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Upholding the T cell immune-regulatory function of CD31 inhibits the formation of T/B immunological synapses *in vitro* and attenuates the development of experimental autoimmune arthritis *in vivo*

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ABSTRACT

CD31, a trans-homophilic inhibitory receptor expressed on both T- and B-lymphocytes, drives the mutual detachment of interacting leukocytes. Intriguingly, T cell CD31 molecules relocate to the immunological synapse (IS), where the T and B cells establish a stable interaction.

Here, we show that intact CD31 molecules, which are able to drive an inhibitory signal, are concentrated at the periphery of the IS but are excluded from the center of the IS. At this site, were the cells establish the closest contact, the CD31 molecules are cleaved, and most of the extracellular portion of the protein, including the trans-homophilic binding sites, is shed from the cell surface.

T cells lacking CD31 trans-homophilic binding sites easily establish stable interactions with B cells; at the opposite, CD31 signaling agonists inhibit T/B IS formation as well as the ensuing helper T cell activation and function. Confocal microscopy and flow cytometry analysis of experimental T/B IS shows that the T cell inhibitory effects of CD31 agonists depend on SHP-2 signaling, which reduces the phosphorylation of ZAP70.

The analysis of synovial tissue biopsies from patients affected by rheumatoid arthritis showed that T cell CD31 molecules are excluded from the center of the T/B cell synapses *in vivo*. Interestingly, the administration of CD31 agonists *in vivo* significantly attenuated the development of the clinical signs of collagen-induced arthritis in DBA1/J mice.

Altogether, our data indicate that the T cell co-inhibitory receptor CD31 prevents the formation of functional T/B immunological synapses and that therapeutic strategies aimed at sustaining CD31 signaling will attenuate the development of autoimmune responses *in vivo*.

1. Introduction

A variety of receptors bearing the immunoreceptor tyrosine-based inhibitory motif (ITIM) are expressed by T cells, and each receptor plays a crucial and nonredundant role in immunoregulation

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[1]. CD31 is an ITIM receptor [2,3] constitutively expressed by T cells [4,5].

Although initially identified as adhesion molecules [6], CD31 receptors actually exhibit low avidity and transient "adhesive" properties, which correspond to the transhomophilic engagement that is necessary to trigger the outside-in signaling function of this receptor [7]. The trans-homophilic engagement of opposing CD31 molecules contributes to co-clustering of immunoreceptor tyrosine-based activation motif (ITAM) receptors through cis-homophilic interactions, provided that the latter are stimulated [7,8]. The proximity of CD31 molecules to ITAM receptors facilitates the phosphorylation of CD31 ITIMs, as CD31 does not exhibit intrinsic kinase activity [9].

The archetypal formation of the immunological synapse (IS) involves the co-clustering of ITAMs on T lymphocytes with other transmembrane receptors on the plasma membrane surface. The supramolecular activation cluster (SMAC) is in intimate contact with MHC molecules on the cognate antigen-presenting cell (APC), and T cell receptor (TCR) concentrations within this IS are indispensable for physiologically activating T cells. The SMAC comprises three regions: 1) the central SMAC (cSMAC), which contains TCR microclusters; 2) the peripheral SMAC (pSMAC), where adhesion molecules are concentrated, which favors synapse stability; and 3) the outmost part, called the distal SMAC (dSMAC), which contains large molecules, such as CD43 and CD45. Upon IS formation, the cytoskeleton of the cell is specifically organized, as reflected by the rapid and robust repositioning (docking) of the microtubule organizing center (MTOC) underneath the T cell plasma membrane and at the cSMAC. The docking of the MTOC allows the microtubule transport of vesicles containing effector molecules (e.g., cytokines or lytic molecules) to be directed toward the bound APC for subsequent polarized secretion. The effective activation of TCR signaling in the cSMAC requires the recruitment and phosphorylation of ZAP70 to the TCR ITAM, which is represented by the CD3ζsubunit. On the other hand, the presence of CD40L in the cSMAC drives CD40-dependent signaling in the APC [10]. In the case of T/B cell synapses, this interaction leads to B cell proliferation, class switch recombination, somatic hypermutation and the production of soluble IgM and IgG by B cells [11]. All of these activating signals at the IS must be tightly balanced by immunoregulatory signals to avoid inappropriate immune responses. Typically, T/B cell interactions are critically involved in rheumatoid arthritis (RA) [12]. Remarkably, CD31 knockout mice develop hyperactive T [13] and B [14] cell responses and are more sensitive to collagen-induced arthritis (CIA) [15,16].

The ITIM receptor CD31 is gaining much attention due to its immunoregulatory properties and for its peculiar trans-homophilic receptor engagement [17]. Interestingly, both T cells and professional APCs, including B cells, express CD31, which exerts important co-inhibitory functions upon trans-homophilic interactions [14,18]. Although it has been shown that CD31 molecules relocate to the interface between T and B cells upon stimulation with a superantigen [19], neither the spatial distribution of CD31 across the SMAC nor its potential regulatory role in the IS is known.

The fact that CD31 co-clustering with the TCR can inhibit $CD4^+$ T cell activation [18] suggests that this ITIM receptor exerts a regulatory role in IS formation. However, we recently showed that the cleavage and shedding of CD31 occurs rapidly upon T cell activation following antibody-mediated TCR crosslinking [20]. Furthermore, these processes abrogate potential CD31 inhibitory signaling and can explain why the regulatory role of CD31 in T cell activation is transitory [18].

Herein, we show that intact and functionally inhibitory CD31 molecules accumulate on T cells at the periphery of the IS but are excluded from the cSMAC. The trans-homophilic portion of CD31, which is located at the most membrane-distal Ig-like domains, is selectively missing in the cSMAC, where the CD31 molecules appear truncated upon effective T/B IS formation. The absence of intact CD31 molecules in the cSMAC favors the stable formation of cell–cell contacts between T/B cell conjugates, which are prevented by intact CD31 molecules [21] and concomitantly facilitate TCR-mediated T cell activation. The latter mechanism is based on the results obtained in the present study, in which the use of a synthetic peptide to sustain ITIM signaling downstream of CD31 [20] dampened the rate of IS formation, the degree of T cell activation and their helper function toward B cells. Moreover, we show that T cell CD31 molecules are excluded also from the center of the T/B cell synapses occurring in the inflamed joints of patients with rheumatoid arthritis and that the administration of CD31 agonists in experimental mice significantly reduces the development of collagen-induced arthritis.

2. Materials and methods

Detailed method protocols are provided in the Supporting Material

2.1. Cells, reagents and antibodies

Jurkat and Raji cells were cultivated in RPMI-1640 Glutamax™ medium supplemented with 10% fetal calf serum and 50 μM β-mercaptoethanol. The synthetic CD31 peptide was prepared and used as previously described [20]. Recombinant CD31 protein was from R&D Systems and sodium stibogluconate from Merck-Millipore. The following antibodies were used: CD31 (clone JC70A, epitope within domain 1, DAKO); CD31-PE (clone MCB78.2, epitope within domain 6, Life Technologies); CD31 (goat polyclonal IgG, R&D Systems), CD31 (clone SP38, Spring Bioscience); CD3-V450®, SHP2 pY542-PE, ZAP70 pY292-PE, ß-tubulin-Cy3™, ERK pT202/pY204-PE, CD69-PE-Cy™7, CD19-AlexaFluor 700®, CD154-PE (all from BD Biosciences); CD20 (clone L26, DAKO); CD3 (clone F7.2.38, DAKO); and CD4 (clone SP35, Spring Bioscience). Immunofluorescence staining of mouse ankle joints was performed using a rabbit polyclonal anti-CD3 antibody (DAKO) and two anti-mouse monoclonal antibodies directed against the extracellular (clone MEC 13.3, BD Biosciences) and intracellular (clone SP38 Spring Bioscience) portions of mouse CD31. Fluorescently labeled Fcγ fragment-specific AffiniPure® $F(ab')_2$ fragments from Jackson ImmunoResearch Laboratories were used to detect positive staining of unconjugated primary antibodies. Soluble IL-2 (Human IL-2 FlexSet) and CD31 (as previously described [20]) were measured with the CBA® technology (BD Biosciences).

