracellular region is composed of a membrane-distal V-type Ig-like domain (D1) and a membrane-proximal C2-type Ig domain (D2). JAM-A can undergo homophilic binding (JAM-A-JAM-A) under recruitment of D1 domains, or heterophilic binding through D2 domain interactions (JAM-A-LFA-1) or through interactions yet to be identified (JAM-A-CD9, $\alpha_{IIb}\beta_3$ or JAM-B) [4]. JAM-A plays a central role in leukocyte migration by modulating leukocyte-endothelial cell interactions; however, JAM-A has not previously been implicated in interactions between immune cells.

During $CD4^+$ T cell priming, DCs induce T cell activation, clonal expansion and differentiation into effector and memory cells. Several molecules present on the surface of T cells and DCs contribute to T cell activation. Among these are adhesion molecules, that not only ensure a strong cell-cell interaction, but can also trigger pathways that will dictate the fate of a naïve $CD4^+$ T cell, such as the CD2-CD48 co-stimulatory pathway [5] and TIGIT-CD155 [6], a co-inhibitory pathway. Given the potential of these pathways for therapeutic intervention in diverse pathological conditions, there is increasing interest in discovering molecules that would promote or disrupt $CD4^+$ T cell-DC interaction.

Considering that JAM-A is expressed by DCs and in view of its potential relevance in several pathological conditions in which $CD4^+$ T cells play dominant roles (e. g. autoimmune diseases and cancer) [4], we hypothesized that JAM-A may play a role in $CD4^+$ T cell-DC interactions. We demonstrate that JAM-A is recruited into the immune synapse formed between $CD4^+$ T cells and DC. We also demonstrate that JAM-A has a functional role in this interaction, as antagonism affected T cell activation, proliferation, differentiation and cytokine secretion.

2. Materials and methods

2.1. Mice

Six to 12-week-old male and female C57BL/6 mice were purchased from Envigo (UK). OT-II transgenic mice containing T cells expressing T cell receptor (TCR) that recognizes the peptide 323–339 from chicken ovalbumin (OVA) bound to MHC-II molecule $I\text{-}A^b$ [7] were bred in-house (Central Research Facilities, University of Glasgow). All animals were specific pathogen free and maintained in accordance with local and home office regulations.

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2.2. DC-T cell co-culture and stimulation

Bone marrow-derived DCs (BMDC) were prepared by culture in GM-CSF, as previously described [8]. On day 6, BMDCs were stimulated with LPS (100 ng/mL) for 24 h. Mature day 7-BMDCs were then pulsed with previously standardized optimal (1 μg/mL) (OVA^{high}) or suboptimal (0.05 μg/mL) (OVA^{low}) concentrations of OVA peptide 323–339 (pOVA) (Sigma-Aldrich) and incubated with isolated CFSE-labelled $CD4^+$ T cells from OT-II mice (1:10 DC:T cell ratio) under treatments with anti-JAM-A mAb or Rat IgG2bk isotype control (20 μg/mL). Cells were harvested 24, 48 h or 72 h after the co-culture set-up for confocal microscopy (1×10^5 DCs, 1×10^6 T cells), brightfield microscopy (4×10^3 DCs, 4×10^4 T cells) or flow cytometry (2×10^5 DCs, 2×10^6 T cells) analysis, respectively. Co-culture supernatants were used for detection of cytokines by ELISA.

2.3. Imaging

For confocal images, DC-T cell co-cultures were fixed with 2% PFA for 20 min before being incubated with Fc block for 10 min and stained with fluorophore-conjugated antibodies anti-CD3-FITC (145–2C11), anti-CD11c-APC (N418), anti-MHC-II-eFluor 450 (HL3) and anti-JAM-A-PE (H202–106). Cells were added to an uncoated μ-slide I (Ibidi) to be analyzed with a ZEISS Cell Observer Spinning Disc Confocal Microscope (Carl Zeiss) using a 63x objective lens. For widefield images, cells were cultured in a 384-well black tissue culture-treated plate with flat clear bottom (Corning) and treated with anti-JAM-A mAb BV11 (Merck) or its isotype control (BioLegend) (20 μg/mL). Cell clusters were visualized using the brightfield of an EVOS FL Auto 2 microscope (Invitrogen). Acquisition of images was automated and tile scans from a 10x objective lens were merged using the microscope's built-in software. All images were analyzed using Fiji open-source software [9]. Confocal images of individual BMDC-T cell clusters had their synapse area and the rest of the cell membrane determined using Fiji's Polygon Selection tool. The fluorescence intensities of both areas were then measured, and the ratio was obtained by dividing the intensity from the synapse area by the rest of the cell membrane. Fiji's Ellipse tool was used to detect the limits of all visible cell clusters. The area of the regions of interest were then measured and exported to a spreadsheet. In Microsoft Excel, a formula was used to automate the application of a threshold to exclude cell aggregates smaller than 2000 μm^2, the minimum area used to define a cluster [10]. Selections of cell aggregates smaller than 2000 μm^2 were then excluded from sample images.

2.4. Flow cytometry

Single-cell suspensions were incubated with Fc Block for 10 min before adding fluorochrome-conjugated antibodies, as previously described [11]. Antibodies used (eBioscience, BD Biosciences or BioLegend) were anti-CD4-eFluor 450 (RM4–5) or -FITC (GK1.5), anti-CD44-PE or -APC (IM7), anti-RORγt-APC (AFKJS-9), anti-T-bet-PE-Cy7 (4B10), anti-IFN-γ-PE (XMG1.2), anti-JAM-A-PE (H202–106) and CD45.1-eFluor 780 (A20). Prior to intracellular cytokine staining, cells were stimulated with phorbol myristate acetate (PMA) (Sigma-Aldrich) (20 ng/mL) and ionomycin (Sigma-Aldrich) (1 μg/mL) for 4 h in the presence of brefeldin A (BD Biosciences). For intracellular cytokine and transcription factor staining, a Cytofix/Cytoperm (BD Biosciences) and a FoxP3 (eBioscience) kit was used, respectively, according to the manufacturers' instructions. Viability was assessed using a fixable cell viability dye (eBioscience) according to the manufacturer's instructions. Cells were run on an LSR Fortessa (BD Biosciences) or LSR-II (BD Biosciences) and analyzed using FlowJo software (TreeStar). For generating tSNE plots, events were first downsized, and biological replicates were concatenated.

2.5. ELISA

Cytokine concentrations were measured from the co-culture supernatants using ELISA MAX Standard Sets for Mouse IL-17 and IFN-γ (BioLegend) according to the manufacturer's instructions. The absorbance was measured using a Sunrise Absorbance Reader (Tecan) at 450 nm with subtraction of wavelengths from readings at 570 nm.

2.6. Statistics

Data is shown as mean ± SD. Specific tests and significance levels are indicated in the correspondent figure legends. Statistical analysis of results was performed using Prism version 6 (GraphPad Software, Inc, CA, USA).

