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J Immunol published online 7 June 2010
http://www.jimmunol.org/content/early/2010/06/07/jimmunol.0902658

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The Journal of Immunology is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Slam Haplotypes Modulate the Response to Lipopolysaccharide In Vivo through Control of NKT Cell Number and Function

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CD1d-restricted NKT cells make up an innate-like T cell subset that plays a role in amplifying the response of innate immune leukocytes to TLR ligands. The Slam locus contains genes that have been implicated in innate and adaptive immune responses. In this study, we demonstrate that divergent Slam locus haplotypes modulate the response of macrophages to the TLR4 ligand LPS through their control of NKT cell number and function. In response to LPS challenge in vivo, macrophage TNF production in Slam haplotype-2* 129S1/SvImJ and 129X1/SvJ mice was significantly impaired in comparison with macrophage TNF production in Slam haplotype-1* C57BL/6J mice. Although no cell-intrinsic differences in macrophage responses to LPS were observed between strains, 129 mice were found to be deficient in liver NKT cell number, in NKT cell cytokine production in response to the CD1d ligand a-galactosylceramide, and in NKT cell IFN-c production after LPS challenge in vivo. Using B6.129c1 congenic mice and adoptive transfer, we found that divergent Slam haplotypes controlled the response to LPS in vivo, as well as the diminished NKT cell number and function, and that these phenotypes were associated with differential expression of signaling lymphocytic activation molecule family receptors on NKT cells. These data suggest that the polymorphisms that distinguish two Slam haplotypes significantly modulate the innate immune response in vivo through their effect on NKT cells. The Journal of Immunology, 2010, 185: 000–000.

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Abbreviations used in this paper: aGalCer, a-galactosylceramide; BMDM, bone marrow-derived macrophage; DC, dendritic cell; IHL, intrahepatic leukocyte; MFI, median fluorescence intensity; NIH, National Institutes of Health; ODN, oligodeoxynucleotide; SLAM, signaling lymphocytic activation molecule.

Published June 7, 2010, doi:10.4049/jimmunol.0902658
BALB/cJ, and SJL/J mice, and *Slam* haplotype-1 is present in C57BL/6, C57BR/6J, and C57L/J mice, among others (36). In this study, we investigated whether genetic regulation of NKT cell number and function by *Slam* haplotypes would affect the ability of NKT cells to modulate innate immune function.

We identified 129S1/SvImJ and 129X1/SvJ strains as being severely deficient in liver, but not thymus or spleen, NKT cell number and in the response in vivo to the prototypical NKT cell agonist glycolipid, αGalCer. We found that these two 129 strains, as well as other strains with low liver NKT cell numbers, responded poorly to LPS in vivo, as measured by macrophage TNF production. Interestingly, we found no difference between the C57BL/6J and 129 strains in the response in vitro of macrophages and dendritic cells (DCs) to LPS. To investigate whether diminished NKT cell number and function were responsible for the poor response to LPS in vivo, we assessed the response to LPS in B6.129c1 congenic mice, which possess the 129-derived extended *Slam* locus introgressed onto the C57BL/6 background. B6.129c1 mice exhibited a deficiency in liver NKT cell number and an impairment in NKT function. Interestingly, we found that these mice also exhibited impaired in vivo macrophage TNF production in response to LPS and that adoptive transfer of C57BL/6J NKT cells to 129X1/SvJ mice resulted in increased macrophage TNF production after LPS challenge. These data suggest that *Slam* haplotypes significantly modulate the LPS response in vivo and that this phenotype is driven through its control of NKT cell number and function.

**Materials and Methods**

**Mice and reagents**

C57BL/6J, 129S1/SvImJ, 129X1/SvJ, LGJ, SJL/J, and NOD/ShiLtJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129-JA18 mice were a gift from Dr. Mark Exley (Beth Israel Deaconess Medical Center, Boston, MA). B6.129c1 congenic mice were described previously (36). Single nucleotide polymorphism genotyping indicated that the 129-derived interval extends from rs8245216 to rs30562373 (∼6.5 Mb). All mice were housed in the specific pathogen-free barrier facility at the University of Vermont. All procedures involving animals were approved by the University of Vermont Institutional Animal Care and Use Committee. The α-GalCer (Axxora Pharmaceuticals, San Diego, CA) was prepared as described (30) and was administered i.p. (100 µg/kg) in a 100-µl volume. LPS from *Salmonella enterica* (Sigma-Aldrich, St. Louis, MO) was diluted in sterile PBS. For in vivo experiments, 20 µLPS was administered i.p. in a 100-µl volume. CpG ODN 1826 (InvivoGen, San Diego, CA) was resuspended in PBS, and 50 µg was administered i.p. in a 100-µl volume.