2.2. Jurkat–Raji conjugates

Raji cells were pre-incubated with 100 ng/ml staphylococcal enterotoxin E (SEE, Toxin Technology) prior to mixing the cells with Jurkat cells on polylysine-coated coverslips. Intracellular staining was carried on fixed (PFA) and permeabilized (0.5% Triton X-100) cells. For flow cytometry the conjugates were formed in solution and cells were distinguished by pre-labeling with either CFSE or CellTrace™ Violet (Life Technologies). Primary human FACS-purified $CD19^+$ cells were pre-incubated with a cocktail of superantigens (SEE, SEA and TSSP-1, 100 ng/ml) prior to mixing with $CD4^+$ cells.

2.3. Synovial tissue

Synovial tissue biopsies from inflamed synovial sites were obtained through arthroscopy from six RA patients. The protocol (00/

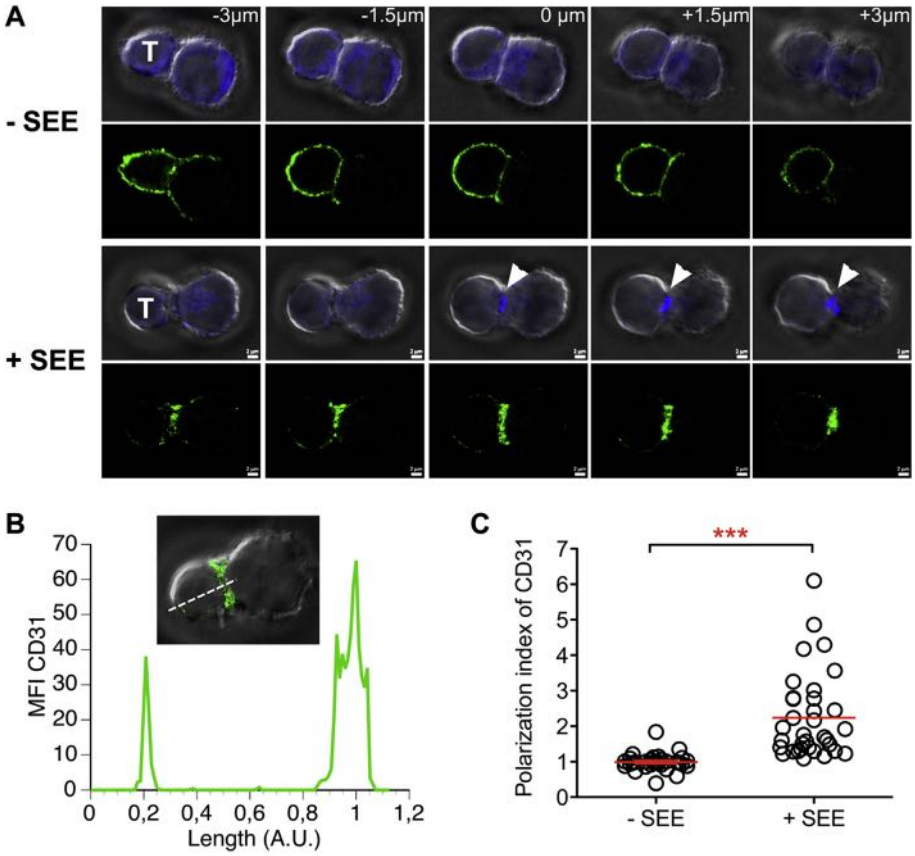

Fig. 1. T cell CD31 is recruited to the immunological synapse. Confocal microscopy of interacting Raji cells (B cells) and Jurkat cells (T cells) in the absence or presence of superantigen SEE. Images were captured after 20 min of reaction; green = CD31 domain 1, blue = TCR (CD3 molecules). A. In the absence of superantigen SEE, the interacting cells did not form true ISs (no TCR cluster at the interaction site), and the CD31 molecules were distributed on the T cell surface. In contrast, in functional ISs, in the presence of superantigen SEE, CD31 molecules were concentrated at the site of TCR clustering (white arrowhead) along the T-B cell interaction site. B. To quantify CD31 polarization, the mean fluorescence intensity (MFI) in the green channel was analyzed along the dotted white line across random conjugates. C. CD31 polarization at the site of T-B cell interaction was dramatically increased in conjugates formed in the presence of superantigen SEE. $n = 31$ "-SEE" and $n = 34$ "+SEE". Red lines represent the means.

149 HP) was approved by the regional ethics committee (CPP Nord-Ouest 1, formerly CCPPRB Haute-Normandie, France), and all participants provided written informed consent. Cryosections were air-dried and fixed in 4% PFA prior to processing for microscopic analysis. Screening of lymphocyte conjugates was performed by computer-assisted image analysis of hematoxylin-eosin-stained sections. Each section was entirely acquired as a mosaic image using the NanoZoomer 2.0-HT (Hamamatsu) and individual Tiff images were processed by computer-assisted image analysis using the QWIN® program (Leica) and a custom macro (threshold on eosin-positive tissue and hematoxylin-positive nuclei, exclusion of elliptical nuclei, counts of total round nuclei and of contiguous round nuclei separated by less than 0.1 µm).

2.4. Collagen-induced arthritis (CIA)

DBA/1J male mice (15 weeks-old, $n = 10$/group) were purchased from Charles River France Laboratories. All experiments conformed to Directive 2010/63/EU of the European Parliament, and formal approval was granted through the Local Animal Ethics Committee (Comité d'éthique Bichat – Debré, Paris, France). CIA was actively induced through immunization with bovine type II collagen (BD Biosciences, cat. N° 354257) in an emulsion with complete Freund's adjuvant (CFA) containing heat-inactivated *Mycobacterium tuberculosis* H37RA (Difco Laboratories) as previously described [22]. Plasma mouse TNF was analyzed using the CBA® Mouse TNF FlexSet (BD Biosciences), and plasma anti-type II collagen IgG titers were analyzed by ELISA. The ankle joints of mice were excised 4 weeks after immunization and immersed for 4 h in DC1 (Labonord; active agents: formic acid and formaldehyde), sectioned, and stained with hematoxylin and eosin.

2.5. Statistical analysis

All *in vitro* experiments included triplicates of each condition and were repeated at least three times. CIA experimental sets were repeated twice and yielded similar results. Data are expressed as the means ± SEM.

The differences between groups were evaluated using one-way (group) or two-way (group and time) ANOVA, as appropriate. The differences were considered significant when the P value was <0.05. The analyses were performed using JMP® 6.0 software (SAS Institute Inc.). $^{*}p < 0.05$; $^{**}p < 0.01$; and $^{***}p < 0.001$.

3. Results

3.1. CD31 molecules are recruited to the immunological synapse

The distribution of CD31 molecules at the site of interaction between Jurkat (T cells) and Raji (B cells) cells was evaluated using confocal microscopy. As shown in Fig. 1, in the presence of superantigen SEE, CD31 molecules translocated to and became strongly

concentrated at the IS, suggesting that CD31 molecules are recruited to the IS that is formed between T and B cells.