3. Results

3.1. JAM-A is expressed by conventional DCs, but not naïve $CD4^+$ T cells

Prior to analyzing the effects of JAM-A blockade on the crosstalk between $CD4^+$ T cells and DCs, we first examined the expression of JAM-A on the surface of these cell types from C57BL/6 J mice (identified as shown in Supplementary Fig. 1). Spleens and lymph nodes (LN) were digested in Collagenase D for a higher yield of adherent cells. LN resident JAM-A^+ leukocytes ($CD45^+$) were mostly composed of $MHCII^+$, $CD11c^+$ and $CD11b^+$ cells, and only a small part of $B220^+$ cells expressed JAM-A, suggesting that the JAM-A-expressing population is mainly formed by DCs and partly by B cells (Fig. 1A). In addition, NK ($NK1.1^+$) and T cells ($CD3^+$) did not appear to express relevant levels of JAM-A. When analyzing specific immune cell subsets, $CD4^+$ T cells and plasmacytoid DCs (pDC) from both spleens and LNs expressed undetectable to low levels of JAM-A (Fig. 1B). On the other hand, conventional DCs (cDC) expressed JAM-A. Among these, LN cDC2 was the subset that expressed the highest levels, allowing the identification of a $JAM\text{-}A^{high}$ population. LN resident cDC1 and cDC2 seemed to express higher levels than splenic cDC1 and cDC2, respectively. As cDCs express JAM-A, but not naïve $CD4^+$ T cells, we analyzed the presence of a potential ligand for JAM-A on the surface of $CD4^+$ T cells. CD11a (LFA-1α) was highly expressed in naïve $CD4^+$ T cells from LNs and spleens (Fig. 1C).

3.2. JAM-A is present in the site of interaction during $CD4^+$ T cell priming

During T cell priming, both DCs and $CD4^+$ T cells coordinate translocation of molecules that are relevant for the cells' crosstalk to the immunological synapse (IS) [12]. To analyze if JAM-A is translocated to the IS during T cell priming, we first confirmed that JAM-A is also expressed on BMDCs (Fig. 2A). Surface expression of JAM-A by naïve (unstimulated) or activated (72h-antigen-stimulated) $CD4^+$ T cells was also analyzed (Fig. 2B). Then, OT-II $CD4^+$ T cells that were cultured with pOVA-pulsed-BMDCs for 24 h were analyzed with confocal microscopy for the expression of MHC-II, as a positive translocation control, CD11c, as a negative control, and JAM-A (Fig. 2C). The analysis of the MFI ratio between the inside and outside of the IS (IS/non-IS) (Fig. 2D) showed a higher concentration of MHC-II inside the IS in comparison with CD11c, but no difference between CD11c and JAM-A (Fig. 2E). Although JAM-A is not translocated to the IS, we show that this molecule is still present in the IS during T cell priming.

3.3. JAM-A blockade during priming disrupts $CD4^+$ T cell-DC cluster formation

During T cell activation, DCs and T cells interact to form clusters, and interventions that affect cluster formation may reflect subsequent changes in T cell activation and function [13]. Given that JAM-A is a molecule that promotes cell-cell adhesion, we analyzed the capacity of

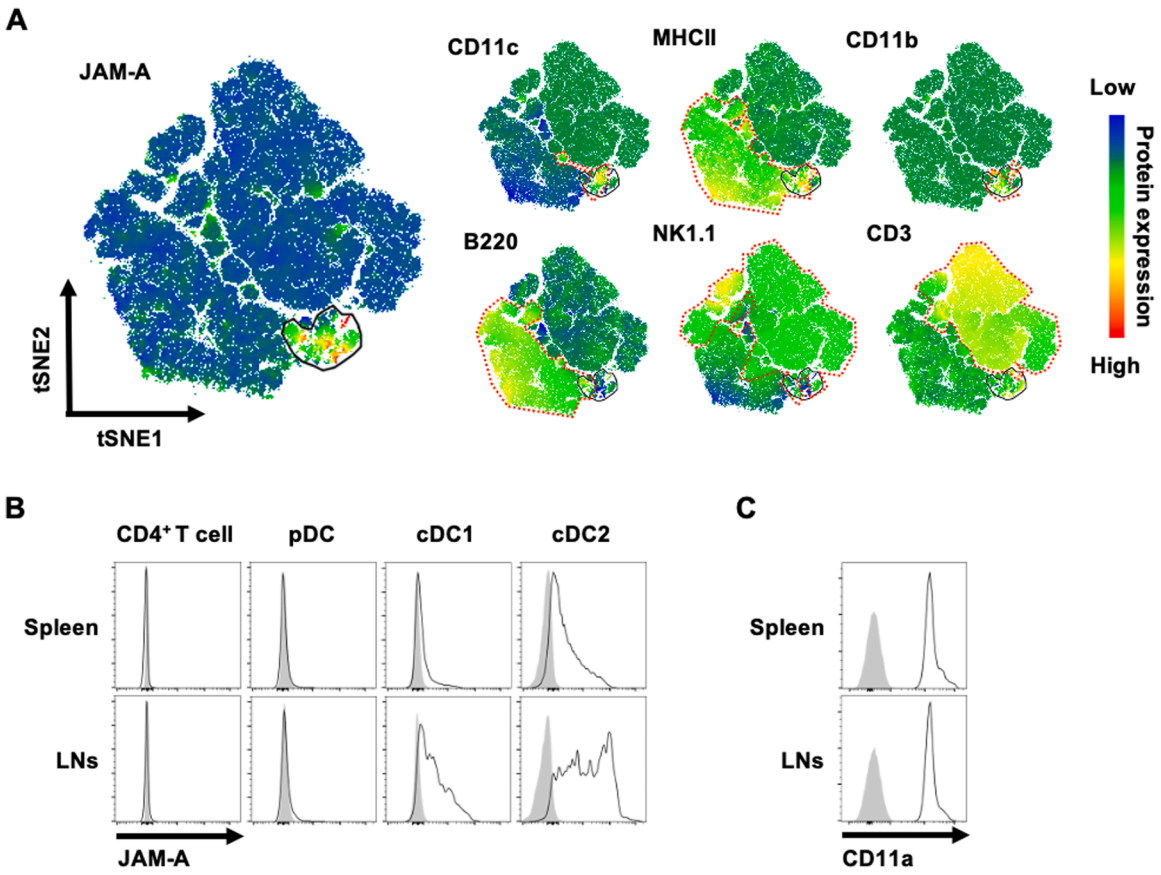

Fig. 1. JAM-A is expressed by murine conventional DCs. Immune cells from C57BL/6 mice were analyzed by flow cytometry for the expression of JAM-A. (A) tSNE analysis of immune cells ($CD45^+$) from LNs based on polychromatic flow cytometry data including JAM-A, CD11c, MHCII, CD11b, B220, NK1.1 and CD3 expression profiles, highlighting the population expressing the highest levels of JAM-A (solid black line) and the populations expressing respective lineage markers (dashed red line). (B) Representative histograms of JAM-A expression (solid line) or its isotype control (gray-shaded) on $CD4^+$ T cells ($B220^-$ $CD11c^-$ $CD3^+$ $CD4^+$), pDCs ($CD3^-$ $NK1.1^-$ $CD11c^{low}$ $PDCA1^+$ $B220^+$ $CD11b^-$), cDC1 ($CD3^-$ $NK1.1^-$ $CD11c^{high}$ $MHCII^+$ $CD8^+$ $CD11b^-$) and cDC2 ($CD3^-$ $CD11c^{high}$ $MHCII^+$ $CD8^-$ $CD11b^+$), from spleens and LNs. (C) Representative histograms of CD11a expression (solid line) or its isotype control (gray-shaded) on $CD4^+$ T cells from spleens and LNs. Data are representative of an experiment performed in biological triplicates.

cells to form clusters during T cell priming under JAM-A blockade. As the literature describes similar *in vitro* effects in BMDCs under treatment with anti-JAM-A mAb BV11 and in JAM-A-deficient BMDCs (*i.e.* increased random motility) [2], we used this mAb antibody to block JAM-A intercellular ligation in T cell-DC cultures. OT-II $CD4^+$ T cells were cultured with BMDCs pulsed with suboptimal or optimal concentrations of pOVA for 48 h under treatment with anti-JAM-A mAb or its isotype control and analyzed with widefield microscopy (Fig 3A). Under suboptimal conditions, groups treated with anti-JAM-A had lower numbers of clusters and total cluster area, but similar mean area of clusters in comparison with isotype-treated groups (Fig. 3B). Under optimal conditions, groups treated with anti-JAM-A had lower numbers of clusters but similar total cluster area, due to an increase in mean cluster size. The frequency distribution analysis showed that anti-JAM-A decreased the number of clusters smaller than 4000 μm^2 (Fig. 3C). These data demonstrate that JAM-A plays a functional role in the interaction between $CD4^+$ T cells and DC.

