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Formation of Productive Immunological Synapses

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CXCL12 Regulates through JAK1 and JAK2 Formation of Productive Immunological Synapses

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The adaptive immune response requires interaction between T cells and APC to form a specialized structure termed the immune synapse (IS). Although the TCR is essential for IS organization, other factors such as chemokines participate in this process. In this study, we show that the chemokine CXCL12-mediated signaling contributes to correct IS organization and therefore influences T cell activation. CXCR4 downregulation or blockade on T cells caused defective actin polymerization at the contact site with APC, altered microtubule-organizing center polarization and the IS structure, and reduced T cell/APC contact duration. T cell activation was thus inhibited, as shown by reduced expression of CD25 and CD69 markers and of IL-2 mRNA levels. The results indicate that, through Gi and JAK1 and 2 kinases activation, CXCL12 signaling cooperates to build the IS and to maintain adhesive contacts between APC and T cells, required for continuous TCR signaling. The Journal of Immunology, 2015, 194: 5509–5519.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM-DC, bone marrow–derived DC; cSMAC, central supramolecular activation cluster; cSMAC, central supramolecular activation cluster; DC, dendritic cell; GAG, glycosaminoglycan; IS, immune synapse; MFI, mean fluorescence intensity; MTOC, microtubule-organizing center; pSMAC, peripheral SMAC; PTx, pertussis toxin; siRNA, small interfering RNA; siTyk2, siRNA pool for Tyk2; wt, wild-type.

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downregulation of JAK1/2 expression, OT-II CD4+ T cells showed defective IS formation when cocultured with OVA peptide–loaded bone marrow–derived DC (BM-DC). These T cells also showed reduced F-actin accumulation as well as defects in MTOC polarization to the contact site and in IS formation. Time-lapse videomicroscopy analysis showed significantly reduced mean contact duration, reflected as T cell activation defects. Our results indicate that whereas T cell–APC interactions are Ag dependent (18, 19), these Ag-specific interactions coincide with Ag-independent, chemokine-promoted adhesive contacts between these cells, which help to build a productive immune synapse.

Materials and Methods

Mice

Male and female 3- to 5-mo-old C57BL/6 mice were purchased from Harlan Laboratories. OVA-specific TCR-transgenic mice (OT-II) were donated by Dr. C. Ardavin (Centro Nacional de Biotecnologia, Madrid, Spain) and Cxcl12Gagtm mice by Dr. F. Arenzana (Institut Pasteur, Paris, France). The phenotype of OT-II mouse offspring was confirmed by flow cytometry using anti–Vα2 TCR Ab (BD Pharmingen). Cxcl12Gagtm/wt and Cxcl12Gagtm/mice were genotyped by PCR amplification on genomic DNA with specific primers (20). Mice were housed in pathogen-free conditions at the animal facility at the Centro Nacional de Biotecnologia/Consejo Superior de Investigaciones Científicas. All animal experiments were approved by the appropriate ethics committees and carried out according to national and European Union guidelines.

Abs and reagents

For Western blot assays, we used the following Abs: anti-JAK1, -Tyk2 (Santa Cruz Biotechnology), -JAK2 (Upstate Biotechnology), -Vav-1 mAb (Cell Signaling Technology), -ZAP-70 mAb (BD Pharmingen), -P–Y (Promega), -β-tubulin mAb, and -CXCL12 mAb (Sigma-Aldrich). HRP-labeled anti-mouse and -rabbit Ig (DakoCytomation) were used as secondary Abs. Cells purity was assessed by flow cytometry using anti-B220-FITC and –CD11c-PE (eBioscience). Surface marker expression was determined by flow cytometry using anti-CD69–PE and –CD25-PE (BioLegend) and –CXCR4-biotin mAb (BD Pharmingen). CXCL12 was purchased from PeproTech. CellTrace CFSE and seminaphtharhodafluor-1 (BioLegend), and –CD11c-PE (eBioscience). Surface marker expression was determined by flow cytometry using anti–B220-FITC and –CD11c-PE (eBioscience). Surface marker expression was determined by flow cytometry using anti–B220-FITC and –CD11c-PE (eBioscience). Surface marker expression was determined by flow cytometry using anti–B220-FITC and –CD11c-PE (eBioscience).

Cell culture and small interfering RNA nucleofection

CD4+ naive T cells were obtained from OT-II mouse spleen and lymph nodes and purified by negative selection with a mouse T cell negative isolation kit (MACS; Miltenyi Biotec); T cell purity was routinely evaluated by flow cytometry using anti–CD11c-FITC and anti–Vα2 TCR Ab (BD Pharmingen). Cxcl12 Gagtm/wt and Cxcl12 Gagtm/mice were genotyped by PCR amplification on genomic DNA with specific primers (20). Mice were housed in pathogen-free conditions at the animal facility at the Centro Nacional de Biotecnologia/Consejo Superior de Investigaciones Científicas. All animal experiments were approved by the appropriate ethics committees and carried out according to national and European Union guidelines.

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cocultured in RPMI 1640 medium with 10% FCS (24, 48, or 72 h), and proliferation was determined by flow cytometry using dye dilution evaluation in a Gallios flow cytometer (Beckman Coulter). The percentage of dividing cells was calculated using Flowcyto (Tree Star).

T cell activation was evaluated in parallel at indicated time points using anti-CD3-PE and anti-CD25-PE or anti-CD69-PE mAb in flow cytometry, as above.

Immunoprecipitation and Western blot analysis
CXCL12 (50 nM)-activated OT-II CD4+ T cells (5 × 10^6) were lysed in 200 µl detergent buffer (1% Nonidet P-40, 50 mM Tris-HCl [pH 8], 150 mM NaCl, 0.5 mM EDTA, 10 mM sodium pyrophosphate, 1 mM PMSF, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 10 mM sodium orthovanadate; 30 min, 4˚C) and immunoprecipitated using anti-PTyr Ab (1 µg/sample; Promega), and cell extracts were analyzed in Western blot, as described previously (25). When needed, densitometry analyses were performed using ImageJ.