Confocal Z-stacks subjected to 3D reconstruction and isosurface analysis confirmed that when T cells did not form an IS with interacting B cells (non-clustered TCR molecules in the absence of the superantigen), the CD31 molecules were intact, as detected by the presence of both the membrane-distal domain 1 and the membrane-proximal domain 6. Furthermore, these molecules were evenly distributed and overlapped with the CD3 molecules along the interaction site (Fig. 2A). Instead, when, in the presence of superantigen SEE, the TCR-CD3 complexes were concentrated at the center of the interaction site (forming the cSMAC), and the intact CD31 molecules were excluded from these clusters. Remarkably, the cSMAC did contain CD31 (positively stained for the domain 6), but all of these molecules were truncated because they lacked domain 1 (Fig. 2A and B). Western blot analysis using the same monoclonal antibodies (Fig. 2C) on the lysate of T/B cell conjugates confirmed the immunofluorescence data: the relative

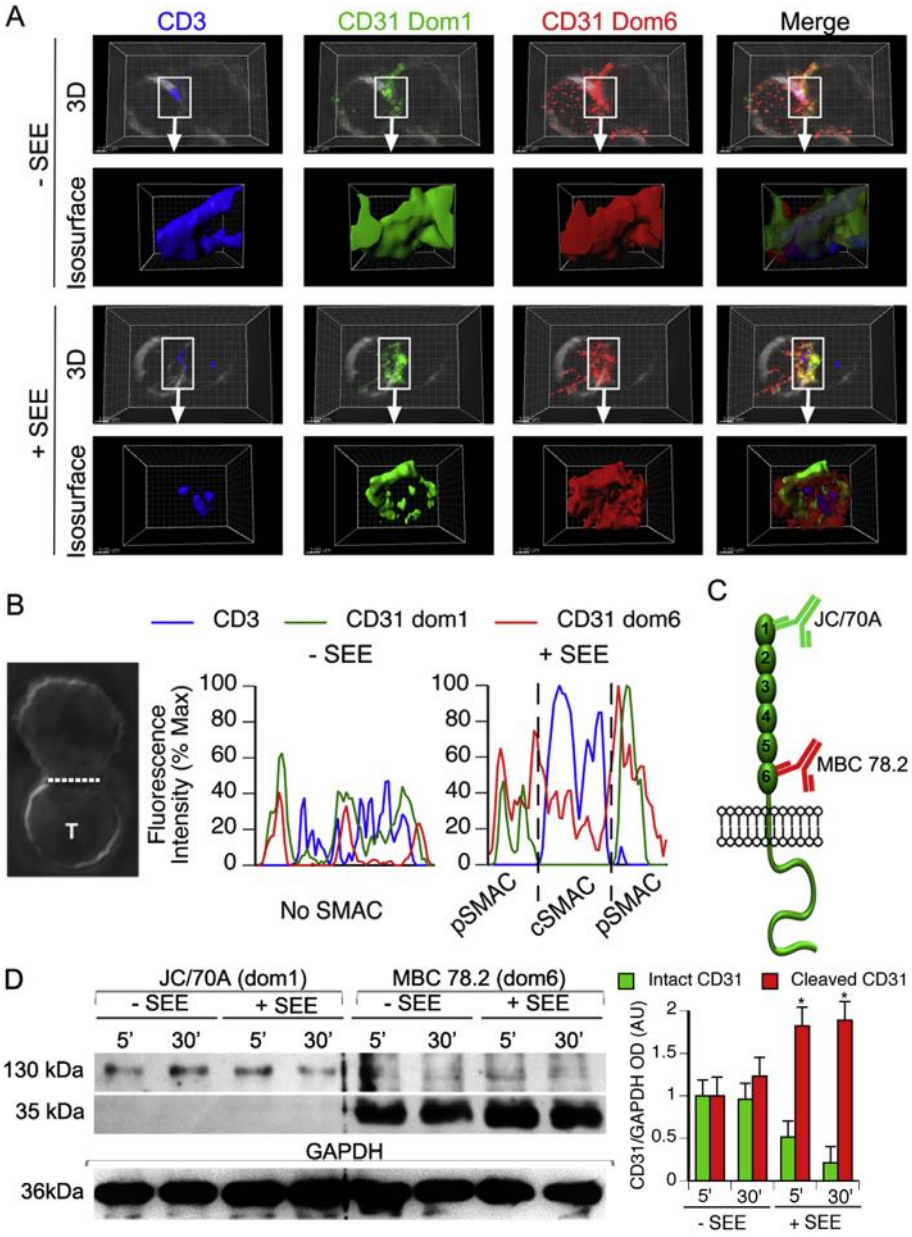

Fig. 2. CD31 molecules are truncated within the immunological synapse. Immunofluorescence and western blot analysis of the CD31 molecules at the IS. A. In the absence of superantigen SEE, the CD3 staining was not concentrated in microclusters (no true IS) and the recruited CD31 molecules were intact (CD31 dom 1, green; CD31 dom 6, red; overlap with CD3, blue). In contrast, the presence of superantigen SEE induced the formation of a true IS, as detected by the focal accumulation of CD3 (blue microclusters). The *en face* analysis of CD31 domains 1 and 6 revealed that intact molecules were concentrated around the CD3 cluster whereas the center of the synapse contained truncated CD31 molecules based on the absence of green fluorescence and the presence of uniform red staining. B. Analysis of the mean fluorescence intensity (MFI) in the three channels along the T cell border confirmed that only domain 6 of CD31 was present within the CD3 cluster (cSMAC), whereas intact CD31 molecules (positive green signal) were concentrated in the pSMAC and excluded from the cSMAC. C. Schematic representation of the CD31 epitopes targeted by the anti-domain 1 (clone JC/70A, green) and anti-domain 6 (clone MBC 78.2, red) antibodies. D. Western blot analysis of cell membrane protein lysates deriving from cells co-incubated in the presence or in the absence of superantigen SEE for 5 and 30 min. The relative amount of intact CD31 molecules, which was detected as 130-kDa bands that were positive for both JC70A (domain 1) and MBC 78.2 (domain 6) clones, is reduced and accompanied by a reciprocal increase of cleaved CD31 molecules (mass = 35 kDa; domain 1-negative and domain 6-positive) in the presence of IS-favoring conditions (+SEE) as a function of time ("-SEE" $n = 3$; "+SEE" $n = 3$).

amount of intact CD31 molecules (mass = 130 kDa; double-positive for domain 1 and 6) was reduced and accompanied by a reciprocal increase in cleaved CD31 molecules (mass = 35 kDa; negative for domain 1 and positive for domain 6) in the presence of IS-favoring conditions (+SEE) as a function of time (Fig. 2D).

3.2. CD31 signaling modulates conjugate formation and functions

Cytometric analysis of CellTrace™ Violet-stained Raji cells and CellTrace™ CFSE-stained Jurkat cells that did or did not react with the superantigen SEE showed that these T/B cell conjugates ($CFSE^+CTV^+$ events, Fig. 3A) rapidly formed and reached steady state after 30 min (Fig. 3A). The presence of the superantigen (+SEE) increased the rate of conjugate formation, but the steady state was reached within the same time period (±30 min, Fig. 3A). We used our previously described custom CBA® method [20] to assess whether the loss of the extracellular portion of CD31, comprising the membrane-distal domain 1, that was observed in the cSMAC at the T cell surface resulted from the cleavage and shedding of this receptor as soluble CD31. The amount of T cell-derived soluble and truncated CD31 protein, consisting of domains 1 to 5 [20], in the supernatant was proportional to the percentage of the conjugates in the same tube (Fig. 3A), suggesting that CD31 molecules are actively cleaved and shed from the surface of the cells involved in T/B conjugate formation. The consequent loss of the trans-homophilic CD31 domain 1 affected the rate of T/B conjugate formation because the latter was accelerated when the T cells were negative for CD31 domain 1 $CD31^{shed}$ (Fig. 3B). Instead, upholding CD31 signaling reduced the formation of T/B cell conjugates (Fig. 3C). Interestingly, the recombinant CD31 protein (recCD31), which induces CD31 signaling through trans-homophilic domains [23], reduced the formation of T/B cell conjugates more efficiently when it was added before conjugate formation. In contrast, the synthetic agonist peptide, which also triggers CD31 signaling on $CD31^{shed}$ cells, reduced the proportion of conjugates equally well when added after the beginning of conjugate formation (Fig. 3C). The inhibition of conjugate formation by CD31 agonists was accompanied by a reduced proportion of MTOC docking, as detected by confocal analysis (Fig. 3D).