3.4. JAM-A blockade during priming attenuates $CD4^+$ T cell activation and proliferation

To analyze if blockade of JAM-A pathways during T cell priming *in vitro* could promote changes in activation and proliferation of $CD4^+$ T cells, BMDCs pulsed with optimal or suboptimal concentrations of pOVA were cultured with CFSE-labelled OT-II naïve $CD4^+$ T cells for 72 h and analyzed by flow cytometry using CFSE dilution and CD44 expression as measures of cell proliferation and activation respectively. Treatment with anti-JAM-A mAb attenuated CD4+ T cell activation (Fig. 4A) and proliferation (Fig. 4B) under both concentrations of antigens in comparison with the isotype control groups, suggesting that JAM-A plays a role in T cell priming.

3.5. JAM-A blockade during priming impacts $CD4^+$ T cell differentiation and cytokine secretion

T cell differentiation is key for successful T-cell responses, as different subsets secrete distinct cytokines that play specific roles during infection and in several pathological conditions. To analyze JAM-A blockade effects on $CD4^+$ T cell differentiation *in vitro*, we used our T cell-DC *in vitro* assay to examine expression of key transcription factors regulating subset differentiation, T-bet (Th1) (Fig. 5A) and RORγt (Th17) (Fig. 5B). Anti-JAM-A mAb treatment decreased the proportion of $CD4^+$ T-bet+ cells and the MFI of T-bet under both concentrations of antigen. A minor increase in the proportion of $CD4^+$ $ROR\gamma t^+$ cells was found under both activation conditions, while the suboptimal condition, but not optimal, also had an increase in RORγt MFI. To address the bias of decreased T cell activation/proliferation in the analysis of T cell differentiation, we also analyzed the expression of T-bet in antigen-experienced T cells (Supplementary Fig. 2). The proportion of $T\text{-}bet^+$ cells and T-bet MFI in $CD44^{high}$ cells were also significantly decreased, consistent with the data in Fig. 4. Cell supernatants were analyzed for the presence of key cytokines secreted by these specific T cell subsets, IL-

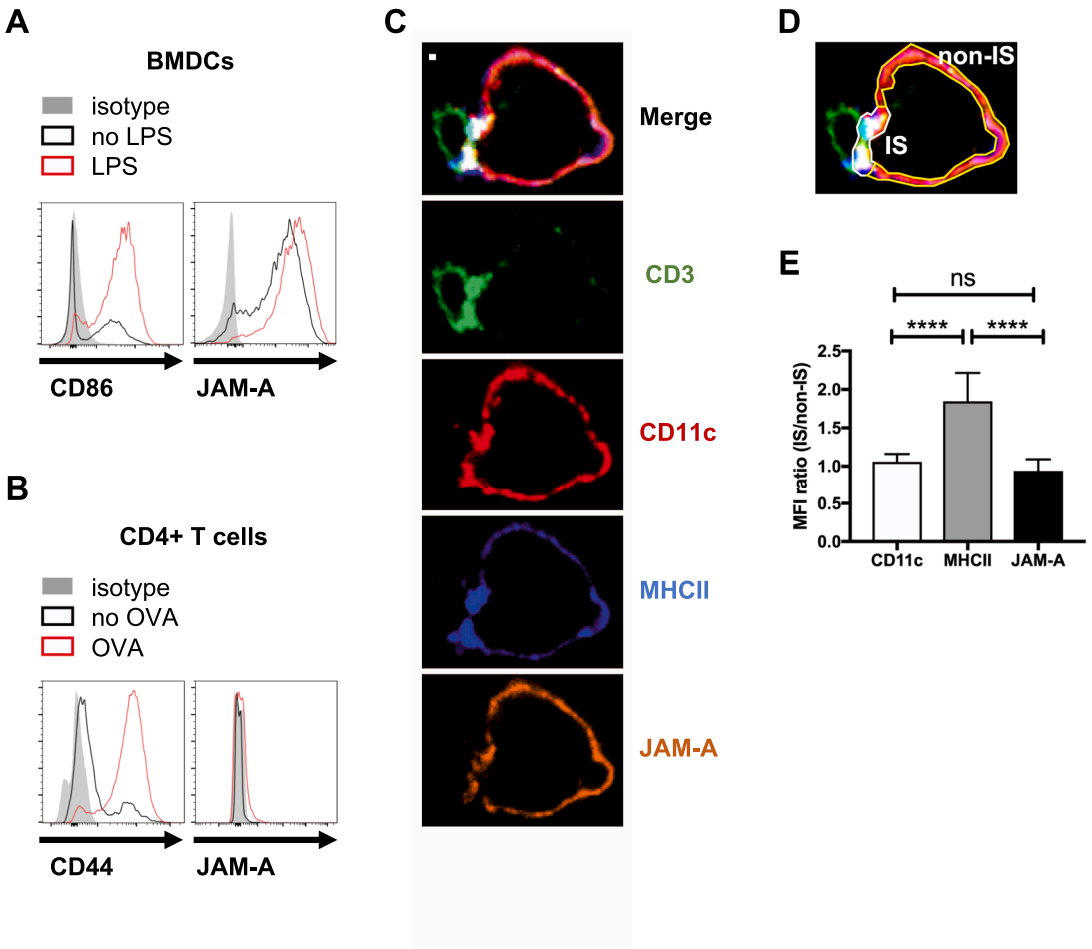

Fig. 2. JAM-A is present in the site of interaction during CD4$^+$ T cell priming. CD4$^+$ T cells from OT-II mice were cultured with pOVA-pulsed BMDCs for 24 h and analyzed by confocal microscopy for the expression of CD11c, MHC-II and JAM-A. (A) Representative histograms of CD86 or JAM-A expression on immature (no LPS) or mature (LPS) BMDCs or in mature BMDCs stained with respective isotype controls. (B) Representative histograms of CD44 or JAM-A expression on unstimulated/naïve (no OVA) or 72h-antigen-stimulated/activated (OVA) CD4$^+$ T cells or in activated CD4$^+$ T cells stained with respective isotype controls. (C) Representative confocal image stacks of CD4$^+$ T cell recorded in the green channel (CD3) in contact with a BMDC identified by its expression of CD11c recorded in the red channel and MHC-II recorded in the blue channel and JAM-A recorded in the yellow channel. The scale bar represents 5 μm. (D) Representative confocal image stack with identification of the immunological synapse (IS) and the BMDC membrane outside the immunological synapse (non-IS). (E) Quantification of the ratio between the BMDC's membrane MFI from the inside and the outside of the IS and comparison between JAM-A and MHC-II or CD11c. Results are expressed as mean ± SD of eight doublets with translocation of MHC-II to the IS. Statistical differences were determined using a one-way ANOVA. ns = non-significant, ****$p \leq 0.0001$.

