CD18 is required for optimal lymphopenia-induced proliferation of mouse T cells

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Sarin R, Abraham C. CD18 is required for optimal lymphopenia-induced proliferation of mouse T cells. Am J Physiol Gastrointest Liver Physiol 303: G851–G860, 2012. First published July 19, 2012; doi:10.1152/ajpgi.00520.2011.—Lymphocyte numbers are tightly regulated in the periphery, and during periods of acute lymphopenia, T cell numbers, diversity, and functionality are reestablished through lymphopenia-induced proliferation. In contrast to the costimulation requirements of antigen-driven proliferation, a number of costimulatory molecules are not required for lymphopenia-induced proliferation. However, the requirement for major histocompatibility complex (MHC)-T cell receptor (TCR) interactions and the enhanced lymphopenia-induced proliferation in T cells with higher TCR affinity argue for a role for surface molecules that contribute to efficient MHC-TCR interactions, in particular adhesion molecules. CD18 is an integrin that contributes to the activation of peripheral and intestinal T cells through adhesive and costimulatory mechanisms. We found that CD18 is required for optimal polyclonal and monoclonal CD4+ T cell lymphopenia-induced proliferation in recombination-activating gene 1-deficient (RAG−/−) mice; this requirement persisted over time. Uniquely, the dependency on CD18 in CD4+ T cells is in the rapid proliferation in RAG−/− recipients and in the slow homeostatic proliferation in irradiated BALB/c recipients. Consistent with the proposed role for intestinal microbiota in lymphopenia-induced rapid proliferation in RAG−/− mice, we observed a significant reduction in rapid proliferation upon treatment of mice with antibiotics; however, the dependency on CD18 for optimal lymphopenia-induced proliferation persisted. Moreover, the dependency for CD18 is maintained over a wide range of numbers of initially transferred T cells, including a low number of initially transferred T cells, when the drive for proliferation is very strong and proliferation is more rapid. Overall, these data argue for an essential and broad role for CD18 in lymphopenia-induced proliferation.

T lymphocytes; costimulation; adhesion molecules; cell trafficking

LYMPHOCYTE NUMBERS ARE TIGHTLY regulated in the periphery, and during periods of acute lymphopenia, T cell numbers, diversity, and functionality are reestablished through lymphopenia-induced proliferation (10, 12). The precise regulation of this process is important, in that inadequate proliferation can lead to low T cell numbers and an immunocompromised state, while excessive proliferation of inappropriate T cell subsets can lead to autoimmunity and inflammatory disorders. Importantly, a commonly utilized experimental mouse model of colitis is based on transfer of naive T cells to lymphopenic hosts, where T cells undergo lymphopenia-induced proliferation and, in the absence of cotransferred regulatory T cell populations, eventually, intestinal inflammation (9). As a result, understanding the factors and mechanisms that contribute to lymphopenia-induced proliferation is critical to our ability to address defects in this proliferation process.

Two forms of lymphopenia-induced proliferation are recognized: 1) homeostatic lymphopenia-induced proliferation, which is characterized by a slow and steady proliferating T cell population, and 2) “spontaneous” lymphopenia-induced proliferation, which is characterized by a distinct, rapidly proliferating T cell population in a recombination-activating gene (RAG) 1-deficient (RAG−/−) host and is thought to be driven by intestinal microbiota (7, 18, 23, 35) (we refer to this population as lymphopenia-induced rapid proliferation). Lymphopenia-induced proliferation has shared and distinct requirements relative to antigen-driven T cell proliferation. Shared requirements include a dependence on cytokines such as IL-7 and IL-15 (29, 31) and on T cell receptor (TCR) recognition of peptide in the context of major histocompatibility complex (MHC) (10, 12). On the other hand, in contrast to antigen-driven proliferation, various costimulatory molecule interactions, such as OX40-OX40L, CD40-CD40L, and 41BB-41BBL, are not required for lymphopenia-induced proliferation (27, 38). Moreover, in contrast to the broad requirement for CD28-B7 interactions during antigen-driven proliferation, CD28-B7 interactions are only required for the rapidly dividing population undergoing lymphopenia-induced proliferation (14).

Lymphocyte function-associated antigen (LFA)-1 (αLβ2 or CD11a/CD18) plays a critical role in trafficking of naive T cells to peripheral and intestinal secondary lymphoid organs and in antigen-specific T cell activation (1, 2, 5, 13, 16, 30, 32). Moreover, polymorphisms in LFA-1 have recently been associated with ulcerative colitis (6), and individuals with LFA-1 dysfunction can develop chronic intestinal inflammation (37), pointing to an important role for LFA-1 in intestinal immune homeostasis and human inflammatory bowel disease. Despite the lack of requirement for a number of costimulatory interactions assessed in lymphopenia-induced proliferation (27, 38), we hypothesized that LFA-1 may have a modulatory role in lymphopenia-induced proliferation for several reasons. The requirements for its MHC-TCR interaction in lymphopenia-induced proliferation (10) and improved lymphopenia-induced proliferation in T cells with a higher TCR affinity (4) argue for the role of surface molecules, in particular adhesion molecules, in efficient MHC-TCR interactions. LFA-1 enhances the efficiency of MHC-TCR engagement and is particularly important in optimizing antigen-specific T cell activation under conditions of low-affinity MHC-TCR interactions (1, 3). Therefore, we hypothesized that, in contrast to other costimulatory molecules, CD18 would contribute to optimal T cell proliferation during lymphopenic conditions.