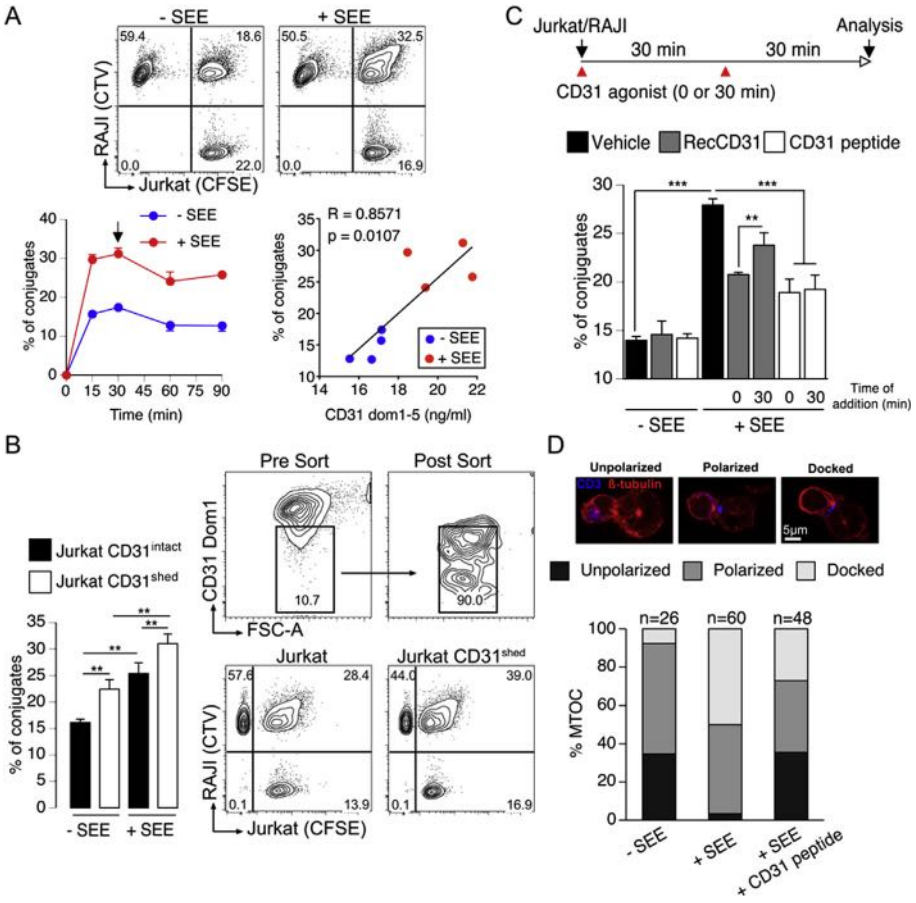

Fig. 3. CD31 agonists revert conjugate formation and block the MTOC docking phase. Conjugates between Raji (labeled with CTV) and Jurkat (labeled with CFSE) cells were mixed (ratio 2:1) and incubated for 20 min at 37 °C in solution. The percentage of T-B cell conjugates in the presence (+SEE) or absence (−SEE) of the superantigen was analyzed using flow cytometry, and individual culture supernatants were processed to measure the CD31 fragments shed from T cells (captured through domain 1 and detected through domain 5, as previously described [20]). A. The formation of T-B cell conjugates (CTV^+CFSE^+ events) reached steady state at 30 min (black arrow), and the levels were proportional to those of soluble truncated CD31 levels in the supernatant. These trends followed a similar time course under the two conditions. The presence of superantigen SEE increased the amount of conjugates and soluble CD31 in parallel. B. Sorting strategy and flow cytometric analysis of the conjugates formed between FACS-purified CD31 domain 1-negative ($CD31^{shed}$) Jurkat cells. The percentage of conjugates formed between $CD31^{shed}$ Jurkat cells was increased compared with the percentage of those formed between FACS-purified $CD31^{intact}$ (CD31 domain 1^+) cells. C. Full length extracellular recombinant human CD31 aa 1-574 (recCD31, 50 µg/ml) or the CD31 domain 6-derived synthetic peptide ([20], 50 µg/ml) was added to the reaction before or after conjugates formation reached steady state (30 min). Both CD31 agonists reduced conjugate formation at 0 min, but the synthetic peptide was more effective that the recombinant protein at 30 min. D. The conjugates that were formed on coverslips were analyzed for the docking of the microtubule organization center (MTOC) in the presence or absence of the CD31 peptide. The presence of the CD31 agonist peptide resulted in reduced docking of MTOCs (proximity of the β-tubulin cluster, red, to the CD3 cluster, blue). "-SEE" $n = 26$; "+SEE" $n = 60$; "+SEE + CD31peptide" $n = 48$.

3.3. CD31 signaling recruits SHP-2 to the immunological synapse and inhibits ZAP70 activation

SHP-2, a key signaling adaptor of the CD31 pathway [9], could be involved in the observed negative regulatory effect of CD31 agonist peptide on IS formation as suggested by the increased concentration of phosphorylated SHP-2 (pSHP-2) proteins beneath the cell membrane at the site of the T/B cell interaction (Fig. 4A). An enhanced level of intracellular pSHP-2 was observed in the presence of the CD31 agonist in TCR-stimulated T cells (Fig. 4B). In parallel, the recruitment and phosphorylation of ZAP70, elicited by the activated ITAMs of the CD3ζ upon TCR stimulation, was abrogated when the CD31 agonist peptide was present in the solution (Fig. 4C). Flow cytometric analysis of CD3-stimulated Jurkat cells in the presence or absence of the CD31 peptide showed that CD3-induced pZAP70 was inhibited within minutes by the presence of the CD31 agonist peptide (Fig. 4D), which appeared concomitant with the increase in pSHP-2 shown in Fig. 4B.

Because the CD31/ITIM/SHP-2 pathway is involved in T cell survival through activation of the ERK1/2 signaling pathway [13], we also evaluated the level of ERK1/2 phosphorylation (pERK) during the TCR-mediated stimulation of Jurkat T cells in the presence or absence of two CD31 agonists. After 5 min of anti-CD3 stimulation, pERK also became detectable by flow cytometry. The addition of sodium stibogluconate (SSG), a SHP-1/2 inhibitor [24], reduced pERK, supporting previous work showing that the recruitment and activation of these phosphatases contributes to ERK phosphorylation in TCR-stimulated Jurkat T cells [25]. Because CD31 signaling involves the recruitment and activation of SHP-2, we evaluated the effect of two CD31 agonists, recCD31 and the CD31 peptide, on the intracellular levels of pERK after CD3 stimulation. As shown in Fig. 4E, both agonists increased the intracellular levels of pERK in CD3-stimulated Jurkat cells in a SHP-dependent manner. We therefore propose that, in parallel to its inhibitory effects on pZAP70 phosphorylation, the CD31/SHP-2 signaling pathway concomitantly promotes $CD4^+$ T cell survival through activation of the ERK pathway upon TCR stimulation (Fig. 4F).

