CD11ch Dendritic Cells Regulate the Re-establishment of Vascular Quiescence and Stabilization after Immune Stimulation of Lymph Nodes

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CD11c<sup>hi</sup> Dendritic Cells Regulate the Re-establishment of Vascular Quiescence and Stabilization after Immune Stimulation of Lymph Nodes

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Lymph node expansion during immune responses is accompanied by rapid vascular expansion. The re-establishment of quiescence and stabilization of the newly expanded vasculature and the regulatory mechanisms involved have not been well studied. We show that although initiation of vascular expansion in immune-stimulated nodes is associated with upregulated endothelial cell proliferation, increased high endothelial venule trafficking efficiency and VCAM-1 expression, and disrupted perivascular fibroblastic reticular cell organization, the re-establishment of vascular quiescence and stabilization postexpansion is characterized by reversal of these phenomena. Although CD11c<sub>med</sub> cells are associated with the initiation of vascular expansion, CD11c<sub>hi</sub>MHC class II (MHC II) <sup>med</sup> dendritic cells (DCs) accumulate later, and their short-term depletion in mice abrogates the re-establishment of vascular quiescence and stabilization. CD11c<sub>hi</sub>MHC II<sup>med</sup> cells promote endothelial cell quiescence in vitro and, in vivo, mediate quiescence at least in part by mediating reduced lymph node vascular endothelial growth factor. Disrupted vascular quiescence and stabilization in expanded nodes is associated with attenuated T cell-dependent B cell responses. These results describe a novel mechanism whereby CD11c<sub>hi</sub>MHC II<sup>med</sup> DCs regulate the re-establishment of vascular quiescence and stabilization after lymph node vascular expansion and suggest that these DCs function in part to orchestrate the microenvironmental alterations required for successful immunity.

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Abbreviations used in this paper: BMDC, bone marrow-derived dendritic cell; CRM, cross-reactive material; DC, dendritic cell; DT, diphtheria toxoid; FRC, fibroblastic reticular cell; HEV, high endothelial venule; LTβR, lymphotixin β receptor; MHC II, MHC class II; PNA, peripheral lymph node addressin; VEGF, vascular endothelial growth factor.

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early vascular expansion raises the possibility that immune cells also mediate the subsequent re-establishment of endothelial cell quiescence and vascular stabilization.

In addition to proliferation and expansion, the lymph node vasculature also undergoes phenotypic alterations during immune responses. HEV endothelial cells in stimulated lymph nodes appear metabolically activated, with increased polyribosomes and prominent rough endoplasmic reticulum. Within a day after DC injection, CXCL9 on HEV is upregulated and may mediate increased entry of activated T cells and NK cells (2, 3). Similarly, the adhesion molecule CD62P is upregulated and mediates effector memory T cell entry (4). Less well characterized in terms of HEV phenotypic alterations is the general increase in trafficking of naive lymphocytes that occurs in the same time frame (3, 5), although this process may be dependent on DCs, as lymph nodes remain small when CD11c<sup>+</sup> cells are depleted (6). Peripheral lymph node addressin (PNAd) is a group of fucosylated and sialylated proteins that mark peripheral node HEV (15), and downregulated expression of PNAd protein cores and synthesizing enzymes within days of lymph node stimulation is accompanied by upregulated expression of mucosal addressin cell adhesion molecule-1, a cell adhesion molecule normally expressed on mucosal HEV (1, 11). These phenotypic alterations are transient with acute stimulation and correspond to the period of increased endothelial cell proliferation and vascular expansion. Whether there are also other phenotypic alterations and whether HEV phenotype is regulated in conjunction with downregulation of endothelial cell proliferation are open questions.

Lymph node blood vessels are suspended in a reticular network composed of collagen-rich fibrils ensheathed by fibroblastic reticular cells (FRCs). FRCs synthesize the extracellular matrix components of the fibrils and provide a surface upon which lymphocytes migrate (16). FRCs also function to express the chemokines that promote T and B cell compartmentalization and IL-7 that maintains T cell numbers (17, 18). The FRC-covered fibrils form a continuous network with the vessel walls. At HEV, FRCs resemble pericytes of other microvascular beds in that they form a sheath several layers thick around the vessels (19). There, FRCs regulate parenchymal entry of blood-borne cells after they have crossed the endothelium (16) and can potentially regulate vasopermeability. The compartment between the FRCs and the fibrillar core in the reticular fibrils is a conduit for small molecules. These conduits continue to the FRC-HEV interface, and small molecules can reach the endothelial cells through the conduit system, providing a potential means of vascular regulation (19–21). FRCs associated with blood vessel walls and also with nearby fibrils are the main expressers of VEGF mRNA in homostatic and immune-stimulated lymph nodes, suggesting a role for perivascular and nearby FRCs in regulating lymph node endothelial cell proliferation (13). Because of the physical and functional association between the endothelial cells and FRCs, regulation of vascular activity is likely to involve regulation of both endothelial cells and nearby FRCs.

In this study, we characterize the re-establishment of vascular quiescence and stabilization, identify CD11c<sup>+</sup>MHC class II (MHC II)<sup>+</sup> DCs as critical mediators of the process, and show that disruption of this process is associated with attenuated T cell-dependent humoral responses. We show that, after vascular expansion, endothelial cell quiescence is re-established with downregulation of the previously upregulated endothelial cell proliferation, HEV trafficking efficiency, and VCAM-1 expression. The newly expanded vasculature is also stabilized, with the reassembly of FRC around and near by vessels. Although CD11c<sup>+</sup> cells are associated with initiation of vascular expansion, CD11c<sup>+</sup>MHC II<sup>+</sup> DCs accumulate in large numbers near vessels at the onset of vascular quiescence and stabilization. Depletion of CD11c<sup>+</sup>MHC II<sup>+</sup> DCs disrupts this process, and these DCs promote endothelial cell quiescence in vitro. CD11c<sup>+</sup> MHC II<sup>+</sup> DCs mediate the downregulation of endothelial cell proliferation at least in part by regulating lymph node VEGF levels. Disruption of the re-establishment of vascular quiescence and stabilization is associated with attenuated development of T cell-dependent B cell responses. Our results provide new insights into the regulation of the lymph node vasculature and describe a novel function for CD11c<sup>+</sup>MHC II<sup>+</sup> DCs in regulating the lymph node microenvironment.

