Deficiency of CD11b or CD11d Results in Reduced Staphylococcal Enterotoxin-Induced T Cell Response and T Cell Phenotypic Changes

Huaizhu Wu, John R. Rodgers, Xiao-Yuan Dai Perrard, Jerry L. Perrard, Joseph E. Prince, Yasunori Abe, Beckley K. Davis, Greg Dietsch, C. Wayne Smith and Christie M. Ballantyne


http://www.jimmunol.org/content/173/1/297

---

**References**

This article cites 50 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/173/1/297.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Deficiency of CD11b or CD11d Results in Reduced Staphylococcal Enterotoxin-Induced T Cell Response and T Cell Phenotypic Changes

Huaiizhu Wu,* John R. Rodgers,‡ Xiao-Yuan Dai Perrard,* Jerry L. Perrard,* Joseph E. Prince,† Yasunori Abe,* Beckley K. Davis,‡ Greg Dietsch,* C. Wayne Smith,¶ and Christie M. Ballantyne2*¶

The β2 integrin CD11a is involved in T cell-APC interactions, but the roles of CD11b, CD11c, and CD11d in such interactions have not been examined. To evaluate the roles of each CD11/CD18 integrin in T cell-APC interactions, we tested the ability of splenocytes of CD11-knockout (KO) mice to respond to staphylococcal enterotoxins (SEs), a commonly used superantigen. The defect in T cell proliferation with SEA was more severe in splenocytes from mice deficient in CD18, CD11b, or CD11d than in CD11a-deficient splenocytes, with a normal response in CD11c-deficient splenocytes. Mixing experiments showed that the defect of both CD11b-KO and CD11d-KO splenocytes was, unexpectedly, in T cells rather than in APC. Cytometric analysis failed to detect CD11b or CD11d on resting or activated T cells or on thymocytes of wild-type adult mice, nor did Abs directed to these integrins block responses in culture, suggesting that T cells educated in CD11b-KO or CD11d-KO mice were phenotypically altered. Consistent with this hypothesis, T cells from CD11b-KO and CD11d-KO splenocytes exhibited reduced intensity of CD3 and CD28 expression and decreased ratios of CD4/CD8 cells, and CD4+ T cells were reduced among CD11b-KO and CD11d-KO thymocytes. CD11b and CD11d were coexpressed on a subset of early wild-type fetal thymocytes. We postulate that transient thymocyte expression of both CD11b and CD11d is nonredundantly required for normal thymocyte and T cell development, leading to phenotypic changes in T cells that result in the reduced response to SE stimulation. The Journal of Immunology, 2004, 173: 297–306.
Materials and Methods

Targeting construct and generation of mutant mice deficient in CD11d

A genomic λ phage clone containing an 8.3-kb fragment of the mouse CD11d gene was isolated from an SV129 murine genomic library and used to develop a construct for targeted homologous recombination as shown in Fig. 1. A 4.1-kb genomic restriction fragment located upstream of exon 1 and a 1.7-kb genomic restriction fragment located downstream of exon 2 were ligated into the polylinker of pBluescript SK+ (Stratagene, La Jolla, CA). A neomycin cassette driven by the mouse RNA polymerase II promoter was inserted between the two fragments (Fig. 1b). All ligations were confirmed by DNA sequencing and restriction digests. The construct was linearized by digestion with XhoI, which cleaves in the polylinker. The construct was designed so that targeted homologous recombination with the murine CD11d gene would result in the replacement of a 2.2-kb genomic fragment containing exons 1 and 2 with the 1.8-kb neomycin cassette (Fig. 1c).

The AB1 embryonic stem cell line (provided by Allan Bradley, Baylor College of Medicine, Houston, TX) was electroporated with linearized vector (25 pg/ml) as previously described (11). After selection with G418, individual colonies were picked and screened for targeted homologous recombination by Southern blotting using a microextraction procedure (12) with a 257-bp probe derived from 3′-flanking genomic DNA that was not included in the construct, as indicated in Fig. 1c. Cells confirmed by Southern blotting to carry the mutation were injected into day 3.5 C57BL/6 blastocysts and transferred into foster mothers. Chimeric males were mated with C57BL/6 females, and germline transmission was confirmed by Southern blotting of tail DNA after digestion with XbaI as indicated in Fig. 1d.

CD11a-, CD11b-, and CD11c-deficient mice and animal experiments

CD11a-knockout (KO) and CD11b-KO mice were generated in our laboratory as previously described (13, 14), and CD11c-KO mice were also developed in our laboratory by targeted homologous recombination techniques (H. Wu, H. Wang, J. L. Perrard, X.-Y. D. Perrard, Y. Abe, A. R. Burns, D. C. Bullard, C. W. Smith, and C. M. Ballantyne, manuscript in preparation). All CD11-KO mice were backcrossed for at least six generations onto C57BL/6 background (Harlan Sprague Dawley, Indianapolis, IN), and C57BL/6 mice purchased from Harlan Sprague Dawley were used as wild-type (WT) controls. For each experiment, age- and sex-matched CD11-KO mice and WT mice were used. All mice were housed in autoclaved microisolator cages, with autoclaved feed. All animal studies were approved by the Animal Protocol Review Committee of Baylor College of Medicine.