Rho GTPase activation assays
Rho GTPase activity was determined in T cell lysates using RhoA activation assay kits (BK124; Cytoskeleton). T cells were stimulated with CXCL12 (50 nM; various times), and the reaction was terminated by adding ice-cold PBS and centrifugation (470 × g, 5 min, 4˚C); lysis and GTPase activity were measured following G-LISA kit protocols.

Quantitative real-time PCR
IL-2 mRNA levels were analyzed by quantitative real-time PCR with specific primers. Cells (3 × 10^5) were lysed, and RNA was extracted using the RNeasy micro kit (Qiagen) with DNase treatment to digest residual genomic DNA. Equal RNA amounts were reverse transcribed using a reverse transcription system (Promega). cDNA was determined by semiquantitative real-time PCR with the LightCycler480 SybrGreen I Master kit (Roche Diagnostics) using specific primer pairs (IL-2, 5'-CACCAGCTGCTACAGCGGA-3' and 5'-AAAATTTGAAGGTGAGCATCC-3'; β-actin, 5'-GGCACCAACCTTCTACAATG-3' and 5'-TGGATGGCTACGTACATGCCTG-3'). Samples were

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** CXCR4 blockade alters IS formation. (A) Conjugates of OT-II CD4+ T cells, alone or treated with AMD3100 or PTx and OVA peptide–pulsed BM-DC, were paraformaldehyde fixed. F-Actin polymerization was detected using phalloidin–Alexa 488 and quantified, and results were expressed as the percentage of T cells showing accumulated protein at the cell–cell contact site. *p ≤ 0.05, nonparametric test. (B) Conjugates as in (A) were stained with anti–γ-tubulin mAb followed by Cy3-goat anti-mouse IgG Ab. Quantitation of MTOC translocation measured as the distance (in micrometers) of the MTOC to the DC/T cell contact zone. Data shown are pooled from two independent experiments. *p ≤ 0.05, **p ≤ 0.01, nonparametric test. (C) Conjugates of siControl or siCXCR4 OT-II CD4+ T cells with OVA peptide–pulsed BM-DC were paraformaldehyde fixed, stained with anti–γ-tubulin, then quantified, and expressed as in (B). Data shown are pooled from three independent experiments. **p ≤ 0.01, nonparametric test. (D) BM-DC were incubated with OVA peptide and seeded onto fibronectin-coated coverslips. OT-II CD4+ T cells, alone or treated with AMD3100 or PTx, were added to the chambers. Contacts between T cells and DC were monitored by time-lapse videomicroscopy (40 min), and the contact times of individual DC–T cell pairs were measured. T cell–DC interactions were classified into three categories based on duration of the interaction: short (<5 min), medium (5–15 min), and long (>15 min). Data pooled from three independent experiments shown the percentage of each category (mean ± SD). *p ≤ 0.05, nonparametric test. (E) Cells as in (D) were monitored by time-lapse videomicroscopy and 200 BM-DC/OT-II CD4+ T conjugates for each condition monitored for 40 min. A representative experiment is shown of three performed. (F) Representative images of distinct time frames from the video recordings (Supplemental Videos 1–3). Arrows indicate T cell/BM-DC contacts. Scale bar, 5 µm.
analyzed in duplicate and normalized to β-actin using ABI 7900HT SDS 2.3 software.

**Immunofluorescence**

The conjugates (5 × 10^4 OVA peptide–loaded BM-DC/2.5 × 10^5 OT-II CD4^+ T cells) were plated on coverslips precoated with poly-l-lysine (20 μg/ml, overnight, 4°C; Sigma-Aldrich) and cultured (30 min, 37°C). Cells were washed in cold TBS and fixed with 2% paraformaldehyde (10 min, 37°C) and then permeabilized with 0.2% Triton X-100 in TBS (10 min, 37°C). After washing with TBS with 1% BSA, 0.1% goat serum, and 0.05% Tween 20 to avoid nonspecific binding, cells were stained with anti-Ptyr mAb (Millipore) or –γ-tubulin (Sigma-Aldrich) (60 min, 37°C), followed by Cy3-goat anti-mouse IgG (3.5 μg/ml, 45 min, 37°C; Jackson ImmunoResearch Laboratories). When necessary, cells were stained with Alexa 488–phalloidin (60 min, 37°C; Sigma-Aldrich). Coverslips were mounted on slides with Fluormount G medium (Southern Biotechnology Associates), and fluorescence was evaluated using the Olympus IX81 microscope with a PLAPON 40 × 03 objective (aperture 1:40) and FV10-ASW 1.6 software. When needed, MFI was determined using ImageJ64 software; staining intensity is shown as a percentage of total MFI versus MFI at the cell–cell contact site.

**Time-lapse videomicroscopy**

OVA peptide–loaded BM-DC (5 × 10^4 cells) cells were added to fibronectin-coated glass chambers (10 μg/ml, 4°C, overnight; Nunc Lab-Tek), placed on a 37°C stage of a laser-scanning fluorescence microscope (AMi 6000B; Leica), and allowed to settle (60 min). We then added 2.5 × 10^5 OT-II CD4^+ T cells. Images were acquired every 30 s before, during, and after T cell addition. Videos were analyzed with image analysis software (Adobe Photoshop CS5).