Materials and Methods

**Mice**

B6 CD11c-DTR mice originally from The Jackson Laboratory (Bar Harbor, ME) and C57BL/6 mice from The Jackson Laboratory, Taconic Farms (Germantown, NY), or National Cancer Institute (Bethesda, MD) were used at 6–10 wk of age. All experiments were performed in accordance with the regulations of the Institutional Animal Use and Care Committee at the Hospital for Special Surgery (New York, NY).

**Bone marrow-derived DC preparation**

Bone marrow-derived DCs (BMDCs) were generated as described (6). Briefly, bone marrow cells were cultured with 8–10% supernatant from GM-CSF-expressing J558L cells, matured with LPS on day 7, harvested on day 8, washed, and injected at 1 × 10<sup>6</sup> cells per hind footpad.

**Flow cytometry**

Flow cytometry analysis was described (6). Briefly, lymph node lymphocytes were digested with type II collagenase (Worthington, Lakewood, NJ), and cells were stained for flow cytometry. Unless otherwise specified, all Abs were from BD Biosciences (San Jose, CA). For analysis of endothelial cell proliferation, mice received 2 mg of BrdU (Sigma-Aldrich, St. Louis, MO) 18 h and 1 h presacrifice and 0.8 mg/ml BrdU in drinking water between. Cells were stained with indicated endothelial cell markers and with anti-BrdU-Alexa Fluor647 (Invitrogen, Carlsbad, CA). For VCAM-1 expression measurement, cells were stained for endothelial cell markers and with biotinylated anti–VCAM-1 (clone 429 [MVCAM.A1]) followed by streptavidin-allophycocyanin (Invitrogen) or streptavidin-Pacific blue (Invitrogen). The level of VCAM-1 was analyzed by flow cytometry.

Other Abs that were used for flow cytometry were: anti-CD45 (30–F11), anti-CD31 (MEC13.3), anti-PNAd (MECA-79), anti-CD11c (HL3), anti-CD11b (anti-MHC II, clone AF6), and anti-rat IgM (as a secondary Ab for anti-PNAd staining; Jackson ImmunoResearch Laboratories, West Grove, PA).

**Depletion of CD11c<sup>+</sup>MHC II<sup>+</sup> cells**

CD11c-DTR mice are transgenic for a simian DTR-GFP fusion protein that is driven by the CD11c promoter and that allows for the depletion of CD11c<sup>+</sup> cells upon diptheria toxin (DT) injection (22). Unless otherwise specified, mice received ip injections of 100 ng DT (Calbiochem, San Diego, CA) or inactive DT (CRM 197, Calbiochem). For CD11c-DTR bone marrow chimeras, 200 ng DT was injected ip. The increased dose of DT for the chimeras was found by us to be necessary for consistent depletion of CD11c<sup>+</sup>MHC II<sup>+</sup> cells (T-C. Tseng and T.T. Lu, unpublished observations) and is in line with the higher dosing of DT used in CD11c-DTR chimeric mice in other studies (23, 24). For day 7 endothelial cell proliferation studies, BrdU was first injected 8 h after DT injection on day 6.

**Aortic ring culture**

The aortic ring culture was based on that described by Berger et al. (25). One-millimeter aortic rings were placed one ring per well of 12-well tissue culture plates coated with 0.0025 U/ml thrombin solution (Sigma-Aldrich). Fibrinogen (0.1%) (Sigma-Aldrich) and CD11c<sup>+</sup> cell subsets were added slowly and incubated at 37°C for 10 min to promote gel formation. M199 (Invitrogen) with 1% fungizone (Invitrogen), 0.04 mg/ml gentamicin (Mediatech), 0.5% e-amino-caproic acid (Sigma-Aldrich), and 2% B6 mouse serum were added, and samples were incubated at 37°C and 5% CO<sub>2</sub>. On day 3, BrdU was added at 0.025 μg/ml. On day 4, type II collagenase (564 U/ml) with RPMI 1640, 0.5% BSA, and 40 μg/ml DNase I were added, and wells were shaken at 50 rpm for 30 min at 37°C. Three rings were pooled as one sample and digested for another 30 min. The
suspension was triturated 40 times, and EDTA was added to 10 mM. Cells were incubated 5 more min, passed through a 70-μm filter, and prepared for flow cytometry.

**CD11c⁺ cell subset sorting**

Mice were immunized with OVA/CFA at multiple sites. On day 8, draining cervical, axillary, brachial, inguinal, popliteal, and lumbar nodes were pooled and collagenase digested. T and B cells were depleted using biotinylated CD3 and B220 Abs and magnetic separation (MACS; Miltenyi Biotec, Auburn, CA). Live gated CD11c⁺ subsets were then sorted using an FACSVantage (BD Biosciences).

**Immunohistochemistry**

Immunohistochemical staining of fresh-frozen sections was performed as described (6). Abs used included CD31, CD11c, desmin (Labvision/Thermo Scientific, Fremont, CA), and gp38 (clone 8.1.1; Developmental Studies Hybridoma Bank, Iowa City, IA). HRP or alkaline phosphatase-conjugated secondary Abs were from Jackson Immunoresearch Laboratories.

**Quantitation of vessels with disrupted FRC organization**

Sections stained for desmin and CD31 were assessed for percent of T-zone HEV with disorganized FRCs per high-power field (×40 objective). HEV were scored as having disorganized FRCs if the FRC sheath was loosely organized, with splaying of FRCs at angles away from vessel wall or across endothelium.

**Evans blue assays**

For detection of Evans blue permeability in frozen sections, mice received tail vein injections of 200 μl of 3.75 mg/ml Evans blue (Sigma-Aldrich) and were perfused with PBS after 10 min to wash out intravascular dye. Nodes were then harvested and frozen for cryosectioning.

For measuring the Evans blue content in tissues, 250 μl of 2% Evans blue was i.v. injected 4 h before sacrificing mice. Bilateral lymph nodes were harvested and incubated in 150 μl formamide for 24 h at 37°C, and supernatants were measured in a microplate reader at 620 nm. A serial dilution of 2% Evans blue was used to generate a standard curve for each experiment, and the tissue Evans blue content was expressed relative to the content in 2% Evans blue.