3.4. CD31 controls the activation and helper function of T cells within T/B cell IS

To assess whether the negative T cell signaling triggered by CD31 agonists impacted the activation of $CD4^+$ T cells involved in T/B cell IS formation, we evaluated the expression of CD69 and the release of IL-2 by Jurkat cells that were stimulated to form functional IS with superantigen SEE-loaded Raji cells. As shown in Fig. 5A, both IL-2

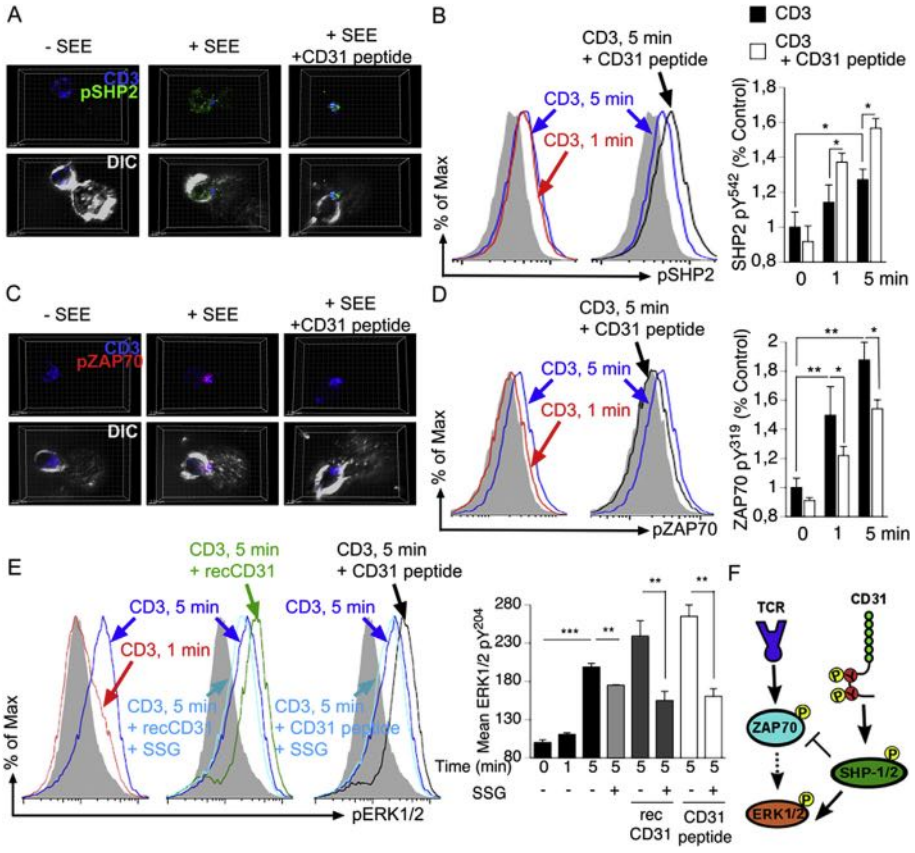

Fig. 4. The induction of CD31 activates SHP-2 and ERK signaling and blocks ZAP70 phosphorylation. Jurkat cells were stimulated for the indicated times using soluble anti-CD3 (clone UCHT1; 2 μg/ml) in the presence or absence of the CD31 peptide (50 μg/ml) at 37 °C. The cells were subsequently fixed, permeabilized and stained for the indicated phospho-proteins and analyzed using confocal microscopy and intracellular flow cytometry. A. The addition of superantigen SEE induced slight SHP-2 phosphorylation at the immunological synapse. The presence of the CD31 agonist peptide resulted in a strong concentration of pSHP-2 proteins at the site of TCR clusters (CD3, blue). B. Flow cytometry confirmed that pSHP-2 was consistently increased by the presence of the CD31 peptide. C. The focal accumulation of pZAP70 (red), which was elicited by superantigen SEE, was reduced using the CD31 peptide. D. The CD31 peptide reliably reduced the relative amount of pZAP70, as detected by flow cytometry. E. Intracellular ERK1/2 phosphorylation following CD3 cross-linking was evaluated using flow cytometry in the presence or absence of CD31 agonists and sodium stibogluconate (SSG, 100 μM), an inhibitor of SHP-1/2 activity. TCR stimulation induced strong ERK1/2 phosphorylation after 5 min, which was reduced by SSG. Both recCD31 and the CD31 peptide enhanced ERK1/2 phosphorylation in a SHP-1/2-dependent manner. The column histogram displays quantitative analysis of cytometric data. F. Schematic representation of the results: the induction of CD31 activates SHP-1/2, which concomitantly blocks TCR signaling while inducing ERK activation.

secretion and the expression of CD69 by Jurkat cells, elicited by their interaction with superantigen SEE-loaded Raji cells, were dampened if the CD31 signaling pathway was upheld in co-cultured Jurkat and superantigen SEE-loaded Raji cells using the CD31 agonist peptide (Fig. 5B). Experiments performed using FACS-purified, primary $CD4^+$ and $CD19^+$ cells from peripheral human blood and stimulated with a cocktail of Sag (TSSP1, SEA, SEE; Fig. 5B) showed that the rate of conjugate formation in this setting was reduced by the CD31 peptide, suggesting that CD31 signaling effectively controls the formation of IS between primary $CD4^+$ T and B cells. The net effect of CD31 signaling on each cell partner was further assessed in separate T or B primary cell preparations. $CD4^+$ T cells obtained from the peripheral blood of healthy donors (Fig. 5C) and incubated with the CD31 agonist peptide showed reduced CD69 expression following TCR stimulation by anti-CD3 and anti-CD28 antibodies (Fig. 5C). Importantly, the expression of CD154 (CD40L) induced by TCR stimulation was also impaired by the CD31 agonist peptide (Fig. 5C), suggesting that CD31 can reduce both the activation and helper function of $CD4^+$ T cells. The latter function is exerted by the expression of CD154 on cognate B cells, which engages the CD40 signaling pathway, leading to the expression of AID and the production of soluble, antigen-specific antibodies. Therefore, we directly evaluated the effect of CD31 signaling on B cell activation via CD40 using soluble recombinant CD40L. As shown in Fig. 5D, upholding CD31 signaling in CD40L-stimulated B cells to mimic the signal provided by a helper T cell in T/B IS resulted in reduced production of soluble immunoglobulins. Altogether, our results suggest that CD31 signaling controls both the activation and helper function of $CD4^+$ T cells and that CD31 signaling agonists have an important regulatory role in T cell-dependent B-cell responses.

3.5. CD31 agonists target T/B cell interactions in RA

The uncontrolled formation of ISs between T and B cells is a key pathological immune process that occurs in autoimmune diseases, such as RA [12,26] and the modulation of the cell–cell interactions involving joint-infiltrated T lymphocytes is an interesting immune-therapeutic approach for the treatment of RA [27]. We therefore aimed to assess whether the regulatory function of CD31 at the IS is relevant in patients with RA. For this purpose, we first screened cryosections of six biopsies of inflamed synovial tissue derived from RA patients for the presence of lymphocyte conjugates. The clinical characteristics and the data from computer-assisted image analysis of lymphoid conjugates on hematoxylin-eosin-stained sections are detailed in Table 1. Consecutive sections of those showing the presence of juxtaposed mononucleated lymphoid cells (Fig. 6A) were processed for co-immunofluorescent staining of CD31 and $CD4^+$ (T) or $CD20^+$ (B) cells. Confocal analyses of T/B cell conjugates (Fig. 6B) showed that intact CD31 molecules surrounded TCR-CD3 clusters (Fig. 6C), which were likely located in the pSMAC, whereas they were excluded from the cSMAC, suggesting that the peculiar distribution of intact and truncated CD31 molecules that was observed *in vitro* with Jurkat and Raji cells also occurred *in vivo* between pathogenic $CD4^+$ T and B human cells. Because the CD31 agonist peptide modulated IS formation and the helper functions of $CD4^+$ T cells *in vitro*, we sought to evaluate its potential therapeutic effects in a mouse model of RA. Interestingly, the development of CIA in mice had previously been reported to be accelerated in the absence of CD31 [15] whereas the administration of CD31-targeting antibodies, which likely crosslink CD31 molecules and act as signaling agonist, protect against joint inflammation and damage in