To determine the role of LFA-1 in T cell lymphopenia-induced proliferation, we dissected its contribution through the adoptive transfer of CD18+/- CD4+ T cells into lymphopenic recipients. LFA-1 comprises the heterodimeric protein CD11a

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and CD18; in the absence of CD18, LFA-1 is not expressed on the surface of T cells. We found that CD18 is required for optimal CD4+ T cell lymphopenia-induced proliferation and accumulation of polyclonal CD4+ T cells and of monoclonal CD4+ T cells from ovalbumin-specific TCR transgenic (DO11.10) mice. This dependency on CD18 is both for rapid and for homeostatic, slow lymphopenia-induced proliferation. Furthermore, the requirement persists over time, with antibiotic treatment, and over a broad range of initially transferred T cell numbers into the lymphopenic host. The importance of LFA-1 in lymphopenia-induced proliferation is similarly observed with use of neutralizing anti-LFA-1 antibodies. Taken together, these findings identify a uniquely broad contribution of CD18 to optimal CD4+ T cell lymphopenia-induced proliferation.

MATERIALS AND METHODS

Mice. CD18−/− mice (generously provided by A. Beaudet) (30) were backcrossed onto the Balb/c background (9 generations) and then to DO11.10 mice. Balb/c mice were obtained from Jackson Laboratories (Bar Harbor, ME). Thy1.1 RAG-1−/− and Balb/c congenic mice were generously provided by Alexander Khoruts (University of Minnesota). Mice were maintained on autoclaved food and irradiated water in a specific pathogen-free facility with filtered air. Experiments were performed in accordance with our Institutional Animal Care and Use Committee and according to National Institutes of Health guidelines for animal use.

Abs and staining reagents. The following Abs (BD Biosciences and/or eBioscience) were used for flow cytometry: allophycocyanin (APC)-Cy7- and APC-labeled anti-CD4, phycoerythrin (PE)- and FITC-labeled anti-CD18, Cy-Chrome-labeled anti-CD3; and PE-Cy5-labeled Thy1.2. PE-and PE-Cy5-labeled KJ1-26 were purchased from Caltag (Burlingame, CA). M1/74 (anti-LFA-1 neutralizing antibody) and 2A3 (rat IgG2a) were purchased from BIO X Cell (West Lebanon, NH).

In vivo T cell activation. Splenic Thy1.2 CD18+/− and CD18−/− total CD4+ T cells were isolated utilizing CD4+ microbeads (Miltenyi, Auburn, CA), stained with 1.0–2.0 μM carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Eugene, OR), and adoptively transferred by intravenous injection (1.5 × 10⁶ cells of each type per mouse, unless otherwise indicated) into Thy1.2, Thy1.1 RAG−/−, or Thy1.1 (irradiated with 650 cGy) Balb/c mice. In some cases, mice were first subjected to 4 wk of treatment with antibiotics [vancomycin hydrochloride (500 mg/l; Hospira, Lake Forest, IL), ampicillin (1 g/l; DAVA Pharmaceuticals, Fort Lee, NJ), metronidazole (1 g/l; Teva Pharmaceuticals, Sellersville, PA), and neomycin sulfate (1 g/l; MP Biomedicals, Solon, OH)] in their drinking water, as described elsewhere (28). In other cases, mice were given 2% (wt/vol) dextran sodium sulfate (DSS; MP Biomedicals) in their drinking water. The spleen and mesenteric and peripheral lymph nodes (MLN and PLN) were harvested and stained with anti-CD4, anti-CD3, and anti-CD18, along with KJ1-26 or Thy1.2, and analyzed on a FACSCalibur or FACSCanto (BD Biosciences).

T cell homing. Freshly isolated CD18+/+ and CD18−/− splenic T cells were stained with two different concentrations of CFSE. CD18+/+ and CD18−/− CD4+ T cells were adoptively cotransferred at a 1:1 ratio (1.5 × 10⁶ T cells each per mouse). At 16 h after transfer, the spleen, MLN, and PLN were harvested and stained for CD4+, and the homing index was calculated as the ratio of CD18+/+ CD4+ T cells to CD18−/− CD4+ T cells adjusted for the initial input ratio.

Statistical analyses. Statistical comparisons of cell divisions or accumulation between treated groups were assessed using a one-tailed Student’s t-test. P < 0.05 was considered significant.