![FIGURE 2. CXCR4 inhibition triggers defective IS organization.](http://www.jimmunol.org/)

(A) Conjugates of OT-II CD4^+ T cells, alone or treated with AMD3100 or PTx, and OVA peptide–pulsed BM-DC, were paraformaldehyde fixed, permeabilized, stained with anti–CD4-FITC and -Ptyr mAb, followed by Cy3-goat anti-mouse IgG Ab and evaluated by flow cytometry. The figure shows Ptyr staining on CD4^+ T cells. A representative experiment is shown of four performed. (B) OT-II CD4^+ T cells were isolated and pretreated with AMD3100 or PTx or untreated. Cells were conjugated to anti-CD3–coated beads (CD3) or control isotype (Ctrl); where indicated, CXCL12 was added at the initiation of bead conjugation. Cells were allowed to conjugate (30 min), fixed, and stained for the indicated proteins. Left, A single slice from a confocal Z-stack. Right, Three-dimensional reconstruction of the IS-like area for talin and CXCR4. Histograms show protein distribution in the merge image of the IS-like reconstruction (red, talin; blue, CXCR4). (C) Ratio of MFI per area calculated at the pSMAC relative to the cSMAC for CXCR4 and talin at the IS-like area. MFI per area was calculated using a Matlab routine for image analysis. Data were analyzed with a one-way ANOVA with a Kruskal–Wallis test, followed by Dunn’s posttest. Means are also shown. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
Statistical analysis

Statistical analysis was performed with Prism software (GraphPad) using the non-parametric Student t test. Multiple comparisons were analyzed with a one-way ANOVA, followed by the Tukey posttest or Dunn’s posttest, where needed.

Results

CXCR4 inhibition impairs IS formation

CXCR4 is recruited to the pSMAC at the IS (6), and its ligand, CXCL12, is secreted by bone marrow stroma cells and endothelial cells (26), among other cell types (27, 28). Using FACS and immunofluorescence techniques, we detected CXCL12 bound to the surface of BM-DC but not on naive OT-II T cells (Supplemental Fig. 1A). To assess the effect of CXCR4/CXCL12 blockade on IS formation, naive OT-II CD4+ T cells were pretreated with AMD3100 (10 μM, 30 min, 37˚C) or PTx (0.2 μg/ml, 120 min, 37˚C) and used to form conjugates with OVA peptide–loaded BM-DC (ratio 5:1, 30 min, 37˚C). Phalloidin staining showed that whereas 87.5 ± 3.5% of untreated OT-II CD4+ T cells showed cytoskeletal rearrangement at the IS contact zone, F-actin accumulation was greatly reduced in T cells treated with AMD3100 (39.7 ± 2.5%) or PTx (20.3 ± 1.5%) (Fig. 1A). Because MTOC translocation toward the contact zone is a hallmark of IS formation (29), we tested for γ-tubulin in the BM-DC/T cell conjugates (Supplemental Fig. 1B). Quantitative analysis showed that AMD3100 or PTx treatment significantly reduced MTOC translocation (Fig. 1B).

To confirm these observations, we used nucleofected OT-II CD4+ T cells with an siRNA pool for CXCR4 (siCXCR4) or control (siControl). Flow cytometry analysis using anti-CXCR4 mAb showed a marked reduction in CXCR4 expression at the cell surface (~85%; Supplemental Fig. 1C). We thus formed conjugates using siCXCR4 and siControl cells and OVA peptide–loaded BM-DC and stained for γ-tubulin as above. The results indicated significant alteration in MTOC translocation to the contact zone in CXCR4-deficient cells (Fig. 1C).

We next determined the effect of AMD3100 and PTx treatments on BM-DC/T cell conjugation dynamics time-lapse videomicroscopy. The majority of untreated T cells formed medium-lived (5–15 min) and long-lived (>15 min) contacts, whereas drug treatments significantly increased the proportion of short-lived (<5 min) contacts (Fig. 1D). Mean contact duration of untreated OT-II CD4+ T cells with BM-DC (14 ± 1 min) was significantly longer than with AMD3100- or PTx-treated T cells (4 ± 0.3 and 4.5 ± 0.2 min, respectively; Fig. 1E, 1F, Supplemental Videos 1–3).

CXCR4 blockade promotes defective IS function

TCR engagement induces phosphorylation and relocation of specific signaling proteins that interact with the TCR-CD3 complex.

FIGURE 3. CXCR4 inhibition promotes defective T cell activation. (A) Conjugates of OT-II CD4+ T cells, alone or treated with AMD3100 or PTx and OVA peptide–pulsed BM-DC, were cultured (12 h, 37˚C), costained with anti–CD3-APC and with anti–CD25-PE or –CD69-PE mAb and evaluated by flow cytometry. Results are shown as a percentage of T cells (mean ± SD). Data are pooled from two independent experiments. *p < 0.05, **p < 0.01, nonparametric test. (B) OT-II CD4+ T cells were labeled with CellTrace violet and then untreated or treated with AMD3100 or PTx before forming conjugates with OVA peptide–pulsed BM-DC. Conjugates were cultured (48 h, 37˚C) and T cell proliferation determined following dye dilution by flow cytometry (left). Quantitative analysis of T cell proliferation using data from three independent experiments (right). Results are shown as a percentage of T cells (mean ± SD). Data are pooled from two independent experiments. *p ≤ 0.05, **p ≤ 0.01, nonparametric test. (C) Conjugates of OT-II CD4+ T cells with OVA peptide–pulsed BM-DC obtained from wt, Cxcl12Gagtm/wt, or Cxcl12Gagtm/Gagtm mice were fixed, permeabilized, and stained with anti–γ-tubulin mAb, followed by Cy3-goat anti-mouse IgG Ab. The figure shows the quantitation of MTOC translocation measured as in Fig. 1B. Data are pooled from two independent experiments. *p ≤ 0.05, nonparametric test. (D) BM-DC as in (C) were incubated with OVA peptide and seeded onto fibronectin-coated coverslips. OT-II CD4+ T cells were added to chambers. Contacts between T cells and DC were monitored by time-lapse videomicroscopy (40 min), and contact times of individual DC–T cell pairs were measured. DC–T cell interactions were classified as in Fig. 1D. Data pooled from two independent experiments shown as the percentage for each category (mean ± SD).
To assess the role of chemokines in DC-triggered signaling events during IS formation, we analyzed phosphotyrosine staining as a T cell activation marker. Untreated or AMD3100- or PTx-treated naive OT-II CD4+ T cells were incubated with OVA peptide–loaded BM-DC for various times; cells were fixed, permeabilized, stained for PTyr, and evaluated by FACS. Treatment with either drug clearly reduced anti-PTyr staining in CD4+ T cells (Fig. 2A).

