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*J Immunol* 2015; 194:5509-5519; Prepublished online 27 April 2015;
doi: 10.4049/jimmunol.1402419
http://www.jimmunol.org/content/194/11/5509

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2015/04/25/jimmunol.1402419.DCSupplemental

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CXCL12 Regulates through JAK1 and JAK2 Formation of Productive Immunological Synapses

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The Journal of Immunology, 2015, 194: 5509–5519.

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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Received for publication September 23, 2014. Accepted for publication March 23, 2015.

This work was supported in part by the Ministerio de Ciencia e Innovación (Grant SAF 2011-23730), the Redes Temáticas de Investigación en Red Program (Grants RD12/0009/009, RD12/0009/001, and R12/0009/006; Red de Inflamación y Enfermedades Reumáticas and Grant RD12/0004/005; Red de Investigación Cardiovascular), and the Gobierno Regional de Madrid (Grant S2010/BMD-2350; Rheumatoid Arthritis: Physiopathology Mechanisms). Optical microscopy was conducted at the Unidad de Microscopia Confocal (Centro Nacional de Biotecnología/Consejo Superior de Investigaciones Científicas) at the Hospital de la Princesa and at the Unidad de microscopia y dinámica de la imagen (Madrid, Spain), which is funded by the Ministerio de Ciencia e Innovación de España and by the Pro Centro Nacional de Investigaciones Cardiovasculares Foundation.

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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; 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-month-old C57BL/6 mice were purchased from Harlan Laboratories. OVA-specific TCR-transgenic mice (OT-II) were donated by Dr. C. Aradiv (Centro Nacional de Biotecnologia, Madrid, Spain) and Cxcl12Gagtm/Gagtm mice by Dr. F. Arenazana (Institut Pasteur, Paris, France). The phenotype of OT-II mouse offspring was confirmed by flow cytometry using anti-CD8 TCR Ab (BD Pharmingen). Cxcl12Gagtm/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 Biotecnología/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), -Vav1 mAb (Cell Signaling Technology), -ZAP-70 mAb (BD Pharmingen), -β-tubulin mAb, and -CXCL12 mAb (Sigma-Aldrich). HRP-labeled anti-mouse and -rabbit Ig (DakoCytomation) were used as secondary Abs. Cell purity was assessed by flow cytometry using anti-B220-FITC and -Gr1-PE/Cy7 (Beckman Coulter), -CD4-PE/Cy5 and -CD3-APC mAb (BioLegend), and -CD11c-PE (eBioscience). Surface marker expression was determined by flow cytometry using anti-CD69-PE and -CD25-PE mAb (BioLegend) and anti-CXCR4-biotin mAb (BD Pharmingen). CXCL12 was purchased from PeproTech and, seminaphtharhodafluor-1 (BioLegend) and anti–CXCR4-biotin mAb (BD Pharmingen). CXCL12 was stained with at least 50 random T cells scored as conjugates only if they contacted no more than one CMAC-labeled BM-DC and no other T cell contacted the scored T cell. F-Actin accumulation at the IS was detected by staining with phalloidin–Alexa 488, and images were analyzed with ImageJ software (National Institutes of Health, Bethesda, MD). Using region of interest of the same area for all measurements, we quantified the signal generated by 1) the BM-DC/T cell contact area (IS), 2) the area of BM-DC membrane not in contact with the T cell (B), 3) an area of the T cell membrane not in contact with the BM-DC (T), and 4) the background. We subtracted the background signal from other measurements, then measured F-actin accumulation at the IS relative to the rest of the T cell using the formula (IS – B)/T. Results were expressed as the percentage of T cells with accumulated F-actin at the contact with DC. Statistical significance was calculated using a two-tailed Student t test.

Analysis of IS architecture

Cell conjugates were observed under a confocal laser scanning unit (TCS SP5; Leica) attached to an inverted epifluorescence microscope (DMi6000; Leica) fitted with an HCX PL APO 40/1.40-0.6 oil objective. Images were acquired and processed with confocal software (LCS; Leica) or WClF ImageJ (http://rsbweb.nih.gov/ij/) to obtain a three-dimensional analysis and maximal projections of T cell–bead contact. Figures were composed with Adobe Photoshop.