RESULTS

CD18 is critical for optimal polyclonal CD4+ T cell lymphopenia-induced proliferation in RAG-1−/− mice. To dissect the role of CD18 in CD4+ T cell lymphopenia-induced proliferation, we isolated splenic CD4+ T cells from CD18+/− and CD18−/− mice. CD18−/− mice were utilized as littermate controls, as flow cytometry shows identical CD18 cell surface expression in CD18+/+ and CD18−/− mice. CD18−/− CD4+ T cells are deficient only in LFA-1, as it is the only β2-integrin expressed on peripheral CD4+ T cells (data not shown). We first determined the role of CD18 in the proliferation of polyclonal CD4+ T cells through adoptive cotransfer of CFSE-labeled CD18+/− and CD18−/− CD4+ T cells into RAG−/− mice. This allows for direct comparison of these T cells within the same mice. Using surface staining for CD18 (16, 22), we were able to clearly distinguish the transferred CD18+/− and CD18−/− CD4+ T cells (Fig. 1A). Consistent with previous reports (39), we observed variable degrees of proliferation in different secondary lymphoid structures (Fig. 1). At 3 days after transfer, 12% (spleen), 10% (MLN), and 23% (PLN) of littermate control CD4+ T cells entered into cell division (Fig. 1, B and C). In contrast, only 1.3% (spleen), 2.0% (MLN), and 3.5% (PLN) of CD18−/− CD4+ T cells entered into cell division (Fig. 1, B and C). Moreover, ~5–15% of littermate control CD4+ T cells had undergone rapid proliferation, as evidenced by dilution of CFSE (Fig. 1, B and D). In contrast, <1% of CD18−/− CD4+ T cells had undergone rapid proliferation in each of the secondary lymphoid organs assessed at 3 days after transfer (Fig. 1, B and D). Some of the cells in the rapidly dividing population further upregulated CD18 expression (Fig. 1B); similar CD18 upregulation can be observed upon antigen-driven T cell activation. The defect in CD18−/− CD4+ T cell entry into cell division and in lymphopenia-induced rapid proliferation was accompanied by a decreased percentage of accumulated CD18−/− CD4+ T cells in the spleen, MLN, and PLN compared with CD18+/− CD4+ T cells (Fig. 1F). The defect in CD18−/− CD4+ T cell entry into cell division (Fig. 1, B and C) and, in particular, in lymphopenia-induced rapid proliferation (Fig. 1, B and D) in the secondary lymphoid organs persisted at 5 and 12 days after transfer, indicating that the defect is not simply one of delayed kinetics. In fact, the defect in accumulation of CD18−/− CD4+ T cells in secondary lymphoid organs became more severe over time (Fig. 1F). In analyzing the T cells that were CFSE+ and had not undergone rapid proliferation, we observed a relative defect in CD18−/− CD4+ T cell progression through the cell cycles, pointing to a defect in slow, homeostatic lymphopenia-induced proliferation (Fig. 1E). Therefore, CD18−/− CD4+ T cells demonstrate a persistent defect in lymphopenia-induced proliferation in RAG-1−/− mice.

CD18 is critical for homeostatic lymphopenia-induced proliferation in an irradiated host. To more clearly define CD18 requirements specifically in homeostatic lymphopenia-induced proliferation, we examined CD4+ T cell lymphopenia-induced proliferation in an irradiated host, where only slow, homeostatic proliferation occurs (18). CD18+/− and CD18−/− CD4+ Thy1.2 T cells were cotransferred into irradiated Thy1.1 Balb/c hosts, and 7 days later we assessed CFSE dilution of T cells in the spleen, MLN, and PLN. We selected this later time point because of the slower rate of proliferation in irradiated hosts.
Fig. 1. CD18<sup>−/−</sup> polyclonal CD4<sup>+</sup> T cells are defective in lymphopenia-induced proliferation in adoptive cotransfer models. A: freshly isolated spleen CD4<sup>+</sup> T cells from CD18<sup>−/−</sup> (solid line) and CD18<sup>+/−</sup> (dashed line) mice were separately stained for surface CD18 expression by flow cytometry to demonstrate clearly distinguishable expression patterns. B: representative flow cytometry plots for carboxyfluorescein succinimidyl ester (CFSE) dilution of CD18<sup>−/−</sup> and CD18<sup>+/−</sup>CD4<sup>+</sup> T cells (surface staining of CD18 on y-axis) at 3, 5, and 12 days after cotransfer of freshly isolated CFSE-labeled spleen CD4<sup>+</sup> T cells from CD18<sup>−/−</sup> and CD18<sup>+/−</sup> mice (1.5 × 10<sup>6</sup> each) into recombination-activating gene 1-deficient (RAG-1<sup>−/−</sup>) mice. Spleen, MLN, and PLN were harvested. C–F: percentage of CD4<sup>+</sup> T cells entering cell division (% divided), percentage of CD4<sup>+</sup> T cells that have fully diluted CFSE (%CFSE<sup>−</sup>; i.e., rapid proliferation), percentage of cells within each division from the CFSE<sup>−</sup>CD4<sup>+</sup> T cell population (homeostatic proliferation), and percentage of accumulated CD4<sup>+</sup> T cells in secondary lymphoid structures. Values are means ± SE of 4 mice per condition; data represent 4 independent experiments. Significance is shown for littermate control vs. CD18<sup>−/−</sup> CD4<sup>+</sup> T cells: *P < 0.05; **P < 0.01; ***P < 0.001; †P < 0.0001; ††P < 0.00001.
than in RAG-1−/− hosts. We observed a defect in CD18−/− CD4+ T cell entry into cell division, progression into later cell divisions, and cell accumulation relative to CD18+/− CD4+ T cells in each of the secondary lymphoid structures (Fig. 2, A–D). Moreover, the requirement for CD18 in lymphopenia-induced proliferation in irradiated hosts persisted through 14 days after transfer, with the defect in progression into late cell divisions being more pronounced at this later time point (Fig. 2, A–D). Complete CFSE dilution in a percentage of T cells was seen at the later time point, consistent with the slow rate of proliferation in an irradiated host. Therefore, in addition to the CD18