We then analyzed in detail the contribution of CXCL12 to the IS structure. OT-II CD4+ T cells were conjugated with anti–CD3-coated latex beads (31), alone or in the presence of soluble CXCL12 (50 nM), and cells were stained for specific IS markers (CD3ζ for cSMAC; talin and CXCR4 for pSMAC) (Fig. 2B, 2C, Supplemental Fig. 1D). We found partial colocalization of talin and CXCR4 in a ring-shaped structure, as shown in three-dimensional reconstitution (Fig. 2B); this colocalization was reinforced by CXCL12 addition, as indicated by the coincidence of red and blue lines in the histograms in Fig. 2B. Talin and CXCR4 redistribution at the pSMAC was clearly increased in the presence of CXCL12 (pSMAC/cSMAC ratio > 1; graphs in Fig. 2C). CXCL12 treatment similarly increased the anti–CD3-triggered IS localization ratio of CD3ζ (Supplemental Fig. 1D). These CXCL12-mediated effects were reduced by treatment of OT-II CD4+ T cells with AMD3100 or PTx (Fig. 2B, 2C, Supplemental Fig. 1D).

We compared the in vitro activation of drug-treated CD4+ cells in the OVA peptide–loaded BM-DC system. Flow cytometry analysis showed that AMD3100 and PTx treatment reduced the stimulatory effect on both CD25 and CD69 expression markers (Fig. 3A, Supplemental Fig. 2A). We therefore used untreated and AMD3100- or PTx-treated live cell tracker–labeled OT-II CD4+ T cells to form conjugates with OVA peptide–loaded BM-DC and determined their ability to proliferate. Flow cytometry detection of cell tracker dilution after 48-h coculture showed lower proliferation of drug-treated T cells (Fig. 3B). These results indicate that CXCL12 binding to CXCR4 in T cells is an important element for IS formation and that blockade of this interaction alters conjugate formation and stability and thus T cell activation.

To determine whether the CXCL12 effect requires chemokine binding to glycosaminoglycans (GAG) on the BM-DC surface, we...

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**FIGURE 4.** CXCL12-triggered Vav-1 and RhoA activation is involved in IS formation. (A) OT-II CD4+ T cells, alone or treated with AMD3100 or PTx, were activated with CXCL12 at indicated times, immunoprecipitated with anti-PTyr Ab, and cell extracts analyzed by Western blot using anti-Zap70 (top) or anti–Vav-1 (middle) Ab. As protein loading control, the membrane was reblotted with anti-PTyr Ab (bottom). OT-II CD4+ T cell lysate was used as positive control. Data are from one representative experiment of three performed. (B) OT-II CD4+ T cells as in (A) were stimulated with CXCL12 (50 nM, 30 s), lysed, and evaluated for RhoA GTPase activity using G-LISA. Data show mean ± SD (n = 3 experiments). *p ≤ 0.05, **p ≤ 0.01, nonparametric test. (C) Conjugates of OT-II CD4+ T cells, untreated or treated with ruxolitinib or tofacitinib, with OVA peptide–pulsed BM-DC, were fixed, permeabilized, and stained with anti–γ-tubulin mAb, followed by Cy3-goat anti-mouse IgG Ab. The figure shows quantitation of MTOC translocation measured as in Fig. 1B. Data are pooled from two independent experiments. ***p ≤ 0.01, nonparametric test. (D) siControl and siJAK1,2 OT-II CD4+ T cells were activated with CXCL12 and immunoprecipitated with anti-PTyr Ab, and cell extracts were analyzed by Western blot using anti–Vav-1 Ab. As protein loading control, the membrane was reblotted with anti-PTyr Ab. OT-II CD4+ T cell lysate was used as positive control. Data shown are from one representative experiment of two performed. (E) siControl and siJAK1,2 OT-II CD4+ T cells were stimulated with CXCL12 (50 nM, 30 s), lysed, and evaluated for RhoA GTPase as in (B). Data show mean ± SD (n = 4 experiments). *p ≤ 0.05, nonparametric test.
prepared BM-DC from Cxcl12mt/mtm mice, which bear a mutated CXCL12 gene (Cxcl12mtmt) (Supplemental Fig. 2B) that precludes CXCL12 interaction with heparin sulfate structures but does not affect CXCR4-dependent CXCL12 cell signaling (20). Conjugates of OVA peptide–loaded Cxcl12mt/mtm BM-DC with OT-II CD4+ T cells (ratio 1:5, 30 min, 37˚C) showed a small but significant reduction in MTOC translocation toward the contact zone that was not detected when we used BM-DC from Cxcl12mt/mtm mice (Fig. 3C). In addition, time-lapse videomicroscopy analysis of conjugation dynamics showed similar levels of short-, medium-, and long-lived contacts compared with those for wild-type (wt) BM-DC (Fig. 3D). These results concur with those using anti-CD3–coated beads and soluble CXCL12, suggesting that in vitro CXCL12–mediated IS stabilization does not require chemokine binding to GAG.