**Generation of bone marrow chimeras**

Wild-type B6 mice were lethally irradiated with 875 rad using an x-ray source, injected with wild-type or CD11c-DTR bone marrow, and then allowed to reconstitute for a minimum of 6 wk (as described in Ref. 13).

**Lymph node VEGF measurements**

Lymph nodes were solubilized in lysis buffer and subject to VEGF measurement using DuoSet mouse VEGF (R&D Systems, Minneapolis, MN) as previously described (6).

**Anti-FBS Ab detection**

ELISA plates were coated with 1% FBS overnight at 4°C and blocked with 1 mg/ml OVA for 2 h. Serum diluted 1000-fold in PBT buffer (0.5% BSA, 0.4% Tween-20, PBS) was added for 2 h. Bound Ab was detected with anti-mouse IgG-HRP (Southern Biotech, Birmingham, AL) and 3,3',5,5'-tetramethylbenzidine substrate (Sigma-Aldrich). Results were normalized to results obtained from a standard batch of reactive serum.

**Results**

**Vascular quiescence and stabilization after lymph node vascular expansion**

We have previously shown that s.c. injection of BMDCs stimulates increased endothelial cell proliferation in draining lymph nodes by day 2 and expansion of endothelial cell numbers by day 5 (6). We further characterized the alterations in endothelial cell activity over time in this system. Upregulated endothelial cell proliferation and vascular expansion was followed after day 5 by a phase of downregulated endothelial cell proliferation and maintenance of endothelial cell numbers (Fig. 1A–C). During this second phase, lymph node cellularity was also reduced (Fig. 1D). We measured the efficiency of HEV cell trafficking by determining the number of injected splenocytes entered per PNAd⁺ endothelial cell. HEV trafficking efficiency was upregulated at day 1 but was downregulated to below baseline by day 6 (Fig. 1E). VCAM-1 expression on PNAd⁺ endothelial cells was analyzed by flow cytometry (Supplemental Fig. 1) and showed a similar pattern of regulation (Fig. 1F), suggesting that alterations in HEV endothelial cell phenotype contributed to alterations in HEV trafficking efficiency. Together, these results suggested that although the initiation of vascular expansion is characterized by upregulated endothelial cell proliferation, HEV trafficking efficiency, and VCAM-1 expression, endothelial cell quiescence is re-established after vascular expansion, with downregulated endothelial cell proliferation, HEV trafficking efficiency, and VCAM-1 expression.

As pericytes contribute to the stabilization of vessels after vascular expansion (8, 10), we examined the organization of the perivascular FRCs. FRCs throughout the lymph node can be identified by their expression of desmin (16, 18, 26). At homeostasis, the majority of T-zone HEVs were ensheathed by desmin⁺ cells arranged in a tightly packed laminar structure (Fig. 1G, left panel, 1I). However, by day 2 after BMDC injection, FRC organization around vessels appeared disrupted. FRCs around most HEV were more loosely organized, and individual FRCs were visible, with some protruding or splayed out at an angle perpendicular to the vessel wall (Fig. 1G, middle panel, 1I). By day 8, FRCs were reassembling around HEVs in a more tightly organized structure (Fig. 1G, right panel, 1I). Staining for gp38, another marker of FRCs (18), showed the same alterations in FRC organization over time (Fig. 1H). In addition to altered perivascular FRC organization, FRCs associated with fibrils near vessels also appeared to undergo organizational alterations, with the fibrillar desmin appearing less continuous and more ragged at day 2 and recovering by day 8 (Fig. 1G). These data suggested that the re-establishment of endothelial cell quiescence after day 5 is accompanied by FRC reassembly that likely contributes to the re-establishment of vascular stabilization.

CD11c⁺ and CD11c⁺MHC II⁺ cells accumulate with different kinetics

We have previously shown that DCs are involved in the initiation of vascular expansion (6) and wanted to ask whether they could also regulate the re-establishment of vascular quiescence and stabilization. We examined whether the presence of different CD11c⁺ cell populations correlated with the initiation of vascular expansion versus the subsequent re-establishment of vascular quiescence and stabilization. CD11c⁺MHC II⁺ cells were composed mostly of CD11c⁺MHC II⁺ and CD11c⁺MHC II⁺ cells at day 0 (Fig. 2A, 2B, 2E), and these two populations accumulated rapidly after BMDC injection, reaching peak or near-peak numbers by day 2 (Fig. 2B). The CD11c⁺ cells that accumulated included the injected BMDCs, which had a CD11c⁺MHC II⁺ phenotype (Fig. 2D). In contrast, the CD11c⁺MHC II⁺ cells accumulated more slowly, with numbers rising more steeply after day 2 and peaking at day 5 (Fig. 2A, 2B). The CD11c⁺MHC II⁺ cells were endogenous cells, as BMDCs remained CD11c⁺ in phenotype through their peak accumulation at day 5 and subsequent reduction in numbers (data not shown) (6). The small population of CD11c⁺MHC II⁺ cells showed relatively low and low levels of accumulation (Fig. 2B). The initiation of vascular expansion, then, was characterized by a relative predominance of CD11c⁺ cells, resulting in a low ratio of CD11c⁺MHC II⁺ cells to CD11c⁺ cells (Fig. 2C). In contrast, the onset of the re-establishment of vascular quiescence and stabilization at day 5 was characterized by increased accumulation of CD11c⁺MHC II⁺ cells and an increase in the CD11c⁺MHC II⁺ cell to CD11c⁺ cell ratio (Fig. 2C).
These results raised the possibility that CD11c<sup>med</sup> cells mediated the initiation of vascular expansion, whereas CD11c<sup>hi</sup>MHC II<sup>med</sup> cells mediated the re-establishment of vascular quiescence and stabilization.