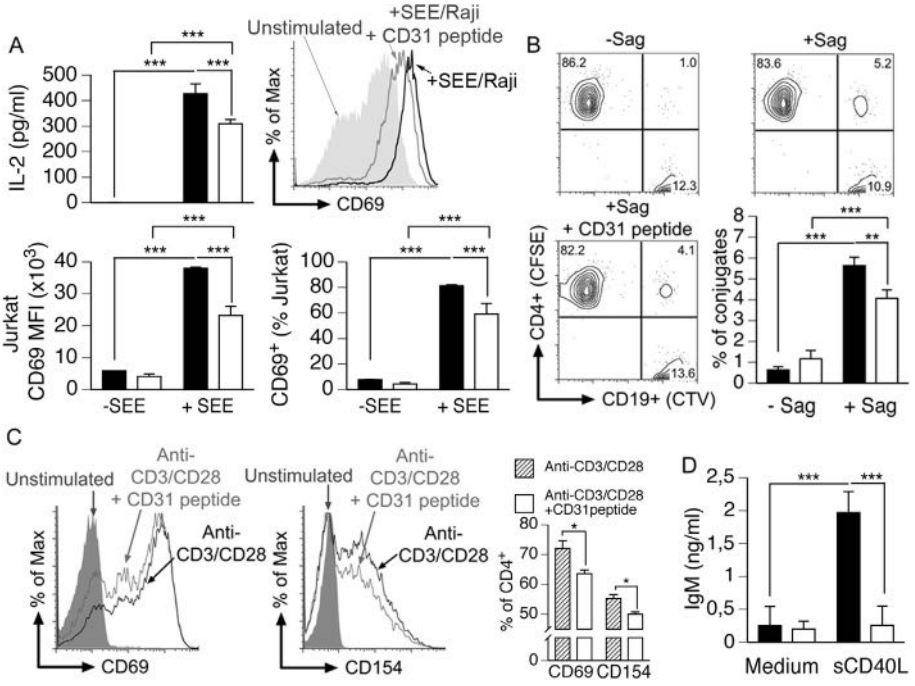

Fig. 5. The CD31 agonist peptide blocks T helper cell function. A. The IL-2 production and CD69 expression on the surface of Jurkat cells stimulated by anti-CD3 and anti-CD28 antibodies were analyzed using CBA® and flow cytometry, respectively. Both results were promptly elicited through co-culture with superantigen SEE-loaded Raji cells (+SEE) and were reduced in the presence of the CD31 agonist peptide. B. Fresh $CD4^+$ T and $CD19^+$ B cells were FACS-purified from human peripheral blood ($n = 3$) and stained with CFSE and CTV as indicated. $CD19^+$ B cells were incubated for 1 h at 37 °C with a cocktail of superantigens ("+Sag" = TSSP1, SEB and SEE 1 μg/ml each) or the vehicle (−Sag) and then washed and incubated with the $CD4^+$ T cells (ratio: B:T = 1:4) in the presence or absence of the CD31 peptide. Conjugate formation was analyzed after 1 h using flow cytometry (representative contour plots from each condition are displayed). Sag-induced conjugate formation was reduced in the presence of the CD31 agonist peptide. C. FACS-purified primary $CD4^+$ T cells were stimulated with anti-CD3/anti-CD28 antibodies for three days and analyzed for the surface expression of CD69 and CD154 by flow cytometry. The presence of the CD31 peptide significantly reduced the expression of CD69 and CD154 in stimulated cells ($n = 3$/condition). D. FACS-purified primary $CD19^+$ B cells were stimulated or not with soluble recombinant CD40L (sCD40L, 500 ng/ml) for 5 days in the presence or absence of the CD31 agonist peptide. Quantitative analysis of soluble IgM in the supernatant (CBA® Human IgM FlexSet, BD Biosciences) showed that the CD31 peptide prevented the CD40L-induced production of IgM by the stimulated B cells.

Table 1
Clinical and morphological data of patient's synovial biopsies.

Biopsy#	Patient age (y)	Sex (M/F)	Duration of disease (y)	Treatment	DAS28	Analyzed fields#	Total surface (mm^2)	Total lymphoid nuclei[a]	Total conjugates[b]	% Conjugates/field (mean ± SD)
1	54	F	7	Etanercept, Ketoprofen	3.5	11	36	2565	469	16 ± 3
2	67	F	4	Prednisone, Methotrexate, Gold salt	5.1	22	63	5726	750	13 ± 3
3	78	F	13	Prednisone, Methotrexate, Aurothiopropanolsulfonate, Celecoxib	3.6	6	9	1127	273	16 ± 4
4	46	F	9	Hydroxychloroquine, Paracetamol	4.2	21	62	3727	628	11 ± 4
5	61	F	21	Prednisone, Ketoprofen	4.4	37	137	14303	2190	15 ± 4
6	70	F	9	Prednisone, Methotrexate, Leflunomide	5.5	79	197	17003	2488	14 ± 4

The symbol # indicates that the number in the column corresponds to the total number [#] of fields that have been analysed for each biopsy.
[a] Lymphoid nuclei were identified as round-shaped, hematoxylin-positive elements.
[b] Conjugates were identified as juxtaposed lymphoid nuclei (<0.1-μm distance between two lymphoid nuclei).

this model [16]. We therefore analyzed the localization of CD31 within the joints of DBA1/J mice subjected to CIA where cell–cell interactions likely take place between the arthritogenic APCs [28] and the cognate T lymphocytes [15] that reach the joints. As in patient biopsies, CD31 molecules were recruited and truncated at the level of the IS on joint-infiltrated T cells, as detected by the dense accumulation of intracellular CD31 and the relative absence of extracellular CD31 beneath the CD3 clusters (Fig. 7A). We therefore sought to evaluate the potential therapeutic effects of the CD31 agonist peptide in this model. DBA1/J mice subjected to CIA were administered the CD31 peptide or the vehicle daily. The treatment began on the day of the booster immunization, when the antigen-