CXCL12-mediated effects on IS formation require Rho/Vav activation

Integrin activation is an essential step in IS stabilization (32); it is triggered by TCR engagement and chemokine binding to their receptors (33, 34), which require ZAP-70 phosphorylation and Rho small GTPase activation (16, 35). In static adhesion assays, CXCL12 stimulation induced OT-II CD4+ T cell adhesion to ICAM-1 and potentiated that triggered by anti-CD3 stimulation (Supplemental Fig. 2C). To evaluate the CXCL12-mediated signaling cascades involved, we used OT-II CD4+ T cells, untreated or treated with AMD3100 (10 μM, 30 min, 37˚C) or PTx (0.2 μg/ml, 120 min, 37˚C). CXCL12 promoted rapid ZAP-70 phosphorylation and RhoA activation; AMD3100 treatment notably decreased both processes, which were reduced to a lesser extent by PTx (Fig. 4A, top, 4B). The nucleotide exchange factor (GEF) Vav is also.

FIGURE 5. CXCL12-triggered JAK activation is involved in IS formation. (A) Conjugates of siControl or siJAK1,2 OT-II CD4+ T cells with OVA peptide–pulsed BM-DC were paraformaldehyde fixed. F-Actin polymerization was detected using phalloidin–Alexa 488 and expressed as in Fig. 1A. **p ≤ 0.01, nonparametric test. (B) Conjugates as in (A) were stained with anti–γ-tubulin mAb, followed by Cy3-goat anti-mouse IgG Ab. Arrows indicate MTOC position. Scale bar, 5 μm. The figure also shows quantitation of MTOC translocation measured as the distance (in micrometers) of the MTOC to the DC/T cell contact zone. Data shown are pooled from two independent experiments. **p ≤ 0.01, nonparametric test. (C) BM-DC were incubated with OVA peptide and seeded onto fibronectin-coated coverslips. siControl or siJAK1,2 OT-II CD4+ T cells were added to chambers. Contacts between T cells and DC were monitored by time-lapse videomicroscopy (40 min), and contact times of individual DC–T cell pairs were measured and classified as in Fig. 1D. Data pooled from three independent experiments show the percentage for each category (mean ± SD). *p ≤ 0.05, **p ≤ 0.01, nonparametric test. (D) Cells as in (C) were monitored by time-lapse videomicroscopy, and 200 BM-DC/OT-II CD4+ T conjugates for each condition were monitored for 40 min. A representative experiment is shown of three performed. (E) Representative images of distinct time frames from the video recordings (Supplemental Videos 4, 5). Arrows indicate T cell/BM-DC contacts. Scale bar, 5 μm. (F) Conjugates of siControl or siTyk2 OT-II CD4+ T cells with OVA peptide–pulsed BM-DC were paraformaldehyde fixed, stained with anti–γ-tubulin, quantified, and expressed as in (B). Data shown are pooled from two independent experiments.
associated with CXCL12-mediated LFA-1 activation (16). We found that, in CXCL12-activated CD4⁺ T cells, Vav-1 was phosphorylated. This activation was abolished by AMD3100 treatment and was not blocked in PTx-treated cells (Fig. 4A, middle). A recent report suggests G protein–independent CXCL12 mediated Vav-1 phosphorylation, which links Rho small GTPase and integrin activation in a pathway that entails rapid CXCL12-mediated JAK activation (17). We thus tested the effect of specific JAK inhibitors on IS formation, including ruxolitinib, which blocks JAK1 and JAK2 activity (36) and tofacitinib, which inhibits JAK3 (37). We calibrated drug concentrations in a CXCL12-triggered OT-II CD4⁺ T cell migration assay. Whereas ruxolitinib treatment (1 μM, 60 min, 37°C) reduced cell migration by 50%, tofacitinib had no effect at any concentration tested (0.3, 1.0, or 3.0 μM, 60 min, 37°C) (Supplemental Fig. 3A). We thus formed conjugates using untreated, ruxolitinib (1 μM)-, or tofacitinib (1 μM)-treated OT-II CD4⁺ T cells, and OVA peptide–loaded BM-DC. γ-Tubulin staining showed significantly less MTOC translocation to the contact zone only in ruxolitinib-treated cells (Fig. 4C).

To rule out nonspecific effects because of the use of chemical inhibitors, we performed the remaining experiments using nucleofected OT-II CD4⁺ T cells with siRNA pools for JAK1/2 (siJAK1,2) or controls (siControl) (10). Western blot analysis confirmed a sharp reduction in protein levels for both kinases (>60%; Supplemental Fig. 3B) in siJAK1,2 T cells. We also observed abrogation of CXCL12-mediated Vav-1 phosphorylation (Fig. 4D) and reduced Rho activation (Fig. 4E) in the siJAK1,2 T cells.

**Naïve OT-II CD4⁺ T cells with reduced JAK levels show altered IS**

To confirm the role of JAK in chemokine-mediated stabilization of the IS, we formed conjugates using siJAK1,2 OT-II CD4⁺ T cells and OVA peptide–loaded BM-DC (30 min, 5:1 ratio, 37°C). Whereas phalloidin staining showed F-actin accumulation cytoskeletal rearrangement at the contact zone in 94.7 ± 2.5% of siControl OT-II CD4⁺ cells, F-actin accumulation was reduced in JAK-deficient CD4⁺ T cells (41.3 ± 3.0%) (Fig. 5A). γ-Tubulin staining indicated a significant alteration in MTOC translocation to the contact zone in JAK-deficient cells (Fig. 5B). Finally, time-lapse videomicroscopy analysis (Supplemental Videos 4, 5) showed that JAK1/2 deficiency significantly increased the percentage of