17 (Th17) and IFN-γ (Th1), as well as IL-6 (Fig. 6). No difference in IFN-γ secretion was found between groups that were stimulated under the same concentration of antigen. Although no difference in IL-17 concentration was found between groups under suboptimal activation conditions, anti-JAM-A treatment increased the secretion of IL-17 under optimal concentrations of antigen. In addition, anti-JAM-A treatment increased the concentration of IL-6 found in the supernatant of cells primed under optimal activation conditions. IL-6 plays an important role in T cell survival and is known to inhibit Th1 differentiation [14]. These results indicate that JAM-A positively regulates Th1 differentiation.

Since anti-JAM-A treatment did not affect IFN-γ secretion but decreased T-bet expression, we analyzed if CD4$^+$ T cells produce more IFN-γ when treated with anti-JAM-A during T cell priming. After being stimulated with antigen for 72 h, T cells were stimulated with PMA/ionomycin and analyzed by flow cytometry for expression of intracellular IFN-γ (Supplementary Fig. 3). Treatment with anti-JAM-A did not affect the proportion of CD4$^+$ IFN-γ$^+$ cells (Supplementary Fig. 3) nor the IFN-γ MFI in CD4$^+$ cells or in populations of IFN-γ producers (not shown).

4. Discussion

We have shown that JAM-A is expressed by cDCs; in higher levels on LN cDC2, a subtype of DC that plays a dominant role in antigen presentation [15,16]. This higher expression of JAM-A on cDC2 populations in comparison with cDC1 also appears to happen in other lymphoid organs besides LNs and spleen, such as Peyer's patches [17]. Similarly, BMDCs express high levels of JAM-A [2,3]. While both naïve and memory CD4$^+$ T cells express undetectable to low levels of JAM-A, homophilic binding of JAM-A in the IS is therefore unlikely. This is supported by transcriptomics analysis that showed very low *f11r* expression in naïve and memory CD4$^+$ T cells from the spleen of C57BL/6 mice (≤ 2 expression value normalized by DESeq2 from ImmGen Open Source Ultra-low-input RNA-seq data) [18]. On the other hand, CD4$^+$ T cells express other potential ligands for JAM-A, such as LFA-1 [19]. We have demonstrated that JAM-A expressed by these cells is present in the IS during T cell priming. While translocation to the IS is well established for MHC-II/CD3 and has been observed in some T cell co-signaling molecules (*i.e.* LFA-1) [12], antigen-specific TCR-induced exclusion from the IS can occur with other molecules, such as CD43 [20,21]. Although JAM-A is not present in a higher proportion in the IS in comparison to the area outside the IS, our data demonstrates that DC

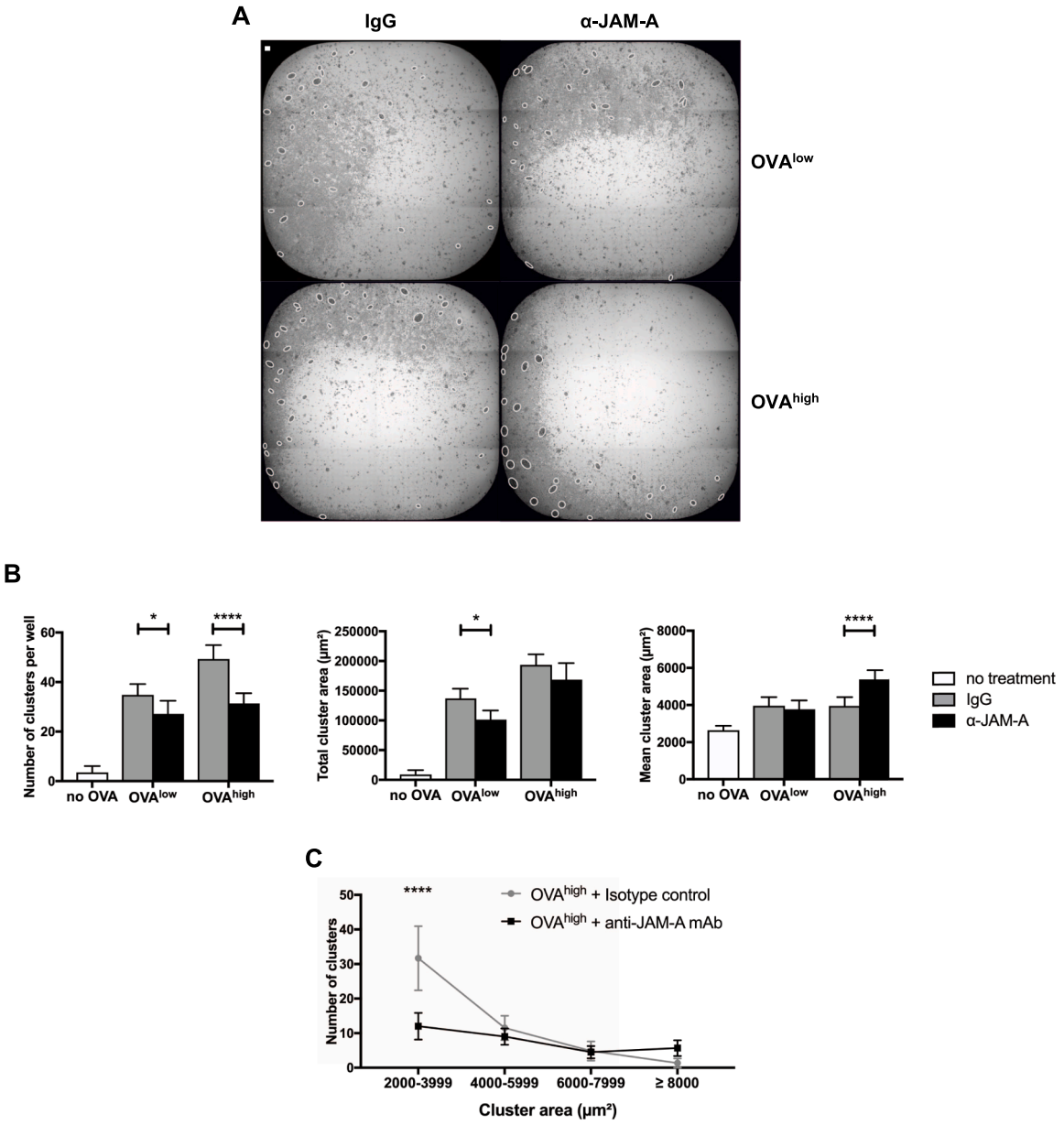

Fig. 3. JAM-A blockade disrupts CD4$^+$ T cell-DC cluster formation. CD4$^+$ T cells from OT-II mice were cultured with BMDCs pulsed with suboptimal (OVA^{low}) or optimal (OVA^{high}) concentrations of pOVA in the presence of anti-JAM-A mAb or its isotype control for 48 h and analyzed by widefield microscopy. (A) Representative images of whole wells containing cell cultures, showing elliptical selections of cell clusters in white. Scale bar represents 500 μm. (B) Quantification of number, mean area and total area of clusters. (C) Frequency distribution of clusters' area under optimal concentration of antigen and treated with anti-JAM-A mAb or its isotype control. Results are expressed as mean ± SD of replicate cultures. Statistical differences were determined using a two-way ANOVA or an unpaired Student's *t*-test. $^*p \leq 0.05$, $^{****} \leq 0.0001$.