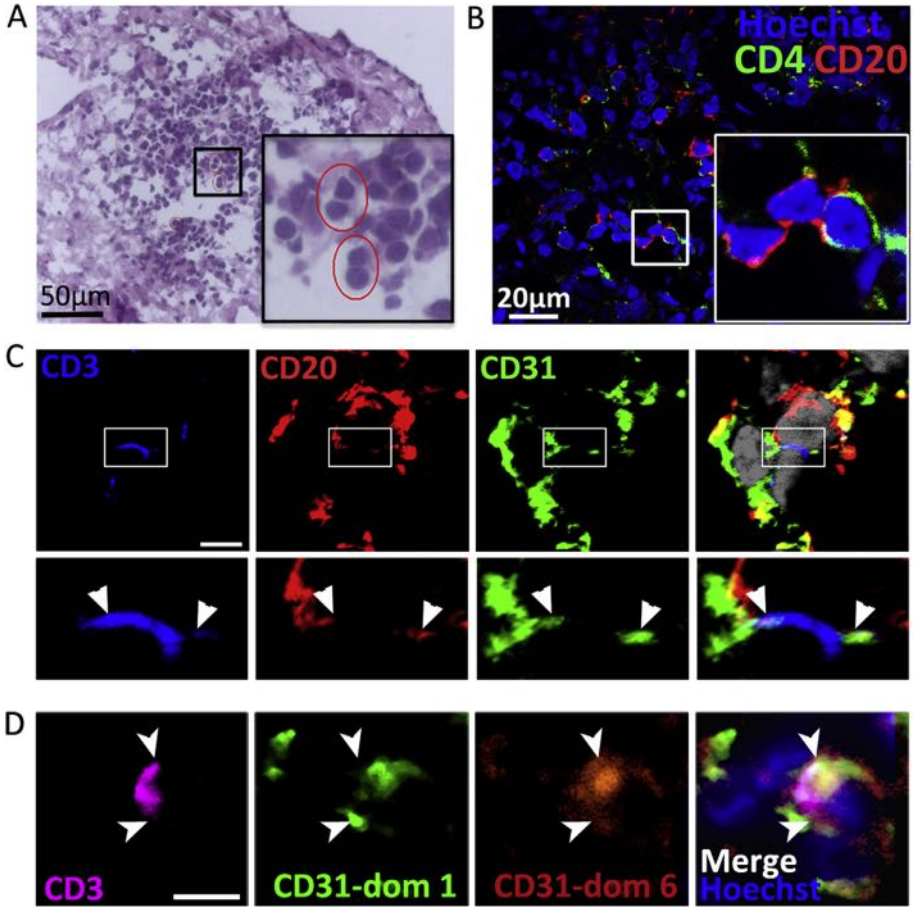

Fig. 6. The CD31 molecules recruited to T/B IS within inflamed synovial tissues of RA patients are truncated. A. The presence of T/B IS was evaluated at sites of lymphoid cell aggregates pre-screened on hematoxylin-staining sections of human synovial tissue biopsies. The inset shows an example of mononuclear cell–cell conjugates within the analyzed cryosections (red circles, $n = 15–20$/sample). B. Immunofluorescence staining with rabbit anti-human CD4 (clone SP35 from Thermo Scientific) and mouse anti-human CD20 (clone L26 from DAKO) monoclonal antibodies revealed that most of these cells were conjugates between T ($CD4^+$, green) and B ($CD20^+$, red) cells, as shown in the representative example (Hoechst, blue = nuclear counterstaining). C. Confocal analysis of CD31 localization at the T/B cell IS. CD3 (blue, rabbit polyclonal antibody from DAKO), CD20 (red, clone L26 from DAKO) and CD31 (green, sheep polyclonal antibody from R&D Systems). CD3 molecules were concentrated in clusters between the cells, which strongly suggested that the interacting T and B cells were involved in active IS within the inflamed synovial tissue. CD31 polyclonal staining surrounded the TCR clusters (arrow heads) and is excluded from the center of the synaptic site. Scale bar, 5 μm. D. Confocal analysis of CD31 integrity at the IS site. CD3 (magenta, rabbit polyclonal antibody from DAKO), CD31 domain 1 (green, clone JC70A from DAKO) and CD31 domain 6 (red, clone MBC1.2 from Life technologies). Whereas CD31 domain 6 staining was positive at the IS, which defined by the CD3 cluster (arrowheads), CD31 domain 1 appeared to be excluded from the IS. Blue = Hoechst. Scale bar, 10 μm.

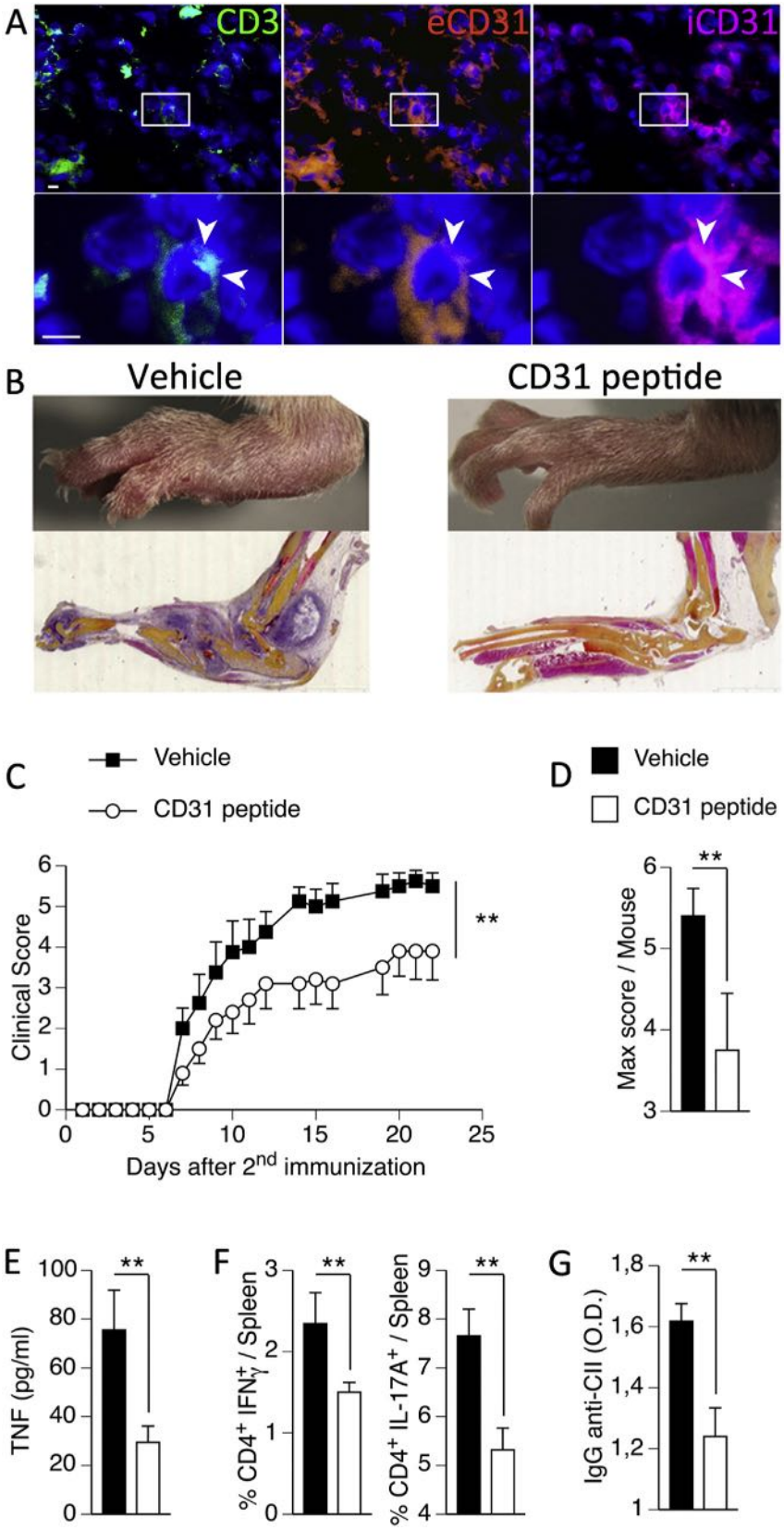

Fig. 7. Therapeutic potential of CD31 agonist treatment in mouse CIA. A. Confocal analysis of CD31 in the ankle joints of DBA1/J male mice subjected to collagen-induced arthritis (CIA). T cells forming ISs were identified via the presence of CD3 clusters (green). The magnification of the inset shows that, as in inflamed human synovial biopsies, the T cell CD31 molecules were recruited and truncated within the IS (arrowheads), as detected by the relative absence of extracellular portion (eCD31, orange) in contrast to the focal concentration of the intracytoplasmic tail (iCD31, magenta) of CD31. B. DBA1/J male mice (male, 15 week-old, $n = 10$/group) were subjected to CIA and treated daily with a subcutaneous injection of CD31 peptide (50 μg/mouse/day) beginning on the day of the second immunization. The clinical and histological signs of arthritis were consistently reduced in mice treated with the CD31 agonist peptide compared with vehicle-treated mice. C. CD31 peptide-treated mice consistently showed a lower clinical score of arthritis throughout the study period. D. The disease severity, as reflected by the maximal clinical score, was significantly lower in CD31-treated mice. E. CBA® analysis of mouse plasma at sacrifice revealed a significant reduction of the pro-inflammatory cytokine TNF in treated mice. F. Effector $CD4^+$ T cells producing either IFNγ or IL-17A were significantly reduced in the spleen of CD31-treated mice as assessed by intracellular cytokine analysis by flow cytometry. G. Plasma levels of anti-collagen type II IgG were measured by ELISA, which showed that they were consistently reduced in CD31-treated mice.