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**FIGURE 6.** JAK contributes to IS architecture. (A) siControl and siJAK1/2 OT-II CD4⁺ T cells were conjugated with anti-CD3–coated beads (CD3) or control isotype (control); CXCL12 was added where indicated and processed as in Fig. 3B. Right, A single slice from a confocal Z-stack showing the focus plane of the IS-like area for talin, CXCR4, and merge (talin/CXCR4). Left, Three-dimensional reconstruction of the IS-like area for talin and CXCR4. Histograms show protein distribution in the merged image of the IS-like reconstruction (red, talin; blue, CXCR4). (B) Ratio of MFI per area calculated at the pSMAC relative to the cSMAC for CXCR4 and talin at the IS-like area. MFI per area was calculated using Matlab for image analysis. Data were analyzed with a one-way ANOVA with a Kruskal–Wallis test, followed by Dunn’s posttest. Means are also shown. ***p ≤ 0.001.
short-lived cell contacts (<5 min; Fig. 5C) and reduced mean contact duration (4 ± 0.3 min; Fig. 5D, 5E). To control biological specificity, we repeated the experiments using OT-II CD4+ T cells nucleofected with a siRNA pool for Tyk2 (siTyk2). These cells showed 90% Tyk2 protein in Western blot with a specific Ab (Supplemental Fig. 3C). siTyk2 cells showed no alteration in MTOC translocation to the contact zone when conjugated with OVA peptide–loaded BM-DC (Fig. 5F).

Detailed analysis of IS structure on siControl and JAK-deficient T cells showed that JAK1 and JAK2 are essential for correct IS formation. siJAK1,2 and siControl OT-II CD4+ T cells were allowed to conjugate with stimulating anti-CD3–coated beads plus soluble CXCL12 (50 nM) (Fig. 6). In the three-dimensional reconstruction of the IS-like area, we observed an increase in talin and CXCR4 colocalization at the pSMAC after CXCL12 addition to the anti-CD3–coated beads (histograms in Fig. 6A). Lack of siJAK1,2 nonetheless prevented correct localization of these molecules; their redistribution at the pSMAC was abolished by JAK1/2 knockdown in the presence of CXCL12 plus anti-CD3–mediated protein kinase C0 redistribution to the contact site was abolished in siJAK1,2 T cells (Supplemental Fig. 4A). JAK1,2-deficient naive OT-II CD4+ T cells conjugated with BM-DC showed a marked reduction in intracellular PTyr staining when evaluated by flow cytometry and by immunofluorescence (Fig. 7A, 7B). These defects led to reduced T cell activation, as shown by lower IL-2 mRNA (Fig. 7C) as well as CD69 levels (Fig. 7D, Supplemental Fig. 4B). The results indicate that, through both Gi and JAK signaling pathways, CXCL12 regulates integrin activation and IS stabilization.

Discussion

For T cell activation, cell membrane and cytoplasmic proteins must be rearranged to form the immunological synapse (32). IS formation depends on TCR-mediated signals that, in concert with costimulatory signals, cause the T cell cytoskeleton, membrane receptors, and certain signaling effectors to polarize toward the APC/T cell interface. Synapse-associated signaling also leads to spatial segregation into organized clusters of the TCR, CD28, LFA-1, and other surface molecules such as chemokine receptors (2). Some reports regard TCR recognition of specific Ags as the starting point of organelle redistribution and IS formation (38–40), and contact duration is thought to control efficient T cell activation in vivo (41). Although guidance of leukocyte trafficking is the principal role of

FIGURE 7. JAK blockade alters IS function. (A) Conjugates of siControl or siJAK1,2 OT-II CD4+ T cells and OVA peptide–pulsed BM-DC were paraformaldehyde fixed, permeabilized, stained with anti–CD4-FITC and -PTyr mAb, followed by Cy3-goat anti-mouse IgG Ab, and evaluated by flow cytometry. A representative experiment is shown of three performed. (B) Conjugates as in (A) were stained with anti-PTyr, followed by Cy3-goat anti-mouse IgG Ab and evaluated by immunofluorescence (right). Scale bar, 5 μm. Quantitation of MFI of PTyr staining using the ImageJ (left). Data shown are pooled from two independent experiments. **p ≤ 0.01, nonparametric test. (C) Quantitative real-time PCR was used to determine relative levels of IL-2 mRNA in siControl or siJAK1,2 OT-II CD4+ T and OVA peptide–pulsed BM-DC conjugates (24 h, 37°C). *p ≤ 0.05, nonparametric test. (D) Conjugates of siControl or siJAK1,2 OT-II CD4+ T cells and OVA peptide–pulsed BM-DC were cultured (12 h, 37°C), costained with anti–CD3-APC mAb and with anti–CD69-PE mAb (right), and evaluated by flow cytometry. Results are shown as the percentage of CD69+ cells relative to total T cells (mean ± SD). Data are pooled from two independent experiments. *p ≤ 0.05, nonparametric test.

FIGURE 8. CXCL12 signaling contributes to maintenance of IS structure. TCR engagement initiates IS formation and triggers protein segregation and integrin-mediated cell adhesion. CXCL12 is retained at the APC surface by binding to GAG; this increases the local chemokine concentration at the APC:T cell contact site. CXCL12 binding to CXCR4 on T cells thus reinforces integrin activation and their binding to adhesion molecules expressed on the APC surface. The molecular mechanism includes CXCL12-mediated Gi protein and JAK activation. Both signaling pathways converge in RhoA activation, which is via Vav-1 in the case of the JAK. The scheme also indicates the inhibitors used in this study and their targets as well as a reference to cSMAC and pSMAC regions.
CXCL12 regulates productive immunological synapses

Chemokines, they also influence T cell recognition of Ag. Chemokines direct T cell tethering to APC, which facilitates scanning and formation of stable Ag-dependent interactions (34). In addition, chemokines promote cell polarization, which increases sensitivity to Ag (42) and costimulate T cell function (5, 43).