Preparation of mouse splenocytes and thymocytes

Mouse splenocytes and thymocytes were prepared as described by Kruisbeek (15), with some modifications. Briefly, after the mice were sacrificed under anesthesia, fresh spleens and/or thymuses were removed. Fetal thymuses were dissected from mouse fetuses under a dissecting microscope on the days indicated in the result section. The day on which vaginal plug occurred was counted as day 0 of gestation. The mouse spleens or thymuses were minced with a sharp sterile blade, placed in a 40-μm nylon cell strainer (BD Biosciences, Franklin Lakes, NJ), and pressed with the plunger of a 6-ml syringe until mostly fibrous tissue remained. The splenocyte or thymocyte suspension in RPMI 1640 supplemented with 5% FBS was collected. The red blood cells were lysed with lysing buffer (0.15 M NH4Cl, 10.0 mM KHCO3, 0.1 mM Na2EDTA, pH 7.4), and the dead cells were removed by a one-step gradient method (15). After being washed once with RPMI 1640 with 5% FBS, the mouse splenocytes or thymocytes were resuspended in RPMI 1640 with 10% FBS.

Purification of T cells from mouse splenocytes

T cells were purified from mouse splenocytes by depletion of non-T cells (negative selection) with a Pan T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). First, mouse splenocytes were stained with biotin-Ab mixture, which includes biotin-conjugated mAbs against mouse CD11b, CD45R, DX5, and Ter-119. Then, the biotin Ab-stained mouse splenocytes were coincubated with anti-biotin microbeads. Finally, purified T cells were obtained by magnetic separation. For T cell purification from CD11b-KO mouse splenocytes, the combination of the Pan T cell isolation kit with mouse MHC class II microbeads was introduced.

In vitro T cell proliferation upon SE stimulation

For the detection of in vitro T cell proliferation in whole splenocytes, whole mouse splenocytes were plated in 96-well round-bottom microtiter plates at 4 × 105 cells/well in a total volume of 200 μl of RPMI 1640 supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. The cells were cultured in the absence (control) or presence of SEs (Toxin Technology, Sarasota, FL) for 72 h, then labeled with 1 μCi of [3H]thymidine (Du Pont-New England Nuclear, Boston, MA) per well for an additional 18 h. After 18 h, the cells were harvested and counted by liquid scintillation spectroscopy (Betaplate; Wallace, Gaithersburg, MD). The results were expressed as mean counts per minute ± SEM of triplicate cultures. Mouse splenocytes were also stimulated with Con A, PHA, and PMA-iodonycin (Sigma-Aldrich, St. Louis, MO). For the Ab-blocking experiment, WT splenocytes were incubated at 4°C for 30 min with 20 μg/ml anti-mouse CD11b (M1/70, rat IgG2b; BD PharMingen, San Diego, CA), CD11d (20SC, hamster IgG; ICOS, Bothell, WA), or CD18 (GAME-46, rat IgG1; BD PharMingen) Abs and then stimulated with SEA for 72 h at 37°C as described above. Rat IgG2b, hamster IgG, and rat IgG1 (BD PharMingen) were used as isotype controls.

For the assessment of purified T cell proliferation, mixing experiments were performed in which purified T cells were coincubated with APC and stimulated with SEs. Purified T cells were obtained from mouse splenocytes as described above. Mouse splenocytes irradiated to 1500 rads were used as APC. Purified T cells (1.3 × 105/well) and irradiated splenocytes (2.6 × 105/well) were cultured in 200 μl of the medium described above in the absence or presence of SEs for 72 h, labeled with [3H]thymidine for 18 h, and counted by liquid scintillation spectroscopy as described above.

In vivo T cell responses to SE stimulation in mice

For the in vivo study, mice were injected i.p. with SEA (100 μg dissolved in 0.2 ml saline for each mouse, with saline only as control), and the responses were compared between CD11-KO mice and WT mice matched for age (8–12 wk old) and sex (both male and female) as described by Marrack et al. (16). Briefly, mice were confined, two to three per cage, for 2 wk before beginning the experiment to allow social relationships to stabilize in the group. To observe the in vivo effect of SEA on T cell responses, mice were sacrificed under anesthesia on day 4 after SEA (or saline as control) administration. The thymuses and splenocytes were removed for cellularity assessment. Differences in average cell numbers were calculated as average splenocyte (thymocyte) numbers of five SEA-inoculated mice/average splenocyte (thymocyte) numbers of five control mice.

Abs and flow cytometric analysis

The following Abs to mouse Ags were used: CD11b (M1/70, FITC, or PE-conjugated); CD11d (20SC); CD3 (17A2, PE-conjugated; BD PharMingen); CD4 (H129.19, rat IgG2a, FITC-conjugated; BD PharMingen); CD8 (53-6.7, rat IgG2a, PerCP-conjugated; BD PharMingen); CD25 (7D4, hamster IgG2, PE-conjugated; BD PharMingen); and CD117 (ACK45, rat IgG2b, PE-conjugated; BD PharMingen). FITC-conjugated goat anti-hamster IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

For flow cytometric analysis, cells (splenocytes or thymocytes, 1 × 106 in a total of 100 μl of PBS) were stained in one of two ways: in the first, the cells were harvested from the above fluorochrome-labeled Abs (1 μg), except for CD11d, which was labeled in two steps with unconjugated hamster anti-CD11d Ab for the first step and FITC-conjugated goat anti-hamster IgG for the second step. After incubation with the indicated Abs for 15 min on ice, the cells were washed three times with cold PBS and finally resuspended in 400 μl of PBS with 1% paraformaldehyde. Data were collected with a FACScan and analyzed with CellQuest software (BD Biosciences, San Jose, CA).