JAM-A is present in the IS and could be available for binding with potential ligands from the T cell surface.

Functional intracellular signaling cascades following JAM-A ligation are reported in non-immune cells [22], whereas the literature lacks studies on these effects in leukocytes [4]. On the other hand, JAM-A ligation to its potential ligands, such as LFA-1, might trigger, for instance, LFA-1-mediated signaling cascades [23]. Based on the evidence of identifying JAM-A in the IS, and given that JAM-A is an adhesion molecule that provides firm attachment to cells during intercellular interactions [1,24], we hypothesized that JAM-A intercellular binding could have a role in T cell/DC interactions. We therefore analyzed the capacity of T cells and DCs to form clusters under treatment with anti-JAM-A mAb. Under suboptimal activation conditions, the treatment decreased the number of clusters and total cluster area. Under optimal conditions, JAM-A blockade also decreased the number of clusters, but because the area of the clusters was in general larger, the total area occupied by them was unchanged in comparison with the isotype-treated group. It is unlikely that the blocking antibody affects DC development, as blockade was performed after differentiation and maturation of the BMDCs. In addition, JAM-A-deficient BMDCs were previously shown to express similar levels of maturation markers (CD80 and CD86), surface molecules related to DC migration (CD11a, CD11b, CD11c, CD62L, JAM-B, and JAM-C) and antigen uptake capacity in comparison to WT BMDCs [2]. On the other hand, our data suggest that JAM-A blockade could be affecting DC adhesion. After priming T cells *in vivo*, DCs have to detach from T cells to present antigen to other cells or to play other immunomodulatory roles. Therefore, blockade of an adhesion molecule such as JAM-A could be modifying parameters as for

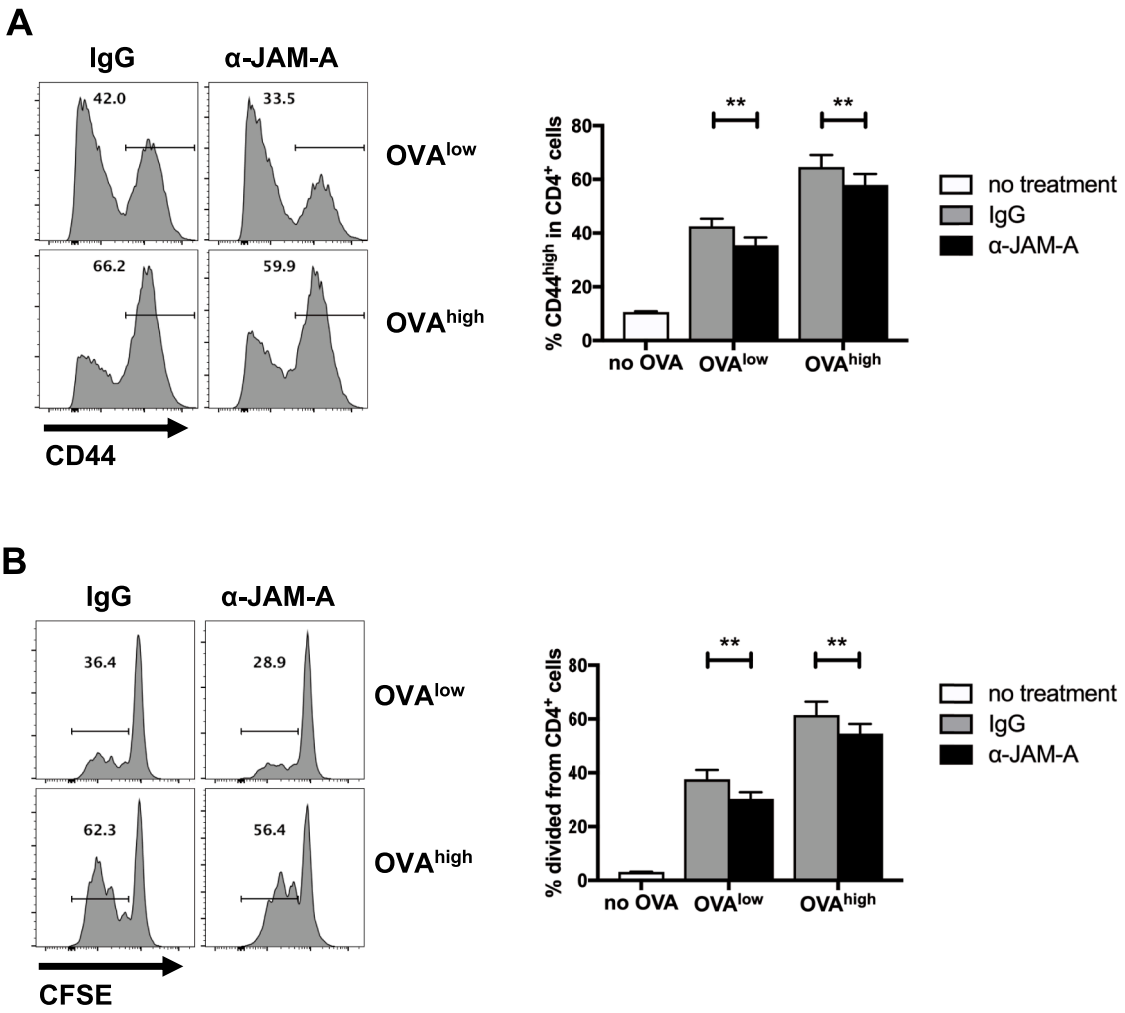

Fig. 4. JAM-A blockade attenuates CD4$^+$ T cell activation and proliferation. CFSE-labelled CD4$^+$ T cells from OT-II mice were cultured with BMDCs pulsed with suboptimal (OVA^{low}) or optimal (OVA^{high}) concentrations of pOVA in the presence of anti-JAM-A mAb or its isotype control for 72 h and analyzed by flow cytometry for CD44 expression and CFSE fluorescence intensity. (A) Representative histograms of CD44 expression on CD4$^+$ cells and quantification of the proportion of CD4$^+$ T cells that are CD44^{high}. (B) Representative histograms of CFSE fluorescence intensity on CD4$^+$ cells and quantification of the proportion of CD4$^+$ T cells that have divided based on the gating of CFSE fluorescence intensity. Results are expressed as mean ± SD of replicate cultures from three independent experiments. Statistical differences were determined using a two-way ANOVA. $^{**}p \leq 0.01$.

example, intensity, area or duration of these intercellular interactions, consequently impairing this process and possibly delaying it. The analysis of clusters from 48h-co-cultures not only reflect cell interaction but also take into account T cell proliferation, as these are already dividing by this time point. To analyze if this physical disruption reflects changes in T cell outcomes, we cultured antigen-pulsed BMDCs with OT-II naïve CD4$^+$ T cells for 72 h and examined their activation status and proliferation. Treatment with anti-JAM-A mAb attenuated CD4$^+$ T cell activation and proliferation both in suboptimal and in optimal concentrations of antigen. The impaired T cell proliferative response under JAM-A blockade might be a reflection of the diminished T cell activation status and seems to be reflected by the disruption in cluster formation. However, whereas the JAM-A ligand LFA-1 has been previously shown to be required for optimal T cell activation [25], we demonstrate that JAM-A only attenuates it, while it still provides support for T cell-DC interactions.