In this study, we show that chemokines also influence IS formation and T cell activation. Membrane CXCR4 concentrates in the IS periphery; drebrin has a function in this relocation and in F-actin reorganization (6). When we blocked CXCL12 binding to CXCR4 or its signaling pathways, IS structure and duration as well as T cell activation were altered. We detected CXCL12 at the BM-DC surface but not on OT-II CD4+ T cells. Our in vitro experiments using anti-C3D–coated beads nonetheless indicated that soluble CXCL12 also influences IS formation. The anti-C3D–coated bead model is often used for T cell activation analysis as it provides the geometry of the cell–cell contact with the APC and, because of the control of the stimuli used, permits dissection of the molecular pathways involved (44, 45). Although these experiments were performed in the absence of antigen ligands, we detected CXCR4 and talin in the pSMAC. Anti-C3D Ab promotes cytoskeletal organization through Vav-1 and talin complexes (46), and Vav-1 is also involved in CXCL12-mediated talin interaction with β1 integrins (16). Talin binding to the cytosplasmic tails of the integrins is thus one of the inside–outside mechanisms that activates integrins and enables their interaction with adhesion molecules.

IS were also formed between BM-DC prepared from CXCL12Gagtm mice and T cells. These mice have a mutant CXCL12 that cannot interact with GAG (20), impeding its retraction at the cell surface. In this mouse model, we nonetheless detected a significant but small defect in MT0C translocation to the contact site, suggesting that soluble CXCL12 is less efficient than GAG-bound CXCL12. Although chemokines act as soluble mediators in vitro, in vivo they are thought to bind GAG at the cell surface (47, 48). These results coincide with data that suggest various roles for chemokines in T cell recognition of APC. Whereas some dominant chemokines such as CCL19 or CCL21 increase T cell motility on DC monolayers (49) and mediate direction and tethering (34), others like CXCL12 might stabilize cell–cell contacts. IS stability is impaired in T cells bearing warts, hypogammaglobulinemia, infections, and myelokathexis syndrome–mutant CXCR4 because of defects in receptor recruitment to the cell–cell contact area (50).

We found defective MT0C translocation in CXCR4-deficient T cells or those with altered CXCR4 function. The MT0C translocation mechanism underlies IS formation, sustains the T cell signaling (29), and increases mitochondrial localization at the cell–cell contact site (51). MT0C positioning during T cell activation facilitates correct effector functions such as cytokine secretion (52) and target cell killing (53, 54). T cell treatment with the inhibitors AMD3100 or PTX or with reduced JAK1 and JAK2 expression levels significantly diminished conjugate duration; these defects might explain the IL-2 production defect as well as the reduced T cell activation and proliferation in these conditions. In T cells, CXCR4 interaction with the TCR–CD3 complex induces ZAP-70 and SLP-76 recruitment, ERK pathway activation, and cytokine synthesis (55–57). Blockade of CXCL12-mediated signaling in T cells during IS formation correlated with less cell activation, as indicated by reduced levels of IL-2 mRNA and of activation markers.

LFA-1 is reported to enhance T cell activation (58–60). Cell adhesion amplifies TCR signaling via several mechanisms, including prolonged inositol phospholipid hydrolysis (61), replenishment of intracellular Ca2+ stores (61), and cAMP increase (62). Chemokine-mediated integrin activation can thus contribute to IS stabilization. We nonetheless observed that PTx treatment did not completely block CXCL12-mediated RhoA activation and Vav-1 phosphorylation in a key signaling pathway involved in integrin activation by chemokines (16), which suggests G protein–dependent and –independent mechanisms. An earlier study indicated that although chemokines increase basal T cell adhesion to DC, the effect on Ag-pulsed T cells is not PTx sensitive (63). In primary T lymphocytes, we previously showed that chemokines control LFA-1– and VLA-4–mediated cell adhesion, both key processes in T cell homing (10) that involve chemokine-mediated JAK activation. A recent report implicates JAKs in CXCL12-mediated integrin activation and lymphocyte trafficking, via RhoA, Rac1, and Rap1 (17). Our findings now link JAK activation to CXCL12-mediated IS stabilization through the Vav-1/RhoA pathway. We cannot entirely rule out a JAK3 function because our experiments to evaluate the role of this kinase were only performed using a chemical inhibitor, tofacitinib. Our results nonetheless show that JAK1 and JAK2 inhibition or knockdown in T cells altered IS formation, reduced conjugate number, contact duration, and T cell activation.

Adhesion molecules, chemokines, and costimulatory and co-inhibitory receptors all take part in the complex process of tuning TCR signaling and T cell activation thresholds. Whereas initiation of integrin-mediated cell adhesion is Ag dependent, our data suggest that through Gi and JAK signaling, CXCL12 helps to maintain the integrin activation at the pSMAC needed for continuous TCR signaling (Fig. 8), a finding with broad implications for cell contact–dependent communication in adaptive immunity.