Assessment of NK cell activity in vitro

The mouse lymphoma cell line YAC, maintained in MEM with 100 μg/ml gentamicin, 2 mM L-glutamine, and 5 mM HEPES, was used as the target. The effectors were splenocytes harvested from mice that received i.p. injections of 100 μg of polynosinate-polycytidylylate (for NK priming) the day before. Target cell lysis was assayed by 51Cr release. YAC cells were incubated with Na104CrO4 for 3 h to internalize the 51Cr. After washing and gradient purification, the 51Cr-YAC cells were plated in triplicate in round-bottom 96-well cell culture plates (5000 cells/well) and coincubated with the mouse splenocytes (effector) at the E:T ratios indicated in Results in a total volume of 200 μl of medium for 4 h at 37°C. Cytotoxicity was determined by the release of 51Cr into the supernatant. Spontaneous release was defined as 51Cr release by target cells in the absence of effector, and maximum release was determined by target cell lysis with SDS. Percent specific lysis was calculated as: specific lysis (%) = ([experimental release – spontaneous release]/maximum release – spontaneous release) × 100.


Differences are considered significant at \( p < 0.05 \).

**Results**

**Generation of mice deficient in CD11d**

ES cells with a targeted event had a 3.5-kb \( Xba I \) fragment identified by the 3′-flanking probe on Southern blot compared with the 8.3-kb fragment in WT 129/Sv mice (Fig. 1d). Targeted ES cell clones were injected into C57BL/6 blastocysts, and male chimeric offspring that were >90% agouti coat color were bred to C57BL/6 mice. Germline transmission was confirmed by Southern blotting studies. Mice heterozygous for the mutation were cross-bred to generate homozygous mice. The expected targeted allele of 3.5 kb was identified by the 3′-flanking probe on Southern blots of DNA from mice carrying the mutation (Fig. 1e).

Homozygous and heterozygous mutant mice were born in expected ratios and were fertile. CD11d mutant mice did not demonstrate any gross abnormalities in growth and development and did not develop an increased rate of spontaneous infections when maintained in autoclaved microisolator cages. CD11d mutant mice had normal peripheral leukocyte counts and the same differentials as WT mice. The basal IgG levels in the serum of CD11d mutant mice were normal (data not shown). Two-color flow cytometric analysis showed that in WT mice, CD11d was predominantly expressed on CD11b-positive cells in splenocytes, and no detectable CD11d expression was found on WT peripheral leukocytes. In CD11d mutant mice, there was no detectable expression of CD11d on either splenocytes or peripheral leukocytes.

**SEA-induced T cell proliferation in splenocytes deficient in CD11d and other CD11/CD18 integrins in vitro**

On the basis of the previous Ab-blocking and transfection studies that showed the involvement of CD11a-ICAM-1 interaction in superantigen-induced lymphocyte proliferation, we first detected SEA-induced T cell proliferation in whole splenocytes from the four CD11(a–d)-KO and CD18-KO mice. As shown in Fig. 2, on in vitro SEA stimulation (0–200 pg/ml), T cell proliferation in CD11a-deficient splenocytes was markedly reduced compared with that in WT mice, which was consistent with previous Ab-blocking studies. At the same time, CD18-deficient splenocytes showed a more severe defect in SEA-induced T cell proliferation, whereas CD11b- and CD11d-deficient splenocytes showed almost the same severe defect in SEA-induced T cell proliferation as CD18-deficient splenocytes. At low concentrations (0–200 pg/ml), SEA induced little T cell proliferative response, with <10,000 cpm of \([3H]\)thymidine incorporation in CD11b-, CD11d-, and CD18-KO splenocytes, compared with high level T cell proliferation with up to >300,000 cpm of \([3H]\)thymidine incorporation in WT splenocytes (Fig. 2). In contrast, CD11c-deficient mice had normal in vitro T cell proliferative response to SEA (Fig. 2). These results indicated that functional genes for both CD11b and CD11d are required nonredundantly for superantigen responses. To test the hypothesis that splenocytes must express CD11b or CD11d at some time during the culture period, we cultured splenocytes separately with Abs targeting CD18, CD11b, or CD11d. As expected, anti-CD18 significantly inhibited T cell proliferation. However, neither anti-CD11d nor anti-CD11b Ab inhibited SEA-induced T cell proliferation in WT splenocytes in vitro (data not shown).