**Serum cytokine analysis**

Serum was prepared from blood collected via cardiac puncture at various times after injection. Samples were frozen at −20°C until analysis. Serum cytokines were measured by ELISA (BD Biosciences, San Jose, CA) or Bio-Plex (Bio-Rad, Hercules, CA), according to the manufacturer’s instructions.

**Cell isolation and culture**

Splenocytes and thymocytes were obtained by gentle pressing through nylon mesh. RBCs were lysed using Gey’s solution. Intraperitoneal leukocyte (IHL) isolation was performed as described (30). Briefly, anesthetized mice were perfused with PBS, after which the liver was removed, minced, and gently pressed through nylon mesh. The resulting cell suspension was washed twice and then resuspended in isotonic 33.8% Percoll (GE Healthcare, Piscataway, NJ). After centrifugation, the IHL cell pellet was resuspended and washed in PBS + 2% FCS.

To obtain neutrophils, bone marrow was flushed with Hanks’ buffered saline solution and layered on a three-step Percoll gradient (72, 64, and 52%), which was centrifuged at 1060 × g for 30 min. Cytospin samples of the 72.64% interface revealed >95% morphologically mature-appearing neutrophils. These techniques were shown not to cause substantial activation or damage to the isolated cells (38, 39).

Peritoneal macrophages were isolated by injecting mice i.p. with 0.8 ml 3% Brewer’s thioglycollate (Sigma-Aldrich). Four days later, peritoneal exudate cells were harvested in RPMI 1640. Cells prepared in this way were ∼75% CD11b+T4/80+ (data not shown).

Bone marrow-derived macrophages (BMDMs) were derived by culturing bone marrow preparations in complete RPMI medium (RPMI 1640 supplemented with 10% FCS [Atlanta Biologicals, Lawrenceville, GA], penicillin-streptomycin, 1-glutamine, nonessential amino acids, 2-ME, and sodium pyruvate) supplemented with 100 ng/ml M-CSF (Ebioscience, San Diego, CA). Cells were used after 6 d and were 95% CD11b+T4/80+ (data not shown). Bone marrow-derived DCs were derived by expanding bone marrow preparations in complete RPMI medium supplemented with 5 ng/ml recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN) for 6–8 d. All incubations were performed at 37°C in a 5% CO2, humidified incubator.

**Flow cytometry**

Cells were stained at 4°C in PBS/2% FCS containing 0.1% sodium azide. F-Block (BD Biosciences) was used in all samples prior to the addition of Abs to block nonspecific Ab binding. Abs used in these experiments were anti-TCR-β (HS7-597), anti-IFN-γ (XM12), anti-IL-4 (11B11), anti-TNF (MP6-XT3), anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD3 (5D-7.3), anti-CD8 (53-6.7), anti-CD11b (1B1), anti-CD49b (DX5), and anti-NK1.1 (PK136) all from BD Biosciences. Anti-Gr1, anti-F4/80, anti-CD229 (Ly9b3), anti-CD48 (HM48-1), and anti-CD44 (mCD84.7) were from BioLegend (San Diego, CA). Anti-CD150 (9D1), anti-CD4 (RM4-5), and anti-CD44 (IM7), and anti-Ly108 (13G3-19D) were from eBioscience. CD14 tetramer loaded with PBS57 was obtained from the National Institutes of Health (NIH) tetramer facility (Emory University Vaccine Center, Atlanta, GA).

For intracellular cytokine staining, cells were isolated from liver or spleen as described above and stained with Abs to surface markers. In all experiments, cells were analyzed directly ex vivo with no cell culture or treatment with brefeldin A or monemurin. After washing in staining buffer, cells were fixed and permeabilized using fixation/permeabilization buffer (BD Biosciences), according to the manufacturer’s instructions. Fixed cells were stained with Alexa Fluor 647-conjugated anti-cytokine mAbs or isotype control mAbs. Data were collected on an LSRII (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

**Ex vivo cytokine analysis**

For ex vivo cytokine analysis by ELISA, splenocytes from αGalCer-injected mice were prepared and incubated in complete RPMI medium for 4 h at 37°C in a humidified 5% CO2 incubator. Supernatants were harvested, and cytokine levels were assessed by ELISA, according to the manufacturer’s instructions (BD Biosciences). Prior to the short-term incubation, the frequency of NKT cells in each preparation was determined by FACS analysis using anti-TCR and CD11d tetramer/PBS57 (data not shown). No significant difference in the percentage of spleen NKT cells between strains was observed, and no difference in the results was observed after normalizing for NKT cell number.