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Disclosures
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Supplemental Fig 1. (A) Analysis of CXCL12 binding to the surface of BM-DC and OT-II T cells by FACS. (B) Conjugates of OT-II CD4+ T cells, alone or treated with AMD3100 or PTx and OVA-peptide pulsed BM-DC were paraformaldehyde-fixed. They were then stained with anti-γ-tubulin mAb followed by Cy3-goat anti-mouse IgG Ab. Arrows indicate MTOC position. (Scale bar: 5 μm). (C) FACS analysis of CXCR4 expression on the surface of OT-II T cells transfected with siControl and siCXCR4. (D) CXCR4 inhibition alter IS organization. OT-II CD4+ T cells pretreated with AMD3100 or PTx or untreated were conjugated to anti-CD3-coated beads (α-CD3) or control isotype (Ctrl); where indicated, CXCL12 was added at the initiation of bead conjugation. Graphs show amounts of talin, CXCR4 and CD3ζ at the IS-like area. The ratio was generated using the ImageJ Synapse Measures plug-in. Means are shown. **p ≤0.01; ****p ≤0.0001. Data were analyzed with a one-way ANOVA followed by Tukey’s post-test.
Supplemental Fig. 2. (A) CXCR4 inhibition promotes defective T cell activation. Conjugates of OT-II CD4+ T cells, alone or treated with AMD3100 or PTx, and OVA-peptide loaded BM-DC were co-cultured (12 h, 37°C), co-stained with anti-CD3-APC and anti-CD25-PE or -CD69-PE mAb and evaluated by flow cytometry. Results show a representative experiment of two performed with the percentage of each cell population indicated. (B) Characterization of Cxcl12<sup>Gag<sub-tm</sub>/Gag<sub-tm</sub></sup> mice. PCR amplification with 5'-TGCCAGCATAAAGACACTCCG-3' and 5'-CAGCCCTTGAA GTAATCACTGC-3' primers on genomic DNA from wild type (WT), Cxcl12<sup>Gag<sub-tm</sub>/wt</sup> (heterozygous) and CXCL12<sup>Gag<sub-tm</sub>/Gag<sub-tm</sub></sup> (homozygous) mice. The figure shows the size of CXCL12<sub>wt</sub> and mutant CXCL12 products. (C) CXCL12 increases anti-CD3-mediated OT-II CD4+ T cell adhesion. Static adhesion assay using OT-II CD4+ T cells on microplates coated with recombinant ICAM-1-Fc alone or with anti-CD3 mAb, CXCL12 or anti-CD3 mAb+CXCL12. Results are expressed as a percentage of cell input. Data are shown as mean ± SD of three independent experiments (duplicates). **p ≤ 0.01; nonparametric test.
Supplemental Fig 3. (A). CXCL12-triggered T cell migration is reduced by JAK1 and JAK2 inhibition. Naïve OT-II CD4+ T cells alone or treated with ruxolitinib (0.1, 0.3 or 1 μM, 60 min, 37°C) or with tofacitinib (0.3, 1 or 3 μM, 60 min, 37°C) were allowed to migrate on ICAM-1-coated chemotaxis chambers toward 50 nM CXCL12. The migration index is shown as mean ± SD of four independent experiments (duplicates). *p ≤0.05, one-way ANOVA. (B) siJAK1,2 cells show reduced JAK1 and JAK2 protein expression. siControl and siJAK1,2 cells were lysed and analyzed by Western blot with anti-JAK1 and -JAK2 antibodies; anti-β-tubulin mAb was used as loading control. Data are from one representative experiment of three performed. (C) siTyk2 cells show reduced Tyk2 protein expression as detected by Western blot with specific antibody; anti-β-tubulin mAb was used as loading control. Data are from one representative experiment of two performed.
**Supplemental Fig 4.** (A) JAK downregulation alter IS organization. siControl and siJAK1,2 OT-II CD4+ T cells were conjugated to anti-CD3-coated beads (α-CD3) or control isotype (Ctrl); where indicated, CXCL12 was added at the initiation of bead conjugation. Graphs show amounts of talin, CXCR4 and PKCθ at the IS-like area in siControl and siJAK1,2 OT-II CD4+ T cells. The ratio was generated using the ImageJ Synapse Measures plug-in. Means are shown. *p ≤0.05; ****p ≤0.0001; ns non-significant. Data were analyzed with a one-way ANOVA followed by Tukey’s post-test. (B) JAK1,2 downregulation promotes defective T cell activation. Conjugates of siControl and siJAK1,2 OT-II CD4+ T cells and OVA-peptide loaded BM-DC were co-cultured (12 h, 37°C), co-stained with anti-CD3-APC and -CD69-PE mAb and evaluated by flow cytometry. Results show a representative experiment of two performed with the percentage of each cell population indicated.
Video legends

Video 1. Live imaging of BM-DC/OT-II CD4⁺ T cell contacts. OVA-peptide-loaded BM-DC were added to fibronectin-coated glass chambers, placed on a 37°C stage of a laser-scanning fluorescence microscope (AMI 6000B, Leica) and allowed to settle before addition of OT-II CD4⁺ T cells. Frames were taken at 30 sec interval for 40 min. Arrows indicate T cell/BM-DC contacts.

Video 2. Live imaging of BM-DC and AMD3100-treated OT-II CD4⁺ T cell conjugates. OVA-peptide-loaded BM-DC were added to fibronectin-coated glass chambers, placed on a 37°C stage of a laser-scanning fluorescence microscope and allowed to settle before addition of AMD3100-treated OT-II CD4⁺ T cells. Frames were taken at 30 sec interval for 40 min. Arrows indicate T cell/BM-DC contacts.

Video 3. Live imaging of BM-DC and PTx-treated OT-II CD4⁺ T cell conjugates. OVA-peptide-loaded BM-DC were added to fibronectin-coated glass chambers, placed on a 37°C stage of a laser-scanning fluorescence microscope and allowed to settle before addition of PTx-treated OT-II CD4⁺ T cells. Frames were taken at 30 sec interval for 40 min. Arrows indicate T cell/BM-DC contacts.

Video 4. Live imaging of BM-DC and siControl-nucleofected OT-II CD4⁺ T cell conjugates. OVA-peptide-loaded BM-DC were added to fibronectin-coated glass chambers, placed on a 37°C stage of a laser-scanning fluorescence microscope and allowed to settle before addition of siControl-nucleofected OT-II CD4⁺ T cells. Frames were taken at 30 sec interval for 40 min. Arrows indicate T cell/BM-DC contacts.

Video 5. Live imaging of BM-DC and siJAK1,2-nucleofected OT-II CD4⁺ T cell conjugates. OVA-peptide-loaded BM-DC were added to fibronectin-coated glass chambers, placed on a 37°C stage of a laser-scanning fluorescence microscope and allowed to settle
before addition of siJAK1,2-nucleofected OT-II CD4\(^+\) T cells. Frames were taken at 30 sec interval for 40 min. Arrows indicate T cell/BM-DC contacts.