**In vitro T cell proliferative response of CD11b- and CD11d-deficient splenocytes to SEB and SEE stimulation**

Among the four types of CD11d-deficient mice, CD11b- and CD11d-deficient splenocytes showed the poorest in vitro T cell response to SEA stimulation (Fig. 2); thus, our studies focused on these two mutations. On the basis of the difference in TCR Vβ specificity among SEB, SEE, and SEA (7, 17), we then determined T cell proliferation in CD11b- and CD11d-deficient splenocytes in response to SEB, SEE, and SEA at a wider range of concentrations. As shown in Fig. 3, compared with WT mice, both CD11b- and CD11d-KO mice had similar defects in T cell proliferative...
response to SEA, SEB, and SEE stimulation. CD11b- and CD11d-KO splenocytes did respond to high concentrations (>1.0 ng/ml SEA and SEB and >10 ng/ml SEE) of SEs. The T cell response in the KO splenocytes approached the maximum level for WT splenocytes (up to >300,000 cpm of [3H]thymidine incorporation) after stimulation with 100 ng/ml SEs (Fig. 3).

In vitro proliferative responses of CD11b- and CD11d-deficient splenocytes to lectin and PMA-ionomycin stimulation

Lectins such as PHA and Con A may activate T cells by indirectly cross-linking the TCR. In comparison with those of WT mice, splenocytes from CD11b- and CD11d-KO mice responded poorly to PHA and Con A stimulation (Fig. 4, a and b). When splenocytes were stimulated with PMA-ionomycin, CD11b- and CD11d-deficient mice did not show defects in the proliferative response as compared with WT mice (Fig. 4c), suggesting that T cells from CD11b- and CD11d-deficient mice were able to proliferate normally if TCR was bypassed.

SEA-induced proliferation of purified T cells in the presence of APC

In mouse splenocytes, both T cells and APC are required for the normal response to SEs. To assess which cells (T cells or APC or both) in CD11b- and CD11d-deficient splenocytes are responsible for the defective proliferative response to SEs, we mixed purified T cells with APC and stimulated with SEA. As expected, purified T cells or APC by themselves did not respond to SEA. When purified T cells from WT splenocytes were coincubated with WT APC, SEA induced high level T cell proliferative responses, with a maximum of >300,000 cpm (Fig. 5a). When coincubated with CD11b- and CD11d-deficient APC, WT purified T cells proliferated normally in response to SEA stimulation, which indicated that CD11b- and CD11d-deficient APC were able to present SEA normally. When coincubated with WT APC, however, purified T cells from both CD11b- and CD11d-KO splenocytes exhibited markedly reduced response to SEA (Fig. 5, b and c), which suggested that T cells rather than APC in CD11b- and CD11d-deficient mice contributed to the defective response to SE stimulation.

In vivo effect of SEA in CD11b-KO and CD11d-KO mice

To determine the in vivo proliferative responses of CD11b- and CD11d-KO mice to SEA stimulation, CD11b- and CD11d-KO mice and WT mice were injected i.p. with 100 μg of SEA per mouse. After SEA injection, spleen and thymus cellularity from the inoculated mice was measured. As shown in Fig. 6a, SEA injection induced a marked increase in the numbers of spleen cells in WT mice (2.7 times greater average spleenocyte numbers for 5 SEA-inoculated mice relative to those for control mice), but a smaller increase in the numbers of spleen cells in CD11b-KO and CD11d-KO mice (1.4 times greater for CD11b-KO and CD11d-KO mice; p < 0.01 compared with WT). In vivo SEA injection, however, as reported previously (16), induced thymus depletion in mice as indicated by a substantial decrease in thymocyte numbers from SEA-treated mice as compared with saline-injected controls. Compared with WT mice (in which the ratio of the average thymocyte numbers of 5 SEA-treated mice relative to

FIGURE 2. T cell proliferation in mouse splenocytes in response to SEA stimulation in vitro. Splenocytes from CD11a-KO, CD11b-KO, CD11c-KO, CD11d-KO, and CD18-KO mice as well as from WT mice were cultured and stimulated in vitro with 1.0 to 200 pg/ml SEA for 72 h. Proliferation was quantitated as described in Materials and Methods and is expressed as mean counts per minute ± SEM of triplicate cultures. Data are representative of three independent experiments with similar results.

FIGURE 3. T cell proliferation in mouse splenocytes in response to SEA, SEB, and SEE in vitro. Splenocytes from CD11b-KO and CD11d-KO mice as well as from WT mice were cultured and stimulated in vitro with 1.0 pg/ml to 1.0 μg/ml SEA (a), SEB (b), or SEE (c), respectively, for 72 h. Proliferation was measured as described in Materials and Methods and is expressed as mean counts per minute ± SEM of triplicate cultures. Data are representative of three independent experiments with similar results.

300 T CELL RESPONSE TO SE IN CD11b/d-NULL MICE

by guest on April 13, 2017 http://www.jimmunol.org/ Downloaded from
those of control mice was 0.31), CD11b-KO and CD11d-KO mice showed less of a decrease in the numbers of thymocytes (thymus depletion) when injected with SEA (ratios of 0.64 and 0.6 for CD11b-KO and CD11d-KO mice, respectively, for 72 h. Compared with WT; Fig. 6).