We further assessed the role of CD11c<sup>med</sup> cells in the initiation of vascular expansion. OVA/CFA injection also led to rapid accumulation of predominantly CD11c<sup>med</sup> cells and slower accumulation of CD11<sup>chi</sup>MHC II<sup>med</sup> cells at day 2 (Fig. 2E), further supporting a role for CD11c<sup>med</sup> cells in initiating vascular expansion. Preliminary experiments had indicated that s.c. and i.p. DT injection in CD11c-DTR mice had different effects on the early accumulation of CD11c<sup>med</sup> cells, and we used these differences to test the role of CD11c<sup>med</sup> cells in the initial upregulation of endothelial cell proliferation. Subcutaneous and i.p. DT at 8 h prior to OVA/CFA at day 0 resulted in a similar fold reduction in CD11c<sup>hi</sup>MHC II<sup>med</sup> cell numbers at day 2 when compared with the respective CRM-treated controls (7.4-fold ± 4.4 SD for s.c. DT and 4.6-fold ± 1.8 for i.p. DT; p = 0.2). In contrast, CD11c<sup>med</sup> populations were less well reduced with i.p. DT injection than with s.c. DT (Fig. 2E,F) for CD11c<sup>med</sup> cells as a whole, 10.8-fold ± 7.2 for s.c. DT and 3.6-fold ± 1.8 for i.p. DT; p < 0.05). Endothelial cell proliferation is up-regulated by OVA/CFA (Fig. 2G) from the basal proliferation rate of ~1% (Fig. 1A), and s.c. DT dramatically reduced the upregulation of endothelial cell proliferation (Fig. 2G). I.p. DT, however, led to a less dramatic reduction of the upregulation (Fig. 2I). These results indicated a correlation between the extent of reduction in CD11c<sup>med</sup> cell numbers and the extent of reduction in the upregulation of endothelial cell proliferation and were consistent with the idea that CD11c<sup>med</sup> cells mediated the initiation of vascular expansion.

CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion disrupts endothelial cell quiescence

We asked whether CD11c<sup>hi</sup>MHC II<sup>med</sup> cells mediated the re-establishment of endothelial cell quiescence that was observed after day 5. I.p. injection of DT in CD11c-DTR mice at day 6 depleted CD11c<sup>hi</sup>MHC II<sup>med</sup> cells but not CD11c<sup>med</sup> cells or the small population of CD11c<sup>hi</sup>MHC II<sup>hi</sup> cells (Fig. 3A, 3B). CD11c<sup>hi</sup>MHC II<sup>med</sup> cells at day 7 consist mainly of CD8<sup>2</sup>CD11b<sup>+</sup> cells (70%) and CD8<sup>+</sup>CD11b<sup>+</sup> cells (16.5%), with 64% of CD8<sup>2</sup>CD11b<sup>+</sup> subset expressing F4/80, and all subsets were equally depleted after DT treatment (Supplemental Fig. 2). The depletion
CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells are localized near vessels and promote endothelial cell quiescence in vitro

We examined the localization of CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells relative to the vasculature. CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells comprised the vast majority of CD11c\textsuperscript{hi} cells from day 5 onward (Fig. 2B), suggesting that the localization of CD11c\textsuperscript{bright} cells in frozen sections reflected the localization of CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells. Consistent with this notion, CD11c\textsuperscript{bright} cells appeared numerous at day 7 but appeared relatively sparse in DT-treated CD11c-DTR mice depleted of CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells (Supplemental Fig. 3). In the T-zone, CD11c\textsuperscript{bright} cells were enriched in regions that were rich in blood vessels and lymphatic sinuses. These regions were under and between follicles and in vessel-rich cords that coursed toward the medulla (Fig. 4A). In the medullary cords, CD11c\textsuperscript{bright} cells aggregated around HEV and small blood vessels, and to a lesser extent, at lymphatic sinus walls (Fig. 4B). Consistent with other studies showing DC-FRC associations (16, 26, 27), CD11c\textsuperscript{bright} DCS were associated with FRCs at both reticular fibrils and vessel walls (Fig. 4C). The localization of CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells around and near vessels suggested that these cells could potentially act directly on local vessels to modulate vascular quiescence and stabilization.

We asked whether CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells could promote endothelial cell quiescence in vitro. Because FRCs may regulate endothelial cell activity (13), we cultured aortic rings that provided a source of both endothelial and fibroblast-type cells. Culturing aortic rings in fibrin gels stimulates proliferative outgrowths of endothelial cells with a microvascular phenotype (28). CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells were added to these cultures and specifically reduced endothelial cell proliferation (Fig. 5A, 5B). The area covered by the outgrowths and total endothelial cell numbers remained unchanged (data not shown). These results further establish that CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells can mediate endothelial cell quiescence and suggest that they are able to act directly on vessels.

CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cell depletion disrupts vascular stabilization

Depletion of CD11c\textsuperscript{hi}MHC I\textsuperscript{med} cells at day 6 also disrupted FRC reassembly. Similar to the appearance of vessels at day 2 after lymph node stimulation (Fig. 1G, 1H), the FRCs around the majority of T-Zone HEVs were more loosely organized, with many cells oriented away from the vessel wall (Fig. 6A–D, Supplemental Fig. 4A). Some FRCs also appeared displaced toward the lumen and were associated with the bodies of endothelial cells.
The vessels were more severely affected than the vessels at day 2, as HEVs tended to look more angular and less rounded (Fig. 6A–C, Supplemental Fig. 4A). FRCs associated with small non-HEV blood vessels, lymphatic sinuses, and reticular fibrils near T-zone HEVs also appeared more disorganized (Fig. 6A, Supplemental Fig. 4A), suggesting a generalized effect on the FRCs of vessel-rich regions in the T-zone. FRCs associated with HEV of the medullary cords were also disrupted (Supplemental Fig. 4B).

We injected Evans blue dye i.v. to test blood vessel permeability. More Evans blue accumulated in the CD11chiMHC IImed cell-depleted lymph nodes, suggesting that the disrupted blood vessels were more permeable to small solutes (Fig. 6E). Together, these results suggested that, in addition to promoting endothelial quiescence, CD11chiMHC IImed cells regulate the re-establishment of vascular stabilization.