CD18 is required for lymphopenia-induced proliferation of monoclonal CD4+ T cells. The kinetics of lymphopenia-induced proliferation vary depending on the affinity of the TCR for its MHC-peptide ligand (4, 11, 17). To determine if CD18 is required for lymphopenia-induced proliferation of a monoclonal TCR population of intermediate affinity, we examined proliferation of CD18−/− DO11.10 CD4+ T cells, in which the affinity of the TCR for IAβ/OVA is on the order of 10−5 M−1 (33). Similar to polyclonal CD18−/− CD4+ T cells, we observed that DO11.10 CD18−/− CD4+ T cells had defects in entry into the cell cycle (Fig. 3A) and in rapid lymphopenia-induced proliferation (Fig. 3B) that were accompanied by a defect in accumulation of DO11.10 CD18−/− CD4+ T cells (Fig. 3C) relative to DO11.10 CD18+/− CD4+ T cells in each of the secondary lymphoid organs. These defects persisted at 3, 5, and 12 days after T cell transfer (Fig. 3, A–C). Therefore, CD18 is required for lymphopenia-induced proliferation over a sustained period of time in monoclonal DO11.10 and polyclonal CD4+ T cell populations.

CD18 requirements for polyclonal CD4+ T cell lymphopenia-induced proliferation are present, but reduced, in the absence of competition from wild-type CD4+ T cells. Cotransfer of CD18−/− and CD18+/− CD4+ T cells directly compares T cell responses within mice. However, it is possible that, in the absence of competition from CD18−/− CD4+ T cells, the CD18−/− CD4+ T cell lymphopenia-induced proliferation defect may be less severe. For example, a requirement for CD28 in rapid lymphopenia-induced proliferation was not uncovered until competitive conditions utilizing cotransfer experiments were conducted (15). Therefore, we adoptively transferred CD18−/− and CD18+/− CD4+ T cells into separate RAG-1−/− mice and assessed proliferation at the intermediate time point (5 days after transfer), when a significant degree of proliferation was already observed under cotransfer conditions. In MLN and PLN of separately transferred RAG-1−/− mice, lymphopenia-induced proliferation and accumulation in CD18−/− and CD18+/− CD4+ T cells were equivalent (Fig. 4, A–D). In contrast, the lymphopenia-induced proliferation defect of CD18−/− CD4+ T cells in the spleen persisted, despite lack of competition from CD18−/− CD4+ T cells, with defects in the percentage of cells entering cell division (75% in littermate controls vs. 32% in CD18−/− CD4+ T cells), as well as in the cells that had undergone rapid lymphopenia-induced proliferation (52% in littermate controls vs. 19% in CD18−/− CD4+ T cells; Fig. 4, A–C). Therefore, in the absence of competition from littermate control CD4+ T cells, CD18−/− CD4+ T cell lymphopenia-induced proliferation is maintained in some, but not all, secondary lymphoid organs.

CD18 on CD4+ T cells is less critical for CD4+ T cell trafficking into secondary lymphoid structures of RAG-1−/− than WT mice. The defect in lymphopenia-induced proliferation of CD18−/− CD4+ T cells in the spleen upon separate transfer of CD18−/− and CD18+/− CD4+ T cells was not accompanied by a decrease in CD18−/− CD4+ T cell accumulation in the spleen (Fig. 4D). To further dissect this outcome, we questioned whether CD18−/− CD4+ T cells demonstrate increased trafficking to RAG-1−/− spleen prior to the onset of proliferation, which might compensate for the decreased numbers resulting from defective CD18−/− CD4+ T cell lymphopenia-induced proliferation. We and others have shown that LFA-1 is important for optimal CD4+ T cell trafficking to MLN and PLN in WT mice with intact secondary lymphoid structures (5, 16). In contrast, CD18−/− CD4+ T cell trafficking is significantly increased to WT spleen, presumably due to redistribu-
tion associated with the defect in entry into other lymphoid structures (5, 16). However, RAG-1−/− mice have altered secondary lymphoid organ cellularity and likely altered chemokine concentrations, in part due to the lack of T and B cells. The role of CD18 in T cell trafficking to secondary lymphoid structures in RAG-1−/− mice has not been defined. Therefore, we isolated CD18+/− and CD18−/− CD4+ splenic T cells, stained them with differential concentrations of CFSE, and adoptively cotransferred the T cells into RAG-1−/− mice. After 16 h, prior to the onset of proliferation, we determined the numbers of migrated CD4+ T cells to the spleen, MLN, and PLN. As controls, we also assessed trafficking to secondary lymphoid organs in WT Balb/c mice. As expected (16), the homing and accumulation of CD18−/− CD4+ T cells relative to CD18+/− CD4+ T cells were decreased to the PLN and MLN, but increased to the spleen, in the control WT Balb/c mice (Fig. 5, A and B). As in Balb/c mice, homing of CD18−/− CD4+ T cells to RAG-1−/− spleen was increased (Fig. 5A), with a corresponding increase in absolute numbers of splenic CD18−/− CD4+ T cells compared with CD18+/− CD4+ T cells (Fig. 5B). However, in contrast to the migration defect to Balb/c PLN, CD18−/− CD4+ T cell homing and accumulation