Assessment of CD11b and CD11d expression on mouse T cells by flow cytometry

As described above, CD11b- and CD11d-deficient splenocytes showed reduced proliferative responses to SE stimulation both in vitro and in vivo, and the in vitro data indicated that the defects in CD11b- and CD11d-deficient splenocytes were due to T cells rather than APC. Therefore, we assessed the expression of CD11b and CD11d on WT T cells by two-color flow cytometry using CD3 as the specific marker for T cells. At baseline (without SE stimulation), few T cells (CD3-positive cells) in WT splenocytes expressed CD11b or CD11d. All the CD11b- and CD11d-positive cells were CD3 negative (data not shown), i.e., at baseline, there was no detected expression of CD11b and CD11d on WT T cells, and both CD11b and CD11d were expressed on non-T cells in WT splenocytes. We then determined CD11b and CD11d expression on WT T cells after SEA stimulation. Similar to baseline, stimulation of WT splenocytes with SEA for 3 days did not significantly enhance the expression of CD11b or CD11d on WT T cells (data not shown).

Phenotyping of T cells from CD11b- and CD11d-deficient mice

Because there was no expression of CD11b or CD11d detected on WT T cells at baseline and SE stimulation did not enhance expression of CD11b or CD11d, the defective responses of CD11b- and CD11d-deficient mouse T cells to SEs were probably not due to the absence of CD11b or CD11d from T cells per se. We therefore investigated whether the absence of CD11b or CD11d may

FIGURE 4. T cell response in mouse splenocytes to PHA, Con A, or PMA-ionomycin stimulation in vitro. Splenocytes from CD11b-KO and CD11d-KO mice and from WT mice were cultured and stimulated with PHA (a), Con A (b), or PMA-ionomycin (c), respectively, for 72 h. Proliferation was measured as described in Materials and Methods, and the results are expressed as mean counts per minute ± SEM of triplicate cultures. Data are representative of three independent experiments with similar results.

FIGURE 5. T cell proliferation in response to SEA stimulation in mixing experiments. a, Purified T cells from WT splenocytes were coincubated with APC from WT, CD11b-KO, and CD11d-KO mice and stimulated with SEA in vitro for 72 h; b, Purified T cells from WT and CD11b-KO splenocytes were coincubated with WT APC and stimulated with SEA in vitro for 72 h; c, Purified T cells from WT and CD11d-KO splenocytes were coincubated with WT APC and stimulated with SEA in vitro for 72 h. T cell proliferation was measured as described in Materials and Methods and is expressed as mean counts per minute ± SEM of triplicate cultures. Data are representative of three independent experiments with similar results.
have caused phenotypic changes in splenocytes or thymocytes that may contribute to the decreased response to SE stimulation. Based on the important roles of CD3 and CD28 in superantigen-induced T cell proliferation (8), we first examined CD3 and CD28 expression on mouse splenocytes by flow cytometry. The percentages of CD3- and CD28-positive cells in CD11b- and CD11d-deficient splenocytes were not different from those of WT splenocytes, which indicated that the T cell percentages in CD11b- and CD11d-deficient whole splenocytes were similar to those in WT splenocytes. As shown in Fig. 7, a and b, however, splenocytes from CD11b-deficient and CD11d-deficient mice had reduced CD3 (with CD3 mean fluorescence intensity (MFI) of 255 for CD11b-deficient and 216 for CD11d-deficient splenocytes) and CD28 (with CD28 MFI of 21 for both CD11b-deficient and CD11d-deficient splenocytes) expression compared with WT splenocytes (with CD3 and CD28 MFI of 366 and 29, respectively). In contrast, CD11a-deficient splenocytes did not show any difference in the intensity of CD3 (with MFI of 379) and CD28 (with MFI of 28) expression compared with WT splenocytes (Fig. 7, a and b).

Because T cells mature in the thymus, we next assessed the expression of CD3 and CD28 on mouse thymocytes. As illustrated in Fig. 7c, CD3 expression on CD11b-deficient and CD11d-deficient thymocytes was also reduced (with CD3 MFI on single-positive cells of 574 for CD11b-deficient and 549 for CD11d-deficient thymocytes, compared with 903 for WT). Because a previous study showed that a mAb to CD4 Ag markedly inhibited superantigen-induced T cell proliferation (8), we then measured CD4 and CD8 expression on mouse splenocytes and thymocytes by two-color flow cytometry, and the CD4-positive:CD8-positive cell ratios were calculated (Table I). The ratios of CD4-positive to CD8-positive cells were significantly lower in both CD11b- and CD11d-deficient splenocytes and thymocytes than in WT mice.

**Assessment of CD11b and CD11d expression on mouse thymocytes by flow cytometry**

There was no detectable CD11b or CD11d expression on WT splenocyte T cells, but T cells from CD11b- and CD11d-deficient splenocytes and thymocytes did show other phenotypic changes.

---

**FIGURE 6.** Changes in the cell numbers of spleens (a) and thymuses (b) of CD11b- and CD11d-KO mice and WT mice after SEA inoculation. CD11b-KO and CD11d-KO mice and WT mice were injected i.p. with 100 μg/mouse of SEA dissolved in saline, or with saline only (control). On day 4 after SEA administration, mice were sacrificed, and the spleens and thymuses were removed for cellularity assessment as described in Materials and Methods. The changes in splen/thymus cell numbers of SEA-inoculated CD11b-KO and CD11d-KO mice as compared with controls were significantly less than those of WT (p < 0.01).