specific T cells that had been primed by the immunogenic APCs upon the first immunization (sensitization phase) were expected to interact with the cognate and disease-effector B cells (effector phase). Interestingly, CD31 peptide-treated mice showed less severe clinical and histological signs of inflammatory arthritis compared with control mice throughout the study period (Fig. 7B). In addition, lower daily (Fig. 7C) and maximal (Fig. 7D) clinical scores were reached by the individual mice. The beneficial effects of the CD31 agonist peptide were associated with reduced immune mediators of RA, such as TNF (Fig. 7E), IFNγ^+ and IL-17A$^+$ CD4$^+$ T cells (Fig. 7F) and anti-collagen type II IgG (Fig. 7G). These results strongly suggest that agents that are able to sustain CD31 signaling, such as the agonist peptide, can considerably dampen T cell-dependent B cell immune responses, such as those in the CIA model.

4. Discussion

CD31 is a neglected yet important ITIM receptor that is expressed by both T cells and APCs [17]. Intriguingly, previous studies have already shown that CD31 is recruited to the IS [19], but the spatial distribution and the role of this ITIM receptor in IS formation as well as the subsequent T cell function remain unknown. We found that CD31 is indeed differentially distributed across the SMAC. Intact CD31 molecules are concentrated at the periphery, whereas they are virtually absent from the cSMAC. Our data show that the cSMAC contains truncated CD31 molecules, which invalidates their trans-homophilic signaling due to the lack of membrane-distal domains. The spatial distribution of the cell surface signaling proteins involved in IS formation and the ensuing cell activation and effector functions (cytokine production, Ig secretion) is an evolving and broad field of research. While the distribution and function of T cell co-stimulatory molecules involved in IS formation have been well characterized, those of the co-inhibitory receptors are less well understood.

Although ITIM receptors require the recruitment of intracellular phosphatases, various T cell inhibitors exert molecule-specific functions and may or may not be present in the cSMAC. For instance, CD45, CD148 and CD43 appear to be excluded from the cSMAC [29–31]. CD45 and CD148 may inhibit close-contact zones between T cells and APCs due to their large size [32]. Whether the large size of CD43 is responsible for its inhibitory function is still a matter of debate, but it is interesting to note that, similar to CD31, its inhibitory function depends on the presence of the cytoplasmic tail [33], and part of its extracellular portion is cleaved from activated lymphocytes [34]. It is therefore tempting to speculate that the absence of CD43 from the cSMAC is due to a site-specific cleavage within TCR microclusters. The cleavage of surface molecules at the site of TCR cluster activation is likely due to the local release of sheddases [35] and other surface molecules, such as L-selectin, are rapidly shed from activating lymphocytes by these types of proteolytic cleavage events [36]. As in other immune cell receptor ectodomain shedding [37], an MMP/ADAM type sheddase is thought to be involved in CD31 cleavage, but the exact identity of the involved enzyme remains unknown [38]. The fact that inhibitory molecules are cleaved but not absent from the cSMAC could allow the use of specific agonists that are able to engage the sequence that remains expressed after cleavage and restore the inhibitory signaling pathway. This very effect could be accomplished by using a homophilic CD31 peptide to stabilize signaling clusters via homo-oligomerization with truncated CD31 on the T cell surface [20]. Sustaining the CD31/SHP-2 signaling pathway using this peptide blocked the formation of T/B cell conjugates and most of the ensuing, TCR-mediated T cell functions (e.g., ZAP70 phosphorylation, MTOC docking, IL-2 production and the expression of CD69 and CD154).

Other T cell inhibitors, such as CTLA-4, PD-1 and BTL-A, are located within the cSMAC. However, their appearance occurs late after the formation of the IS because they require active gene transcription to be expressed. At variance, CD31 is constitutively expressed at the surface of both naive CD4$^+$ T cells and APCs, supporting a physiological role for this trans-homophilic ITIM receptor in setting the threshold for IS formation and the ensuing T cell activation.

Because CD31 is not endowed with intrinsic kinase activity, the phosphorylation of its ITIMs and its signaling function strictly depend on the presence of tyrosine kinases, such as the TCR-associated lymphocyte-specific protein tyrosine kinase (Lck) [9]. This might explain why CD31 molecules need to be co-clustered with TCR-ITAMs to exert their inhibitory signaling functions. Conversely, the fact that CD31 molecules appear truncated within the TCR microclusters suggests that the activation threshold for TCR stimulation is overcome by the T cell, which leads to the rapid cleavage and shedding of nearby CD31 molecules.

Effective ISs are those in which the MTOC is docked beneath the TCR clusters because this strongly promotes the oriented secretion of effector cytokines, including those required for B cell activation (T “helper” function). Our data show that CD31 signaling agonists prevent polarization of the MTOC in T/B cell conjugates, implying that the T/B cell conjugates, even if not dissolved, are effectively functionally inhibited by CD31 agonists. The CD31-mediated inhibition of T/B IS function is reflected by the reduced expression of CD40L by T cells *in vitro*, the reduced production of antigen-specific soluble antibodies *in vivo* and the development of clinical signs of arthritis in the CIA model. These findings are of major interest in the field of CD4$^+$ T cell-dependent B cell-mediated autoimmune diseases, such as RA, in which T/B ISs that exclude intact CD31 molecules from the cSMAC are found in inflamed synovial tissues [12].

The results of the experiments involving CIA suggest that CD31 is involved in the production of pathogenic antibodies, and CD31 agonists may limit the resulting inflammatory damage associated with the production of these mediators. Indeed, it has been reported that the administration of CD31-targeting antibodies reduces the development of CIA in mice [39], and similar results were obtained using CD31-Fc chimeras [40]. Of note, both of these agents trigger CD31/ITIM/SHP-2 signaling [20,23]. Herein, we show that the synthetic CD31 peptide is a promising immunoregulatory molecule for RA, which is in agreement with results from previous studies using this peptide in other experimental models [41,42].

Targeting CD31 during T/B IS formation might yield broader benefits compared with the use of biological molecules, such as cytokine-specific blockers, which target downstream immune effectors. Indeed, the effects of CD31 peptide treatment also include a reduction of TNF, a key target of the current biological treatments for RA. Compared with CTLA-4, an agonists that is currently being tested against several autoimmune diseases, pharmacological agonists of CD31 have the advantage of driving inhibitory functions of both T and B cells as well as other professional APCs, such as macrophages [42,43] and dendritic cells [13]. The expected effects of CD31 agonists on all of these cell types make them promising molecules for patients affected by autoimmune diseases. The main effect of this strategy is that CD31 agonists potentially enhance/restore physiological immune regulation resulting in a transient and reversible inhibition of immune responses without depleting a given cell population or blocking of a cytokine pathway.

Interestingly, because the extent of T/B synapse formation is reflected by the amount of truncated CD31 in biological fluids, including plasma [20], the concentration of soluble, shed CD31 could be used as a biomarker to monitor the effect of treatment and to fine-tune the dosing of pharmacological agents or the CD31 agonist.

In conclusion, the results of the present study reveal that CD31 is differentially expressed across different regions of the IS, where it exerts an unanticipated regulatory role. Our study also points to the promising use of CD31 peptide agonists as a novel therapeutic approach to treat autoimmune diseases by modulating the cooperative T/B lymphocyte responses.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jaut.2014.09.002.

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