Fig. 4. Defect in CD18−/− polyclonal CD4+ T cell lymphopenia-induced proliferation is reduced in a noncompetitive environment. Freshly isolated CFSE-labeled spleen CD4+ T cells from CD18+/− and CD18−/− mice were adoptively transferred into separate RAG-1−/− mice. At 5 days after transfer, spleen, MLN, and PLN were harvested, and CD4+/CD3+ T cells were examined. A: representative flow cytometry plots of CFSE dilution for CD18+/− and CD18−/− CD4+ T cells. B: percentage of CD4+ T cells entering cell division. C: percentage of CD4+ T cells that have fully diluted CFSE. D: number of accumulated transferred CD4+ T cells in secondary lymphoid structures (%CD4+/CD3+ T cells × no. of splenic, MLN, or PLN cells). Values are means ± SE of 4 mice per condition; data represent 4 independent experiments. **P < 0.01; †P < 0.0001.

Fig. 3. CD18−/− DO11.10 CD4+ T cells are defective in lymphopenia-induced proliferation. Freshly isolated spleen CD4+ T cells from DO11.10 CD18+/+ and CD18−/− mice (1.5 × 10^6 each) were labeled with CFSE and adoptively cotransferred into RAG-1−/− mice. At 3, 5, and 12 days after transfer, spleen, MLN, and PLN were harvested, and CD4+ / CD3−/DO11.10+ T cells were examined. A: percentage of DO11.10 CD4+ CD18+/+ and CD18−/− T cells entering cell division. B: percentage of DO11.10 CD4+ T cells that have fully diluted CFSE. C: percentage of accumulated transferred DO11.10 CD4+ T cells in secondary lymphoid structures. Values are means ± SE of 4 mice per condition; data represent 3 independent experiments. *P < 0.05; **P < 0.01; †P < 0.0001.
Anti-LFA-1 antibody (M17/4) or an isotype control antibody (%CD4 T cells of CFSE dilution for anti-LFA-1- and isotype-treated mice.*** means calculated as ratio of CD18+/−:CD18−/− T cells adjusted for the initial input ratio). In RAG-1−/− mice, the altered early migration of CD18+/− CD4+ T cells is not as pronounced to other secondary lymphoid structures as in WT mice.

Requirements for LFA-1 in polyclonal CD4+ T cell lymphopenia-induced proliferation are observed with neutralizing anti-LFA-1 antibodies. It remained possible that the defects we observed in CD18+/− CD4+ T cell lymphopenia-induced proliferation could be due to defects in CD4+ T cell development or a bias in the CD4+ T cell subset from CD18−/− mice. To eliminate these two possibilities, we adoptively transferred WT CD4+ T cells into RAG-1−/− mice and evaluated lymphopenia-induced proliferation after injection of neutralizing antibodies to LFA-1. Relative to mice injected with an isotype control antibody, in mice treated with anti-LFA-1 antibodies, CD4+ T cell entry into cell division and into rapid lymphopenia-induced proliferation was decreased (Fig. 6, A–C). As observed in mice in which CD18−/− and littermate control CD4+ T cells were separately transferred compared with separately transferred isotype control antibody mice, the lymphopenia-induced driven proliferation defect upon anti-LFA-1 treatment was not accompanied by a defect in cell accumulation in the spleen (Fig. 6D), likely due to the significantly increased trafficking of CD4+ T cells to the spleen under conditions of LFA-1 blockade or deficiency (Fig. 5). Taken together, blockade of LFA-1 interactions as an independent approach confirms the critical role for LFA-1 in CD4+ T cell lymphopenia-induced proliferation.