**FIGURE 7.** Flow cytometric analysis of mouse splenocytes and thymocytes for CD3 and CD28 expression. Splenocytes (a and b) and thymocytes (c) from WT and CD11-KO mice were stained with Abs to CD3 (a and c) or CD28 (b) and analyzed by flow cytometry. a, MFIs of CD3 on positive cells (M) were 366 for WT, 379 for CD11a-KO, 255 for CD11b-KO, and 216 for CD11d-KO. b, MFIs of CD28 on positive cells (M) were 29 for WT, 28 for CD11a-KO, 21 for CD11b-KO, and 21 for CD11d-KO. c, MFIs of CD3 on single-positive cells (M) were 903 for WT, 574 for CD11b-KO, and 549 for CD11d-KO. Data are representative of three independent experiments with similar results.
Therefore, the expression of CD11b and CD11d on mouse thymocytes was evaluated. No CD11b or CD11d expression was found on adult (6 wk old) WT (Fig. 8, a and b) or newborn (2 days old) WT thymocytes (data not shown). However, as shown in Fig. 8 c, 41.5% of day 12 WT fetal thymocytes were CD11b positive, and the majority of the C-kit-positive population was CD11b positive. At this time point in thymic development, >70% of CD11b-positive cells were C-kit positive. Approximately 13% of the day 12 WT fetal thymocytes were CD11d positive (Fig. 8d), and most of the CD11d-positive cells at this stage were also CD11b positive (Fig. 8e). Analysis of later stage WT fetal thymocytes showed that the expression of CD11b and CD11d on the fetal thymocytes fell

![Figure 8](image-url)

**FIGURE 8.** Flow cytometric analysis of WT mouse thymocytes for CD11b and CD11d expression. Thymocytes from 6-wk-old WT mice (a and b) were double-stained with Abs to CD3 and to CD11b (a) or CD11d (b). Day 12 WT fetal thymocytes were double-stained with Abs to C-kit and CD11b (c) or CD11d (d) and with Abs to CD11b and CD11d (e). The stained thymocytes were analyzed by flow cytometry. Data are representative of three independent experiments with similar results.

<table>
<thead>
<tr>
<th>Positive Cells in Thymocytes</th>
<th>Positive Cells in Splenocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell nos.</td>
<td>%</td>
</tr>
<tr>
<td>WT</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>2410 ± 122*</td>
</tr>
<tr>
<td>CD8⁻</td>
<td>1704 ± 174</td>
</tr>
<tr>
<td>CD4⁺/CD8⁻</td>
<td>1.45 ± 0.19</td>
</tr>
<tr>
<td>CD11b⁻/⁻</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>1257 ± 154**</td>
</tr>
<tr>
<td>CD8⁻</td>
<td>2046 ± 52</td>
</tr>
<tr>
<td>CD4⁺/CD8⁻</td>
<td>0.61 ± 0.058**</td>
</tr>
<tr>
<td>CD11d⁻/⁻</td>
<td></td>
</tr>
<tr>
<td>CD4⁺</td>
<td>1651 ± 56*</td>
</tr>
<tr>
<td>CD8⁻</td>
<td>2370 ± 198</td>
</tr>
<tr>
<td>CD4⁺/CD8⁻</td>
<td>0.71 ± 0.07**</td>
</tr>
</tbody>
</table>

*Mean ± SEM; n = 3.

*Compared with WT: *p < 0.05; **p < 0.005; ***p < 0.0005.
cytotoxicity in CD11b-KO, CD11c-KO, and CD11d-KO mice compared with WT mice (Fig. 9), consistent with previous studies found that NK cytotoxicity was reduced in CD11a-KO mice. The cytotoxicity of the NK cells from CD11(a–d)-KO mouse splenocytes was also determined. Using YAC as the target, and splenocytes was also determined. Using YAC as the target, and cytotoxicity against YAC cells was tested at the indicated E:T ratios. The results are expressed as mean ± SEM of triplicate cultures.

Assessment of NK cell cytotoxicity

The cytotoxicity of the NK cells from CD11(a–d)-KO mouse splenocytes was also determined. Using YAC as the target, and polyniosamine-polycytidylate-induced splenocytes as effectors, we found that NK cytotoxicity was reduced in CD11a-KO mice compared with WT mice (Fig. 9), consistent with previous studies (18–21). At the same time, no difference was found in NK cell cytotoxicity in CD11b-KO, CD11c-KO, and CD11d-KO mice compared with WT mice (Fig. 9).

Discussion

CD11/CD18 integrins have been shown to play an important role in leukocyte adhesion and signaling in a variety of physiological and pathological conditions (1, 2, 13, 14). The development of CD18-KO mice has provided us with a very useful animal model to explore further the in vivo functions of these integrins (2, 22, 23). To define the individual roles of each CD11CD18 integrin, mice genetically deficient in CD11a or CD11b have been generated in our laboratory (13, 14, 24–26) and by others (27, 28). In this report, we describe the generation of mice homozygous for a targeted mutation in CD11d with confirmation of a null phenotype by flow cytometric analysis of splenocytes. Mice deficient in CD11d have normal growth and development as compared with WT littermates, without an increased rate of spontaneous infection when maintained in autoclaved microisolator cages, similar to mice deficient in CD11a or CD11b (14) but in contrast to mice deficient in CD18 (2).