Analysis of CD3ζ, protein kinase Cθ, talin, and CXCR4 accumulation

OT-II CD4+ T cells were nucleofected as above with siRNA pools for JAK1+JAK2 (siJAK1,2) or siRNA pool control (Dharmacon). These cells, alone or treated with AMD3100 (10 μM, 30 min, 37°C) or PTX (0.2 μM, 120 min, 37°C), were conjugated to anti-CD3–coated latex beads or to isotype control–coated latex beads (Sigma-Aldrich) for 20 min, alone or with CXCL12 (50 nM). Cells were then fixed and stained with appropriate Abs, and the IS was analyzed by confocal imaging. We assumed that background signal is homogeneous in the latex beads. To quantify the fluorescence distribution ratio, individual IS were processed with the “Synapse Measures” plugin for ImageJ (http://rsbweb.nih.gov/ij/) (for a detailed description of Synapse Measures, including the algorithms used, see Ref. 24). To analyze talin and CXCR4 distribution at the IS-like complex, three-dimensional reconstructions of the area were generated with the Leica software. Mean fluorescence intensity (MFI) per area was calculated with a Matlab routine for pSMAC and cSMAC regions from three independent experiments (at least 24 cells were measured per experimental condition). Mean values from the pSMAC/cSMAC ratio of MFI per area were calculated.

T cell activation and proliferation

OT-II CD4+ T cells were labeled with CellTrace Violet (0.5 μM, 30 min, 37°C; Molecular Probes) and added to plates containing OVA peptide–loaded BM-DC (3 × 10^5 DC/6 × 10^4 T cells). Cells were
coclutured 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 Flowjo (Tree Star).

**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 PMSE, 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 REasy 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′-CACCTCAAGCTCTACAGGGCA-3′ and 5′-AAATTTGAGGTGAGCATCC-3′; β-actin, 5′-GCGACAGCCTTCTACAATG-3′ and 5′-TGGATGGCTACGTACATGCTG-3′). Samples were

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**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 ≤ 0.05, **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 showed 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 × 10⁴ OVA peptide–loaded BM-DC/2.5 × 10⁵ 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 α-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 Fluoromount G medium (Southern Biotechnology Associates), and fluorescence was evaluated using the Olympus IX81 microscope with a PLAPON 40 x 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 × 10⁴ cells) cells were added to fibronectin-coated glass chambers (10 μg/ml, 4°C, overnight; Nunc LabTek), placed on a 37°C stage of a laser-scanning fluorescence microscope (AM1 6000B; Leica), and allowed to settle (60 min). We then added 2.5 × 10⁵ 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).
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...
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).

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...
prepared BM-DC from Cxcl12_\text{Gagtm/Gagtm} mice, which bear a mutated CXCL12 gene (\text{Cxcl12Gagtm}) (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 Cxcl12_\text{Gagtm/Gagtm} 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 Cxcl12_\text{Gagtm/wt} 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 \mu M, 30 min, 37˚C) or PTx (0.2 \mu g/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

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**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 \leq 0.01, nonparametric test. (B) Conjugates as in (A) were stained with anti–\gamma-tubulin mAb, followed by Cy3–goat anti-mouse IgG Ab. Arrows indicate MTOC position. Scale bar, 5 \mu m. 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 \leq 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 \pm SD). *p \leq 0.05, **p \leq 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 \mu m. (F) Conjugates of siControl or siTyk2 OT-II CD4^+ T cells with OVA peptide–pulsed BM-DC were paraformaldehyde fixed, stained with anti–\gamma-tubulin, quantified, and expressed as in (B). Data shown are pooled from two independent experiments.
associated with CXCL12-mediated LFA-1 activation. 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 (1.0 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.

**Naive 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

![FIGURE 6](http://www.jimmunol.org/)

**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 (Fig. 6A). CXCL12 plus anti-CD3–mediated protein kinase Cζ 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

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**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. Quantification of MFI of P Flag 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 also influences 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-CD3–coated beads nonetheless indicated that soluble CXCL12 also influences IS formation. The anti-CD3–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 integrin ligands, we detected CXCR4 and talin in the pSMAC. Anti-CD3 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 (46). Talin binding to the cytoplasmic 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 CXCL12−/− mice and T cells. These mice have a mutant CXCL12 that cannot interact with GAG (20), impeding its retention at the cell surface. In this mouse model, we nonetheless detected a significant but small defect in MTOC 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 MTOC translocation in CXCR4-deficient T cells or those with altered CXCR4 function. The MTOC translocation mechanism underlies IS formation, sustains T cell signaling (29), and increases mitochondrial localization at the cell–cell contact site (51). MTOC 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 coinhibitory 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), with a finding with broad implications for cell contact–dependent communication in adaptive immunity.

Acknowledgments

We thank Drs. P. Cohen for the JAK inhibitors and F. Arenzana-Seisdedos for CXCL12Gagmi mice and C. Bastos for secretarial and C. Mark for editorial assistance.

Disclosures

The authors have no financial conflicts of interest.

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