Requirement for CD18 persists in lymphopenia-induced proliferation, despite attenuated proliferation of the rapidly proliferating T cell population. The population of rapidly proliferating T cells upon transfer into a RAG−/− host is thought to be

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Fig. 6. Mice treated with neutralizing anti-lymphocyte function-associated molecule (LFA)-1 antibody demonstrate a decrease in polyclonal CD4+ T cell lymphopenia-induced proliferation. Freshly isolated CFSE-labeled spleen CD4+ T cells from Thy1.2 WT mice were adoptively transferred into separate Thy1.1 RAG−/− mice. Mice were injected intraperitoneally on days 0 and 2 with 500 μg of neutralizing anti-LFA-1 antibody (M17/4) or an isotype control antibody (rat IgG2a). At 5 days after transfer, CD4+/Thy1.1+ T cells in spleen were examined. A: representative flow cytometry plots of CFSE dilution for anti-LFA-1- and isotype-treated mice. B: percentage of CD4+ T cells entering cell division. C: percentage of CD4+ T cells that have fully diluted CFSE. D: number of accumulated CD4+ Thy1.1+ T cells in spleen (%CD4+ Thy1.1+ T cells × no. of spleen cells). Values are means ± SE of 4 mice per condition; data represent 2 independent experiments. ***P < 0.001; †P < 0.0001.

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Fig. 5. Migration of CD18−/− CD4+ T cells to secondary lymphoid organs is less impaired in RAG-1−/− than WT mice. Freshly isolated spleen CD4+ T cells from CD18+/− and CD18−/− mice were stained with differential concentrations of CFSE and adoptively cotransferred to RAG-1−/− or WT Balb/c mice. After 16 h, spleen, MLN, and PLN were harvested, and percentage of CD18−/− and CD18+/− within each organ was determined. A: homing index (calculated as ratio of CD18−/−:CD18+/− T cells to CD18−/−:CD18+/− T cells adjusted for the initial input ratio). B: number of CD18−/− and CD18+/− CD4+ T cells within spleen, MLN, and PLN (calculated as percentage of transferred cells detected in organs multiplied by absolute cell counts). Values are means ± SE of ≥4 independent experiments of 4 mice each. *P < 0.05; **P < 0.01; †P < 0.0001; ††P < 0.00001.

in RAG-1−/− PLN were only slightly decreased and migration to RAG-1−/− MLN was not significantly decreased (Fig. 5, A and B). In RAG-1−/− mice, the absolute number of accumulated CD4+ T cells for each of the secondary lymphoid structures was significantly decreased compared with Balb/c mice (Fig. 5B). Therefore, the increased trafficking of CD18−/− CD4+ T cells relative to that of CD18+/− CD4+ T cells to RAG-1−/− spleen likely compensates for the decreased cell accumulation associated with the defect in CD18−/− CD4+ T cell lymphopenia-induced proliferation, thereby accounting for the lack of the cell accumulation defect in Fig. 4D. In summary, although an increased number of CD18−/− compared with CD18+/− CD4+ T cells traffic to RAG-1−/− spleen, the altered early migration of CD18−/− CD4+ T cells is not as pronounced to other secondary lymphoid structures in RAG-1−/− as in WT mice.
driven by intestinal microbiota (18, 35). To confirm this notion and determine whether attenuation of these rapidly proliferating T cells was affected by CD18 expression, we compared untreated RAG-1−/− mice with RAG-1−/− mice subjected to a 4-wk antibiotic regimen prior to adoptive cotransfer of CFSE-labeled CD18−/− and CD18+/− CD4+ T cells. The antibiotic treatment resulted in a 98.5% reduction in bacterial 16S rRNA levels in the feces (data not shown). At 5 days after adoptive transfer, we observed significant attenuation of T cells entering into cell division (Fig. 7, A and B) and lymphopenia-induced rapid proliferation (Fig. 7, A and C) in the spleen and MLN of mice treated with antibiotics compared with untreated mice. However, the requirement for CD18 persisted in the CD4+ T cells that did undergo proliferation, as indicated by a significant defect in CD18−/− CD4+ T cell proliferation in the antibiotic-treated animals (Fig. 7, A–C).

Given these findings, we next questioned whether 1) lymphopenia-induced rapid proliferation was at maximum levels or 2) increasing exposure to intestinal microbiota by increasing intestinal permeability might increase lymphopenia-induced rapid proliferation, thereby altering the requirement for CD18 on T cells. We adoptively cotransferred CFSE-labeled CD18−/− and CD18+/− CD4+ T cells into RAG-1−/− mice and then administered DSS in their drinking water, which results in epithelial injury and disruption of epithelial barrier function. As T cells have undergone substantial lymphopenia-induced proliferation by 5 days after transfer (Fig. 1), we examined an earlier time point (4 days after transfer) to allow for a window of observation for potential increased lymphopenia-induced proliferation under conditions of disrupted barrier function. We did not observe an increase in T cell entry into cell division (Fig. 8, A and B) or lymphopenia-induced rapid proliferation (Fig. 8, A and C) in the spleen or MLN of DSS-treated mice. Moreover, the requirement for CD18 on T cells in lymphopenia-induced rapid proliferation persisted upon treatment with DSS (Fig. 8, A–C). We similarly did not observe an increase in lymphopenia-induced rapid proliferation when we examined mice at earlier time points (2 and 3 days) after adoptive cotransfer or when mice were first treated with DSS for a full 7 days prior to adoptive transfer, at which time they had developed clear signs of colitis, with bloody diarrhea and significant weight loss (data not shown). Therefore, while reduction of intestinal microbiota attenuates lymphopenia-in-
duced rapid proliferation in secondary lymphoid organs, increased exposure to intestinal microbiota does not further increase the rapidly proliferating population. Furthermore, the requirement for CD18 in lymphopenia-induced proliferation persists, despite perturbation of intestinal microbiota.