Vascular quiescence and stabilization is disrupted in CD11c-DTR → wild-type bone marrow chimeras

To exclude the possibility that the disrupted vascular quiescence and stabilization upon DT treatment of CD11c-DTR mice reflected direct effects of DT on nonhematopoietic cells, such as endothelial cells and FRCs, we made bone marrow chimeras using CD11c-DTR donors and lethally irradiated wild-type recipients. Depletion of CD11chiMHC IImed cells on day 6 after BMDC injection led to an increased proliferation of total endothelial cells with a more pronounced effect on PNAd+ endothelial cell proliferation, increased HEV trafficking efficiency, and increased HEV VCAM-1 expression (Fig. 7A–F). Vascular permeability was increased, and FRC reassembly around vessels was disrupted (Fig. 7G, 7H). These results further supported a role for CD11chiMHC IImed cells in re-establishing vascular quiescence and stabilization after lymph node vascular expansion.

FIGURE 3. Depletion of CD11chiMHC IImed cells disrupts endothelial cell quiescence. A–I, Wild-type (WT) or CD11c-DTR (DTR) mice were injected with BMDCs on day 0 and received i.p. injection of 100 ng DT or CRM on day 6. Popliteal nodes were harvested at day 7. A, Flow cytometry plot showing effect on CD11c+ subsets with DT treatment of DTR mice. B, Number of cells in each CD11c+ subset. C, Ratio of CD11chiMHC IImed (R1) cell numbers to CD11cmed (R2 + R3) cell numbers. D, Proliferation of total CD45+ CD31+ endothelial cell population. E, Proliferation of CD45+ CD31+PNAd+ HEV endothelial cells. F, Proliferation of CD45+ CD31+PNAd+ endothelial cells. G, HEV trafficking efficiency. H, VCAM-1 expression level on PNAd+ endothelial cells. I, Total cell number per lymph node. For B–I, n = 6 mice for each condition. For J and K, n = 4 mice for each condition. For J, asterisk refers to comparison with the same CD11c+ subset in CRM-treated mice. *p < 0.05; **p < 0.01; ***p < 0.001 with the Student t test.

FIGURE 4. CD11chiMHC IImed cells localize near vessels. Immunohistochemical staining of day 8 lymph nodes. The CD11c stain was developed lightly so that CD11cbright cells were most apparent. Blood vessels are CD31bright, and lymphatic sinuses are CD31med. Localization of CD11chiMHC IImed cells relative to vasculature in the T-zone (A, original magnification ×10) and medullary cords (B, original magnification ×40). F denotes B cell follicles. C, Localization of CD11chiMHC IImed cells relative to desmin+ FRCs. Original magnification ×160. For A–C, figures are representative of at least three lymph nodes over three experiments.
CD11chiMHC IImed cells also mediate vascular quiescence and stabilization in OVA/CFA-stimulated nodes

We asked whether CD11chiMHC IImed cells could also mediate the re-establishment of vascular quiescence and stabilization in lymph nodes stimulated with OVA/CFA. The initial rapid accumulation of CD11cmed cells (Fig. 2E, Supplemental Fig. 5A) and reduction of the ratio of CD11chiMHC IImed cells to CD11cmed cells (Supplemental Fig. 5B) was followed by accelerated CD11chiMHC IImed cell accumulation and progressive upregulation of the ratio from day 5 on (Supplemental Fig. 5A,5B). Consistent with our previous findings (6), endothelial cell proliferation peaked at days 5–8, corresponding with the plateau of vascular expansion, and was subsequently downregulated by day 10 (Supplemental Fig. 5C,5D). Trafficking efficiency was upregulated early and then, similar to BMDC-stimulated nodes, was downregulated past baseline by day 10 (Supplemental Fig. 5E). VCAM-1 was also downregulated past baseline at day 10, but, in contrast to stimulation with BMDCs, VCAM-1 was also at lower than baseline at day 1 (Supplemental Fig. 5F). Similar to lymph nodes stimulated with BMDC, early disorganization of FRC was followed by reassembly (Supplemental Fig. 5G). CD11chiMHC IImed cells correlated with downregulation of endothelial cell proliferation, reduction of trafficking efficiency and VCAM-1 below baseline, and FRC reassembly.

OVA/CFA-stimulated CD11c-DTR mice were injected i.p. with DT to deplete CD11chiMHC IImed cells at day 9 or 10, a time point just after the peak of endothelial cell proliferation. Twenty-four hours after DT injection, endothelial cell proliferation, trafficking efficiency, and VCAM-1 expression were increased, and FRC reassembly was disrupted (Supplemental Fig. 5H–M). These results suggested that CD11chiMHC IImed cells also mediate vascular quiescence and stabilization upon immunization with OVA/CFA and support a general role for these cells in regulating the re-establishment of vascular quiescence and stabilization upon lymph node stimulation.

CD11chiMHC IImed cells mediate downregulated VEGF levels

We have previously shown that VEGF is upregulated by day 1 after lymph node stimulation and is an important mediator of the initial upregulation of endothelial cell proliferation (6). In comparison with the elevated VEGF level at day 2 after BMDC injection (Fig. 8A), VEGF was downregulated at day 8, raising the possibility that the downregulation of endothelial cell proliferation during the re-establishment of quiescence was in part due to VEGF downregulation. Depletion of CD11chiMHC IImed cells at day 6 led to

FIGURE 5. CD11chiMHC IImed cells mediate endothelial cell quiescence in vitro. Aortic rings were cultured with sorted CD11c+ cell subsets and analyzed by flow cytometry on day 4. A. Flow cytometry plot showing the gating for sorting of CD11chiMHC IImed (R1) or CD11cmed (R2 + R3) cells. B. Proliferation of CD45+CD31+ endothelial cells from the aortic ring culture. Each symbol represents one pooled sample (see Materials and Methods). *p < 0.05 with the Student’s t test; p = 0.7 for comparison between control culture and 15K CD11cmed cell coculture.