To investigate if JAM-A plays a role on CD4$^+$ T cell differentiation *in vitro*, we analyzed the expression of transcription factors that play a key role in this process, as well as cytokines commonly secreted by corresponding subsets. JAM-A blockade promoted a minor increase in the expression of RORγt, as well as a small increase in the secretion of IL-17 by CD4$^+$ T cells stimulated under optimal activation conditions. Interestingly, previous literature describes a 4.5-fold increase on absolute number of CD4$^+$ IL-17$^+$ T cells in the colonic lamina propria of JAM-A-deficient mice with C57BL/6 background in comparison with C57BL/6 mice [26]. Induction of CD4$^+$ T cell differentiation towards specific Th subsets is usually followed by inhibition of other subsets. T-bet, for example, is described to block transactivation *RORC*, responsible for encoding RORγt, and suppressing Th17 differentiation [27]. In addition, the genetic background of the JAM-A-deficient mice used in the previous study, as well as of the T cells used in our assays (C57BL/6), favors Th1 development [28], which facilitates Th1 polarization studies [29]. We demonstrate that JAM-A blockade decreased T-bet expression by antigen-primed CD4$^+$ T cells and increased IL-6 secretion in cultures from cells primed under optimal activation conditions. Although IL-6 can be also produced by DCs, previous reports in the literature showed that BMDCs from JAM-A$^{-/-}$ mice did not alter their secretion of IL-10, IL-12p70, TNFα and IL-6 up to 24 h after LPS stimulation, compared with wild-type (WT) mice [30]. IL-6 plays an important role in CD4$^+$ T cell differentiation, promoting IL-4-dependent Th2 polarization and inhibiting IL-4-independent Th1 differentiation [14]. Altogether, these results suggest that DC JAM-A is required for optimal Th1 differentiation.

T-bet protein expression does not necessarily correlate with IFN-γ

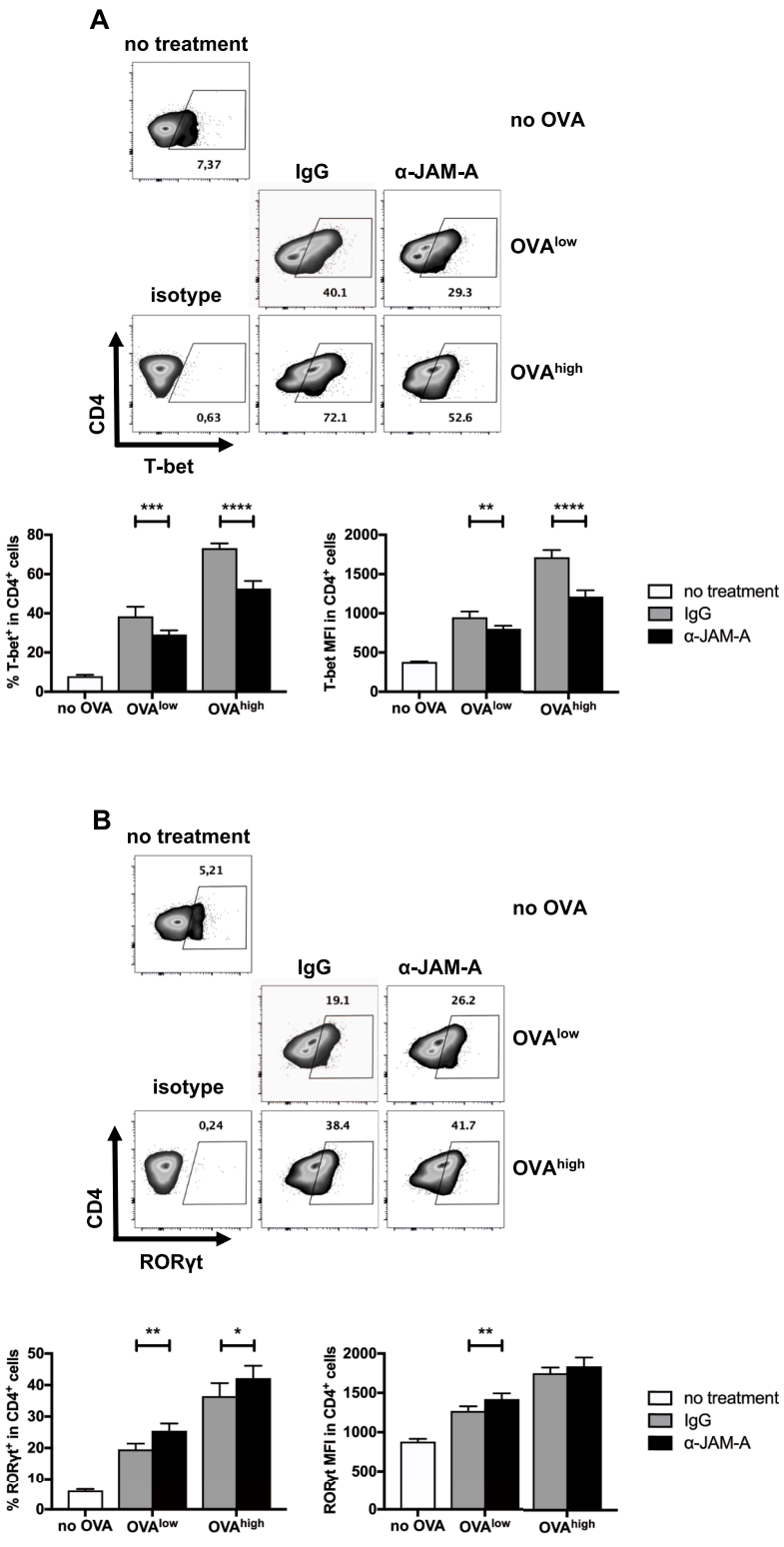

Fig. 5. JAM-A blockade impairs T-bet expression by CD4$^+$ T cells. CD4$^+$ T cells from OT-II mice were cultured with BMDCs pulsed with suboptimal (OVA^{low}) or optimal (OVA^{high}) concentrations of pOVA in the presence of anti-JAM-A mAb or its isotype control for 72 h and analyzed by flow cytometry for the expression of different transcription factors. (A) Representative dot plots of T-bet or (B) RORγt intracellular expression in CD4+ T cells, quantification of the proportion of cells expressing the transcription factors based on the fluorescence emitted by its matched isotype controls and quantification of their MFI. Results are expressed as mean ± SD of replicate cultures. Statistical differences were determined using a two-way ANOVA. *$p \leq 0.05$, **$p \leq 0.01$, *** ≤ 0.001, **** ≤ 0.0001.

production [31], while it is required for this cytokine production [29], making it an early marker for Th1 cells. The role of IFN-γ as a positive regulator of T-bet expression, in addition to the capacity for T-bet to activate IFN-γ expression results in a hypothetical positive feedback loop during Th1 cell differentiation [32]. Anti-JAM-A mAb treatment did not affect the secretion of IFN-γ measured in the culture supernatant nor the production of IFN-γ measured intracellularly in cells from our cultures. This is supported by a study that showed no differences in IFN-γ concentrations from the supernatant of OVA-primed OT-II CD4$^+$ T cells by BMDCs from JAM-A$^{-/-}$ mice in comparison with WT BMDCs [30]. T-bet is expressed in two consecutive waves: while its expression in the first 48 h following CD4$^+$ T cell primary activation requires IFN-γ signaling, later expression of T-bet is IFN-γ-independent [33]. Future investigation by possibly assessing T-bet expression in cells primed under blockade of