**Adoptive transfer**

For adoptive transfer, C57BL/6J liver IHLs were purified as described above, and NKT cells were sorted on a FACS/Aria (BD Biosciences). To avoid activation through TCR cross-linking prior to transfer, NKT cells were sorted using the surrogate markers anti-CD5 and anti-CD11c (22). CD5+CD11c− T cells from the same samples were sorted as non-NKT controls. Two hundred thousand sorted liver NKT cells were administered to 129X1/SvJ recipients by i.v. injection. Eighteen hours after cell transfer, all mice received 20 µLPS i.p.; 45 min later, mice were euthanized, and spleen and liver IHL TNF production was assessed by intracellular cytokine staining.

**In vitro LPS stimulation**

Bone marrow-derived DCs were incubated overnight in 48-well plates in complete RPMI medium and LPS. Supernatants were harvested after 16 h and tested for IL-12p70 by ELISA (BD Biosciences). Bone marrow-derived DCs were derived by expanding bone marrow preparations in complete RPMI medium supplemented with 10 ng/ml LPS for 16 h. LPS was tested for 16 h. Supernatants from these cultures were collected and tested for TNF by ELISA (BD Biosciences). Thiglycollate-elicited peritoneal macrophages were allowed to adhere to wells in 48-well plates for 2 h at 37°C, after which the nonadherent cells were removed. To prime cells, rIFN-γ (10 ng/ml) was added to some wells overnight. The next day, the supernatant was changed, and cells were stimulated with LPS in incomplete RPMI medium for 12 h, after which supernatants were collected. Purified neutrophils were allowed to adhere for 2 h in 24-well plates, after which they were stimulated with 10 ng/ml LPS for 45 min. Supernatants were
collected and tested for TNF by ELISA (BD Biosciences), according to the manufacturer’s instructions.

**Analysis of NF-κB activation**

The activity of NF-κB was assessed by quantitating the phosphorylation of IκB-α using Bio-Plex phosphoprotein assays and Bio-Plex total target bead-based multiplex assays (Luminex xMAP Technology) in cell lysates from BMDMs stimulated with LPS. Cell lysates were prepared using lysis buffer supplemented with protease and phosphatase inhibitors followed by freeze-thaw. Samples, positive-control lysates, and negative-control lysates were incubated overnight with beads coupled with Abs recognizing total IκB-α or phospho-IκB-α (Ser32/Ser36) in a 96-well filter plate. Beads were washed, and biotinylated detection Abs were added to the appropriate wells for 60 min. After another wash, streptavidin-PE was added to all wells for 20 min, the wells were washed, and the beads were resuspended in buffer. Data were acquired using the Bio-Plex suspension array system and Bio-Plex Manager software and are reported as the ratio of phospho-IκB-α to total IκB-α protein.

**Quantitative real-time PCR**

Liver NKT cells were FACs-sorted using anti-TCR and CD1d tetramer/PE-Cy7 (NIH tetramer facility). NKT cells were pooled from 6 C57BL/6J mice or 10 B6.129c1 mice. RNA was extracted using TRIzol LS (Life Technologies, Carlsbad, CA) and a microRNA purification kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. cDNA was synthesized and then amplified according to the manufacturer’s instructions (Ovation RNA Amplification System v2, NuGen Technologies, San Carlos, CA). Slam primers used were: Slam forwards were Slamf1 for 5'-TGGTGATATTGAGTATCATTTT-3', Slamf2 for 5'-TGGTGATATTGAGTATCATTTT-3', Slamf3 for 5'-TGGTGATATTGAGTATCATTTT-3', Slamf4 for 5'-TGGTGATATTGAGTATCATTTT-3'. Primers for Slamf1-3 were used as an endogenous control. The primer sequences for Slam-9 and Slamb2 were synthesized by Eurofins MWG Operon (Huntsville, AL) and a microRNA purification kit (Qiagen, Valencia, CA). An unpaired Student t test or one-way ANOVA was used where appropriate. In all cases, tests were considered significant when p < 0.05.

**Results**

**Severe impairment in NKT cell number and function in 129S1/SvImJ mice**

Previously, we reported the strain-dependent variation in NKT cell number and function among inbred strains of mice (30). To investigate the relationship between NKT cell number and function and the in vivo response to LPS in more detail, we characterized NKT cells in the 129S1/SvImJ strain, a commonly used strain that exhibits particularly low numbers of NKT cells in the liver. The number of NKT cells in different organs was enumerated through FACS analysis of TCRβ+ CD1d tetramer/PE-Cy7+ leukocytes. These data confirmed the deficiency in liver NKT cell numbers observed previously (42,483 ± 19,721; n = 6) compared with C57BL/6J mice (126,500 ± 39,415; n = 6) (Fig. 1A). In contrast, little difference in thymus or spleen NKT cell numbers was observed between the two strains (Fig. 1A).