Splenocytes from mice deficient in CD18 have been demonstrated to have a marked defect in T cell proliferation to SEA, and this was postulated to be due to the absence of CD11a, which is the ligand for ICAM-1 (2). Abs blocking CD18, CD11a, or ICAM-1 each inhibited SE-induced T cell growth and cytokine production (9, 29–31). Additionally, transfected or purified ICAM-1 restored SE-induced T cell proliferation via CD11a binding. Naive or resting T cells were particularly dependent on CD11a stimulation (2, 10). Our present study confirmed the role of CD11a in SEA-induced T cell response as indicated by a defect in T cell proliferation in CD11a-deficient splenocytes stimulated with low dose SEA in vitro. Our new finding was that the defect in SEA-induced T cell proliferation was more severe in CD18-, CD11b-, or CD11d-deficient splenocytes than in CD11a-deficient splenocytes in vitro. The in vivo study showing a smaller increase in the numbers of spleen cells in CD11b-KO and CD11d-KO mice than in WT controls after SEA inoculation confirmed the in vitro finding. Thus, among the CD11/CD18 integrins, CD11b and CD11d, in addition to CD11a, contribute to the proliferative response to SEA stimulation. The reduced thymus depletion in CD11b-KO and CD11d-KO mice after SEA inoculation in vivo may also reflect the reduced T cell activation in these KO mice, because the in vivo pathological effects of SE on mice may be T cell mediated (16).

Each SE reacts with T cells bearing the particular Vβ sequences of TCR, inducing activation of these particular populations of T cells (7, 17). The Vβ specificities of SEA for mouse T cells are 1, 3, 10, 11, 17, whereas the specificities of SEB and SEE are 3, 7, 8.1–8.3, 17, and 11, 15, 17, respectively (17). CD11b- and CD11d-deficient splenocytes showed defects in SEE- and SEE-induced T cell proliferation similar to the defects in the SEA-induced T cell response, indicating that the defects do not result from changes in Vβ subsets. The T cell response to high dose SEs and normal T cell proliferation in response to PMA-ionomycin observed in CD11b- and CD11d-deficient splenocytes indicate that T cells themselves in CD11b-KO and CD11d-KO mice have the ability to proliferate, and they are able to proliferate normally if TCR and costimulatory signals are bypassed.

Both T cells and APC in mouse splenocytes are required for the normal response to SE stimulation. The interaction between CD11a on T cells and ICAM-1 on APC is considered to be essential for the normal response to SE stimulation (29–31). CD11a is expressed predominantly on T cells and serves as a costimulatory molecule for T cell activation (32). Our mixing experiments showed that T cells, rather than APC, in CD11b- and CD11d-deficient splenocytes had the defect that accounted for the reduced response to SE stimulation. This was unexpected, because neither CD11b nor CD11d has been shown to be predominantly expressed on WT T cells. CD11b is considered to be expressed predominantly on monocytes/macrophages, PMN, and NK cells (33–35), whereas CD11d is expressed mainly on subsets of the myelomonocytic lineage (myeloid cells), particularly on monocytes, macrophage foam cells, and splenic red pulpal macrophages (36–40).

In humans, CD11d is expressed on peripheral leukocytes, including neutrophils, eosinophils (especially upon stimulation with PMA or fMet-Leu-Phe), monocytes, and to a lesser extent, lymphocytes (38, 39). In contrast to human leukocytes, no detectable CD11d expression was found on WT mouse peripheral leukocytes in our current study. In humans, 2–10% of T cells (mainly CD8-positive cells) in human peripheral blood express CD11b (35, 41). Brief exposure of resting T cells (both CD4 and CD8) to phorbol ester resulted in an increase of CD11b on the surface of a small subpopulation of human T cells (41). Abs to CD11b inhibited anti-CD3-induced human T cell proliferation as well as IL-2 release (35). In contrast, our two-color flow cytometric analysis of mouse splenocytes showed that few T cells in WT spleen cells expressed CD11b or CD11d at baseline or after SE stimulation, which rules out the possibility that the defect in SE-induced T cell proliferation in CD11b- and CD11d-deficient mice is due to the absence of CD11b and CD11d in responding mouse T cells.