FIGURE 6. Depletion of CD11chiMHC IImed cells disrupts FRC reassembly and vascular barrier function. Mice were injected with BMDCs on day 0, received i.p. DT or CRM on day 6, and were analyzed on day 7. Desmin+ FRC organization around and near T-zone HEV at ×160 (A) and ××40 (B). Arrows highlight examples of perivascular FRCs that are more loosely associated or protruding out from the vessel wall. C, gp38+ FRC organization around HEV. Arrows highlight examples of perivascular FRCs that are protruding out from the vessel wall. Original magnification ×40. Photos are representative of at least three mice per condition. ***p < 0.001.
increased VEGF levels at day 7 (Fig. 8B), suggesting that CD11c\(^{hi}\) MHC I\(^{med}\) cells mediated the relative downregulation of VEGF levels during the period of endothelial cell quiescence. Anti-VEGF abrogated the increase in endothelial cell proliferation that occurred with depletion of CD11c\(^{hi}\)MHC I\(^{med}\) cells (Fig. 8C), suggesting that the increase in VEGF induced by CD11c\(^{hi}\)MHC I\(^{med}\) cell depletion was an important factor in disrupting endothelial cell quiescence. Together, these results suggested that CD11c\(^{hi}\)MHC I\(^{med}\) cells mediate the downregulation of VEGF levels during the period of endothelial cell quiescence.

### Disrupted vascular quiescence does not lead to vascular expansion

We asked whether the increased endothelial cell proliferation induced by CD11c\(^{hi}\)MHC I\(^{med}\) cell depletion resulted in expanded endothelial cell numbers. Endothelial cell numbers remained constant up to 11 d after continuous depletion of CD11c\(^{hi}\)MHC I\(^{med}\) cells (Supplemental Fig. 6A, 6C, 6E), despite increased endothelial cell proliferation at all time points (Supplemental Fig. 6B, 6D, 6F). This is in contrast to the first few days postimmunization when increased endothelial cell proliferation led to expanded endothelial cell numbers (6) (Fig. 1A, 1B). These results suggested that endothelial cell survival was compromised upon CD11c\(^{hi}\)MHC I\(^{med}\) cell depletion at day 6, potentially because of the severity of vessel destabilization, because endothelial cells require CD11c\(^{hi}\)MHC I\(^{med}\) cells for survival after proliferation or because other microenvironmental elements present early postimmunization are lacking at day 6.

**Disrupted re-establishment of vascular quiescence and stabilization is associated with attenuated T cell-dependent B cell responses**

Increased lymphocyte entry upon disruption of vascular quiescence and stabilization could potentially increase competition for lymphocyte survival factors and disrupted FRC organization could potentially interfere with lymphocyte migration and expression of stromal-derived lymphocyte survival factors. We thus asked whether disrupting the re-establishment of vascular quiescence and stabilization was associated with disruption of the developing immune response. The injected BMDCs are cultured with FBS, and they induced the development of germinal centers and anti-FBS IgG titers by day 8. CD11c\(^{hi}\)MHC I\(^{med}\) cell depletion at day 6 led to reduced germinal center B cell numbers and anti-FBS IgG at day 8 (Fig. 9A, 9B), suggesting attenuation of the T cell-dependent B cell response. IgM-secreting plasma cells induced during splenic T cell-independent responses express DTR-GFP fusion protein in CD11c-DTR mice and are directly depleted by DT injection (29), raising the possibility that the reduced anti-FBS IgG

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**FIGURE 7.** Depletion of CD11c\(^{hi}\)MHC I\(^{med}\) cells in CD11c-DTR bone marrow chimeras disrupts vascular quiescence and stabilization. Wild-type mice reconstituted with CD11c-DTR bone marrow received footpad injection of BMDCs on day 0 and 200 ng DT or CRM i.p. on day 6. Popliteal nodes were harvested at day 7. CD11c\(^{+}\) cell subsets were gated as in Figs. 2A, 3A. A. CD11c\(^{hi}\)MHC I\(^{med}\) cell numbers. B. Ratio of CD11c\(^{hi}\)MHC I\(^{med}\) (R1) cells to CD11c\(^{med}\) (R2 + R3) cells. C. Proliferation of total CD45\(^{+}\)CD31\(^{+}\) endothelial cell population. D. Proliferation of PNAd\(^{+}\) endothelial cells. E. Relative HEV trafficking efficiency. Values in DT-treated mice were normalized to that of the CRM-treated controls in each experiment. F. VCAM-1 expression level on PNAd\(^{+}\) endothelial cells. For A–F, n = 4–8 mice per group. G. Vascular permeability as indicated by Evans blue content in lymph nodes. n = 4 mice per group. H. Percent of T-zone HEV that have disrupted FRC organization. Results represent at least 24 fields over five lymph nodes for each condition. For A–H, \(**\) \(p < 0.05; \ast \ast \ast \) \(p < 0.01\) with the Student t test.

**FIGURE 8.** CD11c\(^{hi}\)MHC I\(^{med}\) cells mediate downregulated lymph node VEGF levels. A. Relative VEGF levels in draining lymph nodes with time after BMDC injection. VEGF levels at each time point were normalized to that of unstimulated lymph nodes taken from control mice at the same time. \(n = 4\) mice for each time point. B and C. Mice received BMDCs on day 0, i.p. DT or CRM on day 6, and were examined on day 7. B. Lymph node VEGF levels upon CD11c\(^{hi}\)MHC I\(^{med}\) cell depletion. \(n = 6–9\) mice per group. C. Anti-VEGF blocks the increase in endothelial cell proliferation induced by CD11c\(^{hi}\)MHC I\(^{med}\) cell depletion. Mice received 200 \(\mu\)g anti-VEGF (R&D Systems) or control goat IgG on day 6. Each symbol represents one mouse. Results representative of two similar experiments. \(\ast \) \(p < 0.05; \ast \ast \ast \) \(p < 0.01\) with Student t test when compared with day 0.
reflected direct plasma cell depletion by DT rather than a downstream effect of CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion. Anti-FBS IgM titers were not reduced by DT injection (Fig. 9C), arguing against a significant contribution of direct plasma cell depletion. We observed that ∼25% of IgG<sub>B220<sup>low</sup></sub> plasma cells expressed GFP (Supplemental Fig. 7). However, DT injection led to reduction of both GFP<sup>+</sup> and GFP<sup>−</sup> plasma cell numbers (Supplemental Fig. 7), suggesting that although a percentage of plasma cells may be directly depleted by DT, the reduction in anti-FBS IgG likely also reflects attenuated plasma cell development. Because it was possible that Ag was transferred from BMDCs to endogenous CD11c<sup>hi</sup>MHC II<sup>med</sup> cells (30), CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion may have led to attenuated B cell responses by interfering with Ag presentation and consequent T cell activation. However, the numbers of CD25<sup>+</sup>, CD69<sup>+</sup>, and CD44<sup>+</sup> T cells were unchanged (Supplemental Fig. 8), suggesting that a lack of Ag-presenting function at day 6 did not contribute to the attenuated B cell response at day 8. Together, our results suggested that CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion and disrupted re-establishment of vascular quiescence and stabilization were associated with attenuated development of T cell-dependent B cell responses.