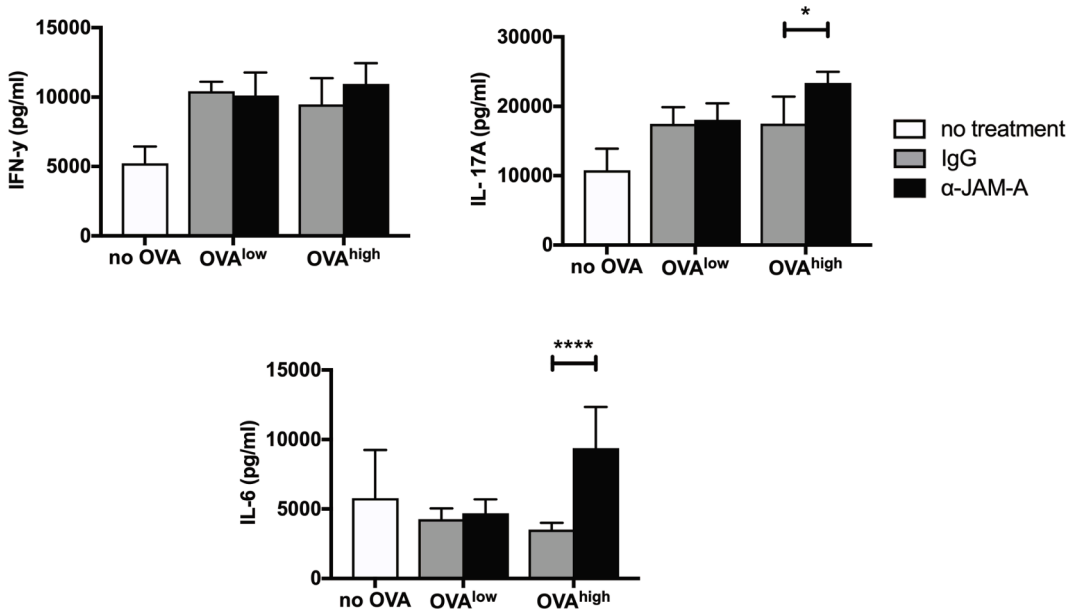

Fig. 6. JAM-A blockade does not affect IFN-γ secretion but increases IL-6 secretion. $CD4^+$ T cells from OT-II mice were cultured with BMDCs pulsed with suboptimal (OVA^{low}) or optimal (OVA^{high}) concentrations of pOVA in the presence of anti-JAM-A mAb or its isotype control for 72 h and analyzed by flow cytometry for the expression of different transcription factors. The graphs show concentrations of IFN-γ, IL-17 and IL-6 from culture supernatants measured by ELISA. Results are expressed as mean ± SD of replicate cultures from three independent experiments. Statistical differences were determined using a two-way ANOVA. $*p \leq 0.05$, **** ≤ 0.0001.

JAM-A pathways at earlier time points will determine the effects of JAM-A blockade in the distinct waves of T-bet expression and further our understanding of JAM-A role in Th1 differentiation and function.

Our data demonstrate that JAM-A expressed by DCs regulates $CD4^+$ T cell-DC interactions during T cell priming. JAM-A is present in the IS formed between T cells and DC during T cell priming. Blockade of JAM-A in vitro disrupted $CD4^+$ T cell-DC cell cluster formation in cells cultured for 48 h and attenuated T cell activation and proliferation in cells analyzed 72 h after priming. In addition, JAM-A blockade *in vitro* decreased the expression of T-bet, supporting a role for JAM-A as a regulator of Th1 differentiation. These findings highlight the relevance of JAM-A in regulating immune responses in the context of inflammation.

Authors' contribution

CSB: Conceptualization, Investigation, Formal analysis, Writing - Original Draft ; RAB: Conceptualization, Investigation, Writing - Review & Editing; HS: Resources, Writing - Review & Editing ; JMB and PG: Supervision, Funding acquisition, Writing - Review & Editing.

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Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

References

[1] I. Martìn-Padura, S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscella, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons, E. Dejana, Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration, J. Cell Biol. 142 (1998) 117–127, https://doi.org/10.1083/jcb.142.1.117.

[2] M.R. Cera, A. Del Prete, A. Vecchi, M. Corada, I. Martin-Padura, T. Motoike, P. Tonetti, G. Bazzoni, W. Vermi, F. Gentili, S. Bernasconi, T.N. Sato, A. Mantovani, E. Dejana, Increased DC trafficking to lymph nodes and contact hypersensitivity in junctional adhesion molecule-A–deficient mice, J. Clin. Invest. 114 (2004) 729–738, https://doi.org/10.1172/jci21231.

[3] N. Ogasawara, T. Kojima, M. Go, J. Fuchimoto, R. Kamekura, J. Koizumi, T. Ohkuni, T. Masaki, M. Murata, S. Tanaka, S. Ichimiya, T. Himi, N. Sawada, Induction of JAM-A during differentiation of human THP-1 dendritic cells, Biochem. Biophys. Res. Commun. 389 (2009) 543–549, https://doi.org/10.1016/j.bbrc.2009.09.024.

[4] C.S. Bonilha, R.A. Benson, J.M. Brewer, P. Garside, Targeting opposing immunological roles of the junctional adhesion molecule-A in autoimmunity and cancer, Front. Immunol. 11 (2020), 602094, https://doi.org/10.3389/fimmu.2020.602094.

[5] J.M. Green, V. Karpitskiy, S.L. Kimzey, A.S. Shaw, Coordinate regulation of T cell activation by CD2 and CD28, J. Immunol. 164 (2000) 3591–3595, https://doi.org/10.4049/jimmunol.164.7.3591.

[6] X. Yu, K. Harden, L.C. Gonzalez, M. Francesco, E. Chiang, B. Irving, I. Tom, S. Ivelja, C.J. Refino, H. Clark, D. Eaton, J.L. Grogan, The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells, Nat. Immunol. 10 (2009) 48–57, https://doi.org/10.1038/ni.1674.

[7] M.J. Barnden, J. Allison, W.R. Heath, F.R. Carbone, Defective TCR expression in transgenic mice constructed using cDNA- based α- and β-chain genes under the control of heterologous regulatory elements, Immunol. Cell Biol. 76 (1998) 34–40, https://doi.org/10.1046/j.1440-1711.1998.00709.x.

[8] A.M. Platt, R.A. Benson, R. Mcqueenie, J.P. Butcher, M. Braddock, J.M. Brewer, I. B. McInnes, P. Garside, The active metabolite of spleen tyrosine kinase inhibitor fostamatinib abrogates the CD4+ T cell-priming capacity of dendritic cells, Rheumatology 54 (2015) 169–177, https://doi.org/10.1093/rheumatology/keu273.

[9] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.-Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (2012) 676–682, https://doi.org/10.1038/nmeth.2019.

[10] N.A. Zumwalde, E. Domae, M.F. Mescher, Y. Shimizu, ICAM-1–dependent homotypic aggregates regulate CD8 T cell effector function and differentiation during T cell activation, J. Immunol. 191 (2013) 3681–3693, https://doi.org/10.4049/jimmunol.1201954.