**Requirement for CD18 in lymphopenia-induced proliferation persists over a wide range of initial transferred numbers of T cells.** In antigen-driven proliferation, the requirement for CD18 is particularly pronounced during suboptimal T cell activation (1, 3), while during high antigen concentrations, CD18 requirements can be eliminated. In lymphopenia-induced proliferation, proliferation decreases as the number of T cells transferred into the lymphopenic host increases (14). This is consistent with finite available TCR ligands (25, 36). Therefore, we questioned whether the requirement for CD18 can be eliminated when the drive for lymphopenia-induced proliferation is high, as under conditions of low numbers of transferred T cells, and, conversely, if the CD18 requirement increases as the drive for lymphopenia-induced proliferation diminishes with higher numbers of initially transferred T cells. Increasing numbers of CD18−/− and CD18+/− CD4+ T cells (0.5, 1.5, 3.0, and 6.5 × 10^6 each) were adoptively cotransferred into RAG-1−/− mice. As expected, at 5 days after transfer, we observed that as the initial number of transferred T cells increased, the percentage of T cells entering the cell cycle decreased, most markedly in MLN and PLN (Fig. 9, A and B). Nevertheless, the defect in CD18−/− CD4+ T cell entry into cell division and in lymphopenia-induced rapid proliferation relative to that in CD18+/− CD4+ T cells was observed throughout the range of transferred T cell numbers (Fig. 9, A–C). Consistent with the lymphopenia-induced proliferation defect in CD18−/− CD4+ T cells, we observed a significant defect in accumulation of CD18−/− CD4+ T cells in each of the secondary lymphoid structures examined over the entire range of transferred CD4+ T cell numbers (Fig. 9D). Therefore, the requirement for CD18 on CD4+ T cells in lymphopenia-induced proliferation persists over a wide range of available TCR ligand doses.

**DISCUSSION**

Lymphopenic proliferation is tightly regulated in vivo and is modulated by local competition for specific resources, such as MHC-TCR interactions. In this study, we demonstrate an essential and uniquely broad-ranging role for CD18 in lymphopenia-induced proliferation that likely reflects the role of CD18 in adhesion and costimulation. We found that CD18 contributes both to rapid and to homeostatic, slow proliferation, in contrast to a number of other costimulation molecules that play a role in antigen-driven T cell activation, but not in lymphopenia-induced proliferation (27, 38). The CD18 requirement was observed in polyclonal CD4+ T populations of varying TCR affinities, as well as in a monoclonal TCR transgenic CD4+ T cell population. Furthermore, the dependency on CD18 continued over time. In antigen-driven proliferation, CD18 contributions have been particularly notable during suboptimal T cell activation (1–3). However, the requirement for CD18 persisted, despite attenuation of the intes-

![Fig. 9. Requirement for CD18 in polyclonal CD4+ T cell lymphopenia-induced proliferation persists over a wide range of initial T cell numbers. Freshly isolated CFSE-labeled spleen CD4+ T cells from CD18+/− and CD18−/− mice were adoptively cotransferred into RAG-1−/− mice at increasing numbers (0.5 × 10^6, 1.5 × 10^6, 3.0 × 10^6, and 6.5 × 10^6 of each CD18+/− and CD18−/− CD4+ T cells). At 5 days after transfer, spleen, MLN, and PLN were harvested, and CD4+CD3+ T cells were examined. A: representative flow cytometry plots for CFSE dilution of CD18+/− and CD18−/− CD4+ T cells in MLN. B: percentage of CD4+ T cells entering cell division. C: percentage of CD4+ T cells that have fully diluted CFSE. D: percentage of accumulated transferred CD4+ T cells in secondary lymphoid structures. Values are means ± SE of 3 mice per condition; data represent 3 independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; †P < 0.0001; ††P < 0.00001.\]
tinal microbiota-dependent rapidly proliferating population through antibiotic administration and over a wide range of increasing numbers of initial T cells in lymphopenic hosts, where the drive for proliferation progressively decreased, arguing that the role for CD18 is not strictly TCR ligand dose-dependent. As the amount of available cytokines also decreases with an increase in the number of initial T cells transferred, this also indicates that the requirement for CD18 persists, despite alterations in access to the cytokines essential to lymphopenia-induced proliferation.