**CD11c<sup>med</sup> cell accumulation is regulated by CD11c<sup>hi</sup>MHC II<sup>med</sup> cells**

We showed that i.p. DT treatment of CD11c-DTR mice at homeostasis depleted CD11c<sup>hi</sup>MHC II<sup>med</sup> cells but not CD11c<sup>med</sup> cells (Fig. 3J) but that the same DT treatment followed by OVA/ CFA immunization resulted in reduced accumulation of CD11c<sup>hi</sup>MHC II<sup>hi</sup> cells at day 2 (Fig. 2H). CD11c<sup>med</sup>MHC II<sup>hi</sup> cells represent DCs that migrate from the skin (31) and, upon immunization, large numbers of these cells accumulate in the first 2 d (Supplemental Fig. 5A). We hypothesized that the reduced CD11c<sup>hi</sup>MHC II<sup>hi</sup> cells with OVA/CFA reflected an indirect effect of CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion on CD11c<sup>med</sup>MHC II<sup>hi</sup> cell migration or survival rather than direct CD11c<sup>med</sup>MHC II<sup>med</sup> cell depletion. We thus tested the effects of i.p. DT on the accumulation of injected wild-type BMDCs in CD11c-DTR mice. BMDC accumulation in the draining lymph nodes on day 2 was lower in DT-treated mice than in control CRM-treated mice (Supplemental Fig. 9A), and there was a corresponding reduction in the upregulation of endothelial cell proliferation (Supplemental Fig. 9B). Together, our data suggest that although i.p. DT injection directly depletes CD11c<sup>hi</sup>MHC II<sup>med</sup> cells, in the setting of a large influx of CD11c<sup>med</sup> cells from the skin (such as during the first 2 d of an immune response), i.p. DT can also indirectly reduce the accumulation of CD11c<sup>med</sup> cells in lymph nodes.

**Discussion**

We delineate a novel role for CD11c<sup>hi</sup>MHC II<sup>med</sup> DCs in mediating lymph node vascular quiescence and stabilization. We show that these cells accumulate in large numbers near vessels upon vascular expansion in immune-stimulated nodes and mediate the subsequent quiescence and stabilization of the newly expanded vasculature. CD11c<sup>hi</sup>MHC II<sup>med</sup> DCs are considered to be classical or conventional DCs that function to stimulate T cells (32-34). Our results suggest the existence of a DC-vasculature axis whereby CD11c<sup>hi</sup>MHC II<sup>med</sup> DCs, in addition to their T cell-stimulating functions, facilitate lymph node function by mediating vascular quiescence and stabilization.

Our studies were mostly conducted in BMDC-stimulated mice, but examination of OVA/CFA-stimulated mice supported a general role for CD11c<sup>hi</sup>MHC II<sup>med</sup> DCs in re-establishing vascular quiescence and stabilization in immune-stimulated nodes. In both BMDC- and OVA/CFA-stimulated nodes, early CD11c<sup>med</sup> cell accumulation was followed by increased CD11c<sup>hi</sup>MHC II<sup>med</sup> cell accumulation and upregulation of the CD11c<sup>hi</sup>MHC II<sup>med</sup> cell:CD11c<sup>med</sup> cell ratio. OVA/CFA-stimulated nodes demonstrated a broader peak of endothelial cell proliferation, delayed plateauing of endothelial cell expansion, and a more progressive rise in the CD11c<sup>hi</sup>MHC II<sup>med</sup> cell:CD11c<sup>med</sup> cell ratio, perhaps reflecting the prolonged stimulation by the deposited OVA/CFA. Generally, however, the kinetics of CD11c<sup>med</sup> cell and CD11c<sup>hi</sup>MHC II<sup>med</sup> cell accumulation and the regulation of vascular quiescence and stabilization were similar to that of BMDC-stimulated lymph nodes. The main difference between the two systems was that, although VCAM-1 was at below baseline after vascular expansion in both systems, VCAM-1 was upregulated early in BMDC-stimulated nodes but was downregulated early in OVA/CFA-stimulated nodes. This suggests that the exact pattern of cell adhesion molecule regulation in the first 1 to 2 d after lymph node stimulation is stimulus specific. Consistent with this thought, we have found that PNAd<sup>+</sup> is upregulated at day 1 in OVA/CFA-stimulated nodes (T.-C. Tzeng and T.T. Lu, unpublished observations), and s.c. OVA/LPS, similar to injection of LPS-stimulated BMDCs, induces early upregulation of VCAM-1 (T.-C. Tzeng and T.T. Lu, unpublished observations). Together, our data indicate that, although some of the parameters associated with early vascular activation may be stimulus specific, the events associated with the re-establishment of vascular quiescence and stabilization seem consistent between systems, and CD11c<sup>hi</sup>MHC II<sup>med</sup> cells have a general role in mediating quiescence and stabilization.

We observed that FRC organization adjacent to and near blood vessels was modulated over time and by CD11c<sup>hi</sup>MHC II<sup>med</sup> cells. Disrupted FRC organization upon CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion was associated with increased vascular permeability, and disrupted FRC organization at day 2 correlates with the early increase in vascular permeability that has been described (7). These results are consistent with the idea that FRCs function at least in part to regulate lymph node vascular permeability. FRCs are also key sources of chemokines (17), IL-7 (18), and VEGF (13), and our results indicating the modulation of VEGF over time and with CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion raise the possibility that multiple FRC functions may be linked to the state of FRC organization.
Our results suggested that, after vascular expansion, CD11c<sup>hi</sup> MHC II<sup>hi</sup> cells mediate downregulation of endothelial cell proliferation at least in part by mediating downregulation of lymph node VEGF levels. VEGF-expressing FRCs (13) and CD11c<sup>lo</sup>MHC II<sup>med</sup> cells are both located around vessels and on nearby fibrils, raising the possibility that CD11c<sup>hi</sup>MHC II<sup>med</sup> cells downregulate endothelial cell proliferation by acting directly on FRCs to downregulate FRC expression of VEGF. This model does not preclude alternate scenarios whereby DC-derived soluble mediators flow through the fibril-associated conduit system (19–21) to modulate endothelial cell activity directly. Indeed, Jung and colleagues (35) have recently shown that uterine CD11c<sup>hi</sup> DCs mediate implantation site angiogenesis and vascular stabilization and that they express TGFβ1 that can potentially downregulate endothelial cell activity (36, 37).