[11] C.T. Prendergast, A. Patakas, S. Al-Khabouri, C.L. McIntyre, I.B. McInnes, J. M. Brewer, P. Garside, R.A. Benson, Visualising the interaction of CD4 T cells and DCs in the evolution of inflammatory arthritis, Ann. Rheum. Dis. 77 (2018) 579–588, https://doi.org/10.1136/annrheumdis-2017-212279.
[12] M.L. Dustin, The immunological synapse, Cancer Immunol. Res. 2 (2014) 1023–1033, https://doi.org/10.1158/2326-6066.CIR-14-0161.
[13] K. Inaba, R.M. Steinman, Accessory cell-T lymphocyte interactions. Antigen-dependent and -Iidependent clustering, J. Exp. Med. 163 (1986) 247–261.
[14] S. Diehl, J. Anguita, A. Hoffmeyer, T. Zapton, J.N. Ihle, E. Fikrig, M. Rincón, Inhibition of Th1 differentiation by IL-6 is mediated by SOCS1, Immunity 13 (2000) 805–815, https://doi.org/10.1016/S1074-7613(00)00078-9.
[15] S.C. Eisenbarth, Dendritic cell subsets in T cell programming: location dictates function, Nat. Rev. Immunol. 19 (2019) 89–103, https://doi.org/10.1038/s41577-018-0088-1.
[16] T. Worbs, S.I. Hammerschmidt, R. Förster, Dendritic cell migration in health and disease, Nat. Rev. Immunol. 17 (2017) 30–48, https://doi.org/10.1038/nri.2016.116.
[17] C.Da Silva, C. Wagner, J. Bonnardel, J.P. Gorvel, H. Lelouard, The Peyer's patch mononuclear phagocyte system at steady state and during infection, Front. Immunol. 8 (2017), https://doi.org/10.3389/fimmu.2017.01254.
[18] T.S.P. Heng, M.W. Painter, K. Elpek, V. Lukacs-Kornek, N. Mauermann, S.J. Turley, D. Koller, F.S. Kim, A.J. Wagers, N. Asinovski, S. Davis, M. Fassett, M. Feuerer, D.H. D. Gray, S. Haxhinasto, J.A. Hill, G. Hyatt, C. Laplace, K. Leatherbee, D. Mathis, C. Benoist, R. Jianu, D.H. Laidlaw, J.A. Best, J. Knell, A.W. Goldrath, J. Jarjoura, J. C. Sun, Y. Zhu, L.L. Lanier, A. Ergun, Z. Li, J.J. Collins, S.A. Shinton, R.R. Hardy, R. Friedline, K. Sylvia, J. Kang, The immunological genome project: networks of gene expression in immune cells, Nat. Immunol. 9 (2008) 1091–1094, https://doi.org/10.1038/ni1008-1091.
[19] I. Puigdomènech, M. Massanella, N. Izquierdo-Useros, R. Ruiz-Hernandez, M. Curriu, M. Bofill, J. Martinez-Picado, M. Juan, B. Clotet, J. Blanco, HIV transfer between CD4 T cells does not require LFA-1 binding to ICAM-1 and is governed by the interaction of HIV envelope glycoprotein with CD4, Retrovirology 5 (2008) 1–13, https://doi.org/10.1186/1742-4690-5-32.
[20] A.I. Sperling, J.R. Sedy, N. Manjunath, A. Kupfer, B. Ardman, J.K. Burkhardt, Cutting edge: TCR signaling induces selective exclusion of CD43 from the T cell-antigen-presenting cell contact site, J. Immunol. 161 (1998) 6459–6462.
[21] J. Delon, K. Kaibuchi, R.N. Germain, Exclusion of CD43 from the immunological synapce is mediated by phosphorylation-regulated relocation of the cytoskeletal adaptor Moesin, Immunity 15 (2001) 691–701, https://doi.org/10.1016/S1074-7613(01)00231-X.
[22] K. Ebnet, Junctional adhesion molecules (JAMs): cell adhesion receptors with pleiotropic functions in cell physiology and development, Physiol. Rev. 97 (2017) 1529–1554, https://doi.org/10.1152/physrev.00004.2017.
[23] B.L. Walling, M. Kim, LFA-1 in T cell migration and differentiation, Front. Immunol. 9 (2018), https://doi.org/10.3389/fimmu.2018.00952.
[24] A. Babinska, M.H. Kedees, H. Athar, T. Ahmed, O. Batuman, Y.H. Ehrlich, M. M. Hussain, E. Kornecki, F11-receptor (F11R/JAM) mediates platelet adhesion to endothelial cells: role in inflammatory thrombosis, Thromb. Haemost. 88 (2002) 843–850, https://doi.org/10.1055/s-0037-1613312.
[25] S. Kandula, C. Abraham, LFA-1 on CD4 + T cells is required for optimal antigen-dependent activation *in vivo*, J. Immunol. 173 (2004) 4443–4451, https://doi.org/10.4049/jimmunol.173.7.4443.
[26] M. Khounlotham, W. Kim, E. Peatman, P. Nava, O. Medina, C. Addis, S. Koch, B. Fournier, A. Nusrat, L. Timothy, C.A. Parkos, Compromised intestinal epithelial barrier induces adaptive immune compensation that protects from colitis, Immunity 37 (2012) 563–573, https://doi.org/10.1016/j.immuni.2012.06.017.
[27] V. Lazarevic, X. Chen, J.-.H. Shim, E.-.S. Hwang, E. Jang, A.N. Bolm, M. Oukka, J. K. Kuchroo, L.H. Glimcher, Transcription factor T-bet represses Th17 differentiation by preventing Runx1-mediated activation of the RORγt gene, Nat. Immunol. 12 (2011) 96–104, https://doi.org/10.1038/ni.1969.Transcription.
[28] J.D. Gorham, M.L. Güler, R.G. Steen, A.J. Mackey, M.J. Daly, K. Frederick, W. F. Dietrich, K.M. Murphy, Genetic mapping of a marine locus controlling development of T helper 1/T helper 2 type responses, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 12467–12472, https://doi.org/10.1073/pnas.93.22.12467.
[29] S.J. Szabo, S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, L.H. Glimcher, A novel transcription factor, T-bet, directs Th1 lineage commitment, Cell 100 (2000) 655–669, https://doi.org/10.1016/S0092-8674(00)80702-3.
[30] M. Murakami, C. Francavilla, I. Torselli, M. Corada, L. Maddaluno, A. Sica, G. Matteoli, I.D. Iliev, A. Mantovani, M. Rescigno, U. Cavallaro, E. Dejana, Inactivation of junctional adhesion molecule-A enhances antitumoral immune response by promoting dendritic cell and T lymphocyte infiltration, Cancer Res. 70 (2010) 1759–1765, https://doi.org/10.1158/0008-5472.CAN-09-1703.
[31] E. Ylikoski, R. Lund, M. Kyläniemi, S. Filén, M. Kilpeläinen, J. Savolainen, R. Lahesmaa, IL-12 up-regulates T-bet independently of IFN-γ in human CD4+ T cells, Eur. J. Immunol. 35 (2005) 3297–3306, https://doi.org/10.1002/eji.200526101.
[32] G. Lugo-Villarino, R. Maldonado-López, R. Possemato, C. Peñaranda, L. H. Glimcher, T-bet is required for optimal production of IFN-γ and antigen-specific T cell activation by dendritic cells, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 7749–7754, https://doi.org/10.1073/pnas.1332767100.
[33] E.G. Schulz, L. Mariani, A. Radbruch, T. Höfer, Sequential Polarization and Imprinting of Type 1 T Helper Lymphocytes by Interferon-γ and Interleukin-12, Immunity 30 (2009) 673–683, https://doi.org/10.1016/j.immuni.2009.03.013.