Differential dependencies on costimulatory molecules for lymphopenia-induced compared with antigen-driven proliferation are being identified. Interactions between 41BB and 41BBL, CD40 and CD40L, and OX40 and OX40L are not necessary for lymphopenia-induced proliferation, despite reports that these interactions contribute to antigen-driven T cell proliferation (27, 38). In contrast, CD24 on T cells is required for lymphopenia-induced proliferation, whereas it is not required for allogeneic CD4+ T cell responses in vivo (20). CD28-B7 interactions are required for antigen-driven proliferation and rapid lymphopenia-induced proliferation in RAG-1−/− mice (14) but not for the slowly dividing T cell population (homeostatic) undergoing lymphopenia-induced proliferation (27). Differential regulation of rapid and homeostatic, slow proliferation has also been observed with respect to cytokines (23, 29, 35), differentiation status (23), and susceptibility to suppression by CD4+CD25Foxp3+ T cells (39). Therefore, of the surface molecules examined to date, CD18 is unique, in that it plays a role not only in antigen-driven CD4+ T cell proliferation, but also in rapid and homeostatic, slow lymphopenia-induced proliferation.

The requirement for CD18 in lymphopenia-induced proliferation was most pronounced under conditions of competition between CD18−/− and littermate control CD4+ T cells in cotransfer experiments. Similar findings, specifically under competitive conditions, have been observed with STAT3 in lymphopenia-induced proliferation (8). Even more dramatic is the case of CD28, where contributions to lymphopenia-induced proliferation were not originally identified in the absence of competition (27), but were subsequently identified specifically in the rapidly proliferating population during competitive conditions (15). The pronounced role for CD18 in proliferative efficiency may be highlighted in cotransfer experiments because of the competition for limited resources required for optimal proliferation, including TCR ligands. In light of in vivo physiological variations in CD18 expression on CD4+ T cell subsets, CD18 may provide selective advantage for lymphopenia-induced proliferation among specific T cell subsets in this competitive environment.

Interestingly, transfer of LFA-1-deficient naive CD4+ T cells into RAG-1−/− mice resulted in less severe colonic inflammation than did transfer of WT naive CD4+ T cells (26). It might be hypothesized that one mechanism contributing to this attenuation is decreased lymphopenia-induced expansion of LFA-1-deficient T cells in the RAG-1−/− recipients, although this was not investigated in the aforementioned study. In fact, although blocking various costimulatory interactions, such as CD40-CD40L interactions (21), attenuates intestinal inflammation in the model of T cell transfer into lymphopenic hosts, these costimulatory molecules do not play a role in lymphopenia-induced proliferation, such that lymphopenia-induced proliferation needs to be specifically examined. Also important in this adoptive transfer colitis model are the trafficking, activation, and differentiation of CD4+ T cells in intestinal lymphoid organs, along with subsequent CD4+ T cell trafficking into the intestinal lamina propria. We previously showed that each of these latter checkpoints is defective in LFA-1-deficient CD4+ T cells in an antigen-dependent system (22). Therefore, each of these checkpoints, combined with our current findings of LFA-1 contributions to lymphopenia-induced proliferation, likely accounts for the decreased LFA-1-deficient CD4+ T cell colitis in RAG-1−/− mice.

Defining mechanisms of lymphopenia-induced proliferation has broad implications for autoimmunity, intestinal inflammatory disorders, cancer therapy, and transplantation. Lymphopenia-induced proliferation, commonly observed after cancer chemotherapy, plays an essential role in maintenance of T cell numbers and function and can contribute to overcoming T cell tolerance, thereby allowing for improved tumor rejection (34). However, lymphopenia-induced proliferation can lead to adverse outcomes, such that the activation and proliferation of T cells with increased reactivity to self-antigens that can occur during lymphopenia-induced proliferation can contribute to development of autoimmunity (19). Moreover, the lymphopenia-induced proliferation during transplantation can increase susceptibility to rejection and to lymphoproliferative complications after transplantation (24, 40). Interestingly, on the one hand, lymphopenia-driven proliferation plays an important role in the intestinal inflammation that develops upon transfer of naive T cells into lymphopenic hosts; on the other hand, nonpathogenic T cells with increased TCR affinity can compete with these naive T cells in a lymphopenic environment, thereby serving a “regulatory” function and preventing intestinal inflammation (4). Therefore, therapeutic interventions to address defective regulation of lymphopenia-induced proliferation, either due to inadequate proliferation and function or excessive proliferation of T cell subsets, require an understanding of the mechanisms regulating this proliferation. In addition to its role in antigen-driven CD4+ T cell activation, we now demonstrate that CD18 contributes to optimal homeostatic and intestinal microbial-dependent lymphopenia-induced CD4+ T cell rapid proliferation in a sustained fashion and over a broad range of conditions. As such, CD18 has a unique potential to be an effective target for modulation under conditions where these combined forms of proliferation are playing a role.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

R.S. and C.A. performed experiments, analyzed data, prepared figures, and approved the final version of the manuscript. C.A. is responsible for conception

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and design of research, interpreted the results of the experiments, drafted the manuscript, edited and revised the manuscript.

REFERENCES