The CD11c<sup>lo</sup>MHC II<sup>med</sup> DCs at day 7 consisted of mainly CD8<sup>+</sup> CD11b<sup>-</sup> and CD8<sup>+</sup> CD11b<sup>+</sup> subsets, and all subsets were equally depleted in CD11c-DTR mice. Either subset could potentially regulate vascular function. Interestingly, recovery of altered HEV PNA-associated molecules and mucosal addressin cell adhesion molecule-1 is dependent on B cells and lymphoxygen β receptor (LTβR) signals (11). B cells express LTβR signals, and LTβR signals can regulate CD8-CD11c<sup>lo</sup>MHC II<sup>med</sup> DC numbers (38), raising the possibility that B cell-DC interactions regulate the re-establishment of vascular quiescence and stabilization and that CD8<sup>+</sup> CD11c<sup>lo</sup>MHC II<sup>med</sup> DCs are the relevant subset.

Our results suggested a role for CD11c<sup>med</sup> cells in mediating the upregulation of endothelial cell proliferation at the onset of vascular expansion. The injected BMDCs that triggered the upregulation had a CD11c<sup>med</sup>MHC II<sup>med</sup> phenotype and stimulated the rapid accumulation of endogenous CD11c<sup>med</sup>MHC II<sup>hi</sup> and CD11c<sup>lo</sup>MHC II<sup>med</sup> cells. OVA/CFA also stimulated the rapid accumulation of predominantly CD11c<sup>med</sup> cells, and, in DT-treated mice, the extent of upregulation of endothelial cell proliferation correlated with the extent of CD11c<sup>med</sup> cell accumulation. CD11c<sup>med</sup>MHC II<sup>hi</sup> cells include skin-derived DCs, and CD11c<sup>med</sup>MHC II<sup>med</sup> cells include skin-derived DCs, blood-borne monocyte-derived DCs, and plasmacytoid DCs (31, 32, 39). Further studies will be needed to identify the exact CD11c<sup>med</sup> subpopulation(s) involved in initiating vascular expansion and also whether they could be related in lineage to the late-accumulating CD11c<sup>lo</sup>MHC II<sup>med</sup> cells.

Our studies mainly focused on the role of CD11c<sup>lo</sup>MHC II<sup>med</sup> DCs in mediating the re-establishment of vascular quiescence and stabilization after vascular expansion. However, our experiments also suggested that they regulate vascular activity at homeostasis, as their specific depletion in homeostatic mice by i.p. DT led to increased basal endothelial cell proliferation. The maintenance of homeostatic vascular activity may be important for the accumulation of skin-derived DCs and the robust upregulation of endothelial cell proliferation that occurs at day 2 after inflammatory stimulation, as i.p. DT at homeostasis attenuated the accumulation of CD11c<sup>med</sup>MHC II<sup>hi</sup> cells after OVA/CFA and of injected BMDCs and also attenuated upregulation of endothelial cell proliferation. We observed that CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion disrupted FRC organization around sinuses, raising the possibility that the reduced accumulation of skin-derived DCs reflects disrupted lymphatic function. CD11c<sup>lo</sup>MHC II<sup>med</sup> cells at homeostasis, then, may regulate vascular quiescence and stabilization that is important for the accumulation of Ag-bearing DCs and for full vascular expansion in the early phase of an immune response.

Our results suggest that the re-establishment of vascular quiescence and stabilization may contribute to the optimal development of T cell-dependent B cell responses. Although it is possible that Ag was transferred from the injected BMDCs to endogenous CD11c<sup>hi</sup>MHC II<sup>med</sup> DCs and the reduced B cell response upon CD11c<sup>hi</sup>MHC II<sup>med</sup> cell depletion at day 6 was related to the lack of Ag presentation to T cells, activation of T cells that participate in the B cell response between days 6 and 8 is likely to have occurred at an earlier time point before CD11c<sup>lo</sup>MHC II<sup>med</sup> cell depletion (40, 41), and we also observed that activated T cell numbers were unchanged. Instead, we hypothesize that the attenuated B cell response is related to the following factors: 1) increased B cell trafficking induced by CD11c<sup>lo</sup>MHC II<sup>med</sup> cell depletion may increase competition for limited survival factors that are needed for optimal germinal center development; 2) FRCs are a source of survival factors (18, 42), and disrupted FRC organization may be associated with attenuated expression of these survival factors; and 3) Ag-specific T cells accumulate under and between B cell follicles and migrate into follicles during germinal center development (41, 43). These areas of accumulation are HEV and CD11c<sup>lo</sup>MHC II<sup>med</sup> cell-rich areas. Potentially, disrupted FRC organization around vessels and nearby fibrils in these areas leads to disrupted T cell migration and follicular entry, thereby limiting the amount of T cell help. Further studies will be needed to determine the mechanism(s) by which vascular quiescence and stabilization may contribute to the developing immune response.

Our findings describe a novel role for DCs in regulating lymph node vascular quiescence and stabilization and have potential implications for treating autoimmune and lymphoproliferative diseases. Autoimmune diseases, such as systemic lupus erythematosus, characterized by pathogenic autoantibodies are associated with enlarged lymph nodes with abundant vasculature (44), and the survival and aggressiveness of lymphomas may be related to the state of the vasculature (45, 46). Targeting lymph node CD11c<sup>lo</sup>MHC II<sup>med</sup> DCs for their effects on the vasculature may be a means to disrupt autoimmune and lymphoproliferative processes.

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