As previously reported, interference with the SE/MHC-CD3/TCR complex (signal 1) by using mAbs to CD3 or MHC class II strongly inhibited T cell proliferation induced by low doses of

---

**FIGURE 9.** Cytotoxicity of NK cells from CD11-KO mice and WT mice. Splenocytes from CD11-KO and WT mice were used as effector, and YAC cells as target. Cytotoxicity against YAC cells was tested at the indicated E:T ratios. The results are expressed as mean ± SEM of triplicate cultures.
superantigen (8). Blocking the CD28 costimulatory pathway (signal 2) with an anti-CD28 mAb also achieved similar inhibition of T cell response to superantigen stimulation (8, 9, 29–31). These studies suggest that each of the molecules makes a major contribution to T cell activation by MHC-superantigen complex stimulation. As an investigation of the defects in T cells of CD11b- and CD11d-KO mice that contribute to the decreased response to SE stimulation, we found reduced intensities of CD3 and CD28 expression on CD11b- and CD11d-deficient splenocytes as compared with those of WT mice. Based on the important roles of CD3 and CD28 in SE-induced T cell proliferation, the decreased number of CD3 and CD28 molecules as determined by FACS on CD11b- and CD11d-deficient T cells might explain at least part of the poor response of these cells to SE stimulation.

In comparison with WT splenocytes, the ratios of CD4+ to CD8+ cells in CD11b-KO and CD11d-KO splenocytes were markedly reduced. It is well known that CD4 and CD8 molecules contribute to the stable interaction of a complex of Ag-MHC molecules with the TCR molecule (42). Because superantigens activate T cells in direct association with MHC II molecules on APC (43–48), it is expected that the toxins would preferentially stimulate CD4+ cells. Although some have suggested that SEs might affect CD4+ and CD8+ cells equally (7), others have found that CD4+ T cells were high responders to SEA, whereas CD8+ T cells were low responders in mice implanted with an SEA pump (42). Furthermore, an Ab to CD4 Ag blocked SEA-induced T cell response >80% (8). In addition, our study showed that CD4+ cells had higher intensities of CD3 expression than CD8+ cells in both WT and KO splenocytes, and the intensities of CD3 expression on both CD4+ and CD8+ cells were reduced in CD11b-KO and CD11d-KO mice compared with WT mice (data not shown). Based on these findings, the significantly lower ratios of CD4+ to CD8+ cells in CD11b-KO and CD11d-KO splenocytes may be another reason for the reduced response of these cells to SE stimulation.

As an investigation in T cell development, cytometric analysis showed that the intensity of CD3 expression on CD11b- and CD11d-deficient thymocytes was also markedly reduced compared with that of WT thymocytes, and the ratios of CD4 to CD8 single-positive cells in CD11b-KO and CD11d-KO thymocytes were significantly lower than in WT thymocytes. In contrast, the ratios of CD4+ to CD8+ cells were normal in CD11a-KO thymocytes (27, 28). Our next expectation was that CD11b and CD11d might be expressed on WT mouse thymocytes and that the absence of CD11b or CD11d from the thymocytes as seen in CD11b-KO and CD11d-KO mice would result in changes in T cell phenotypes. Although expression of CD11b and CD11d was not detected on adult or 2-day-old mouse thymocytes, both CD11b and CD11d were expressed on early stage (day 12) WT mouse fetal thymocytes. In the early stage fetal thymocytes, the C-Kii+ population has been previously described to consist of two populations (CD11b+ and CD11b− subsets) (49). Two major single-positive populations, Thy-1.2 IL-2Rα− and Thy-1.2 IL-2Rα+, appear to emerge from this C-Kii+ population. These two major single-positive populations seem to be derived directly from the CD11b+ rather than from the CD11b− subset of the C-Kii+ population. The fact that deficiency in either CD11b, in which case CD11d is present, or CD11d, in which case CD11b is present, results in similar T cell phenotypic changes indicates that both CD11b and CD11d are essential for early T cell development, and CD11b and CD11d must play distinct roles in this process. Because the majority of CD11d+ cells in mouse early fetal thymocytes are CD11b+, we postulate that this CD11b and CD11d double-positive population is critically important for early thymocyte differentiation. These results suggest that CD11b and CD11d transduce signals inwardly to thymocytes, and/or couple thymocytes to distinct cell partners. Absence of either CD11b or CD11d from the cells would affect early T cell development, resulting in the phenotypic changes in mature T cells shown in CD11b-KO and CD11d-KO mice, with reduced T cell responses to SE stimulation. In support of the role of CD11b and CD11d in early T cell development, we found that anti-CD11b and anti-CD11d Abs did not inhibit SEA-induced T cell proliferation in WT splenocytes in vitro (data not shown). Although previous reports have suggested that β2 integrins play a key role in thymocyte differentiation (50, 51), this is the first report that β2 (CD11/CD18) integrins also play an important role in thymocyte differentiation.

In summary, our study showed that T cells from CD11b-KO and CD11d-KO splenocytes had decreased proliferative response to SE stimulation, which may be related in part to phenotypic changes as evidenced by lower intensity of CD3 and CD28 expression on splenocytes, and lower ratios of CD4+ to CD8+ cells in the KO splenocytes. We also found a lower intensity of CD3 expression and lower ratio of CD4+ to CD8+ cells in the KO thymocytes. Both CD11b and CD11d were expressed on WT early stage fetal thymocytes. We postulate that both CD11b and CD11d are required for early stage thymocyte development, and absence of either CD11b or CD11d from the early stage fetal thymocytes leads to a defect in T cell development, resulting in reduced T cell response to SE stimulation.

Acknowledgments

We acknowledge the technical assistance of Elizabeth Priest, the statistical assistance of E. O’Brian Smith, and the editorial assistance of Kerrie Jara.

References

306