To assess NKT cell function, we examined the response in vivo to the NKT cell agonist glycolipid, αGalCer. A comparison of serum cytokines elicited at various times after administration of αGalCer revealed a significant impairment in the production of TNF, IL-4, and IFN-γ in 129S1/SvImJ mice at all time points (Fig. 1B). Indeed, at peak early time points, serum cytokine concentrations in 129S1/SvImJ mice were as low as 10% of those in C57BL/6J mice. At 12 h, near its peak production in response to αGalCer, there was significantly less serum IFN-γ in 129S1/SvImJ mice compared with C57BL/6J mice (Fig. 1B). Analysis of the kinetics of NKT cell cytokine production after αGalCer administration revealed that a significantly smaller fraction of the 129S1/SvImJ NKT cells were producing cytokine at all times after αGalCer administration (Fig. 1C).

**Strain-dependent variation in the LPS response in vivo**

NKT cells have been implicated in the amplification of the response to TLR ligands, such as LPS (17, 21, 25). To investigate whether there was a correlation between strain-dependent variation in NKT cell number and function and the response in vivo to TLR ligands, we compared serum TNF levels in response to LPS between strains with high (C57BL/6J) and low (SMJ, LGJ, 129S1/SvImJ, and NOD/ShiLtJ) NKT cell numbers. At its peak, 1 h after LPS administration, serum TNF in 129S1/SvImJ mice (1079 ± 1019; n = 6) was <15% of that seen in C57BL/6J mice (7406 ± 1600; n = 5), a level similar to that observed in C57BL/6 NKT-deficient JA18/−/− mice that received LPS (Fig. 2A). Similarly, SMJ, LGJ, and NOD/ShiLtJ mice, previously reported to be deficient in NKT cell number and function (27, 30, 31), exhibited similarly low serum TNF levels in response to LPS administration (Fig. 2B). The strain-dependent variation was not limited to the response of TNF to LPS, because at 5 h after injection, serum IFN-γ levels in 129S1/SvImJ mice were also significantly lower than that of C57BL/6J mice (Fig. 2C). Likewise, the strain-dependent variation was not limited to LPS, because evaluation of the in vivo response to CpG ODN, which was previously demonstrated to result in NKT cell activation via the endogenous pathway (19), revealed significantly lower serum TNF and IL-6 levels in 129S1/SvImJ mice versus C57BL/6J mice (Fig. 2D). To investigate whether differences in NKT cell function could be involved in the impaired response of 129S1/SvImJ mice to LPS, we compared NKT cell function in both strains after an in vivo LPS challenge. These data revealed a striking decrease in NKT IFN-γ production in 129S1/SvImJ mice versus C57BL/6J mice (Fig. 2E).

**Impaired in vivo macrophage TNF production in response to LPS in 129S1/SvImJ mice**

TNF production in response to i.p. administration of LPS is primarily due to myeloid lineage cells (40), and it was demonstrated that macrophage TNF production in response to LPS is diminished in NKT cell-deficient JA18/−/− mice (21). Therefore, we used intracellular cytokine staining to directly compare LPS-induced TNF production between C57BL/6J and 129S1/SvImJ mice. This
FIGURE 1. Impaired NKT cell number and function in 129S1/SvImJ mice. NKT cell number and function were compared between age- and sex-matched C57BL/6J and 129S1/SvImJ mice. Thymus, spleen, and liver NKT cell numbers were assessed using anti-TCR and CD1d tetramer/PBS-57. CD45 was used in liver IHL preparations for gating purposes. A, Representative contour plots depicting percentages of NKT cells (left panels) and NKT cell number (right panels) in different tissues from C57BL/6 and 129S1/SvImJ mice. Each circle represents a single mouse, and the mean number of NKT cells is indicated by a line. **p < 0.01. B, Impaired serum cytokine production in 129S1/SvImJ mice in response to αGalCer. Mice were administered 2 μg αGalCer i.p., and serum was collected from blood at various time points after injection. Data are presented as the mean ± SEM. Each time point represents three or four mice per strain and is representative of three independent experiments. C, Intracellular cytokine staining of NKT cells at various times after αGalCer administration. Representative intracellular cytokine staining of splenocytes 2 h after αGalCer administration (top panels). Splenocytes were stained with anti-TCR and CD1d tetramer/PBS57 to identify NKT cells, and they were stained intracellularly with anti-cytokine or isotype control mAbs. Shaded graphs represent isotype control staining. The percentage of cytokine-positive NKT cells is shown. The mean percent cytokine positive ± SD NKT cells at all time points is shown in the bottom panels. Each point represents three or four mice and is representative of three independent experiments. D, Ex vivo analysis of NKT cell cytokine production. Splenocytes were harvested from mice injected 2 h previously with 2 μg αGalCer i.p. and placed in culture for 4 h, after which IFN-γ and IL-4 in cell culture supernatant was assessed using ELISA. The data are representative of two independent experiments.
analysis was done directly ex vivo, in the absence of any additional culture with brefeldin A or monensin. In liver and spleen, the bulk (85–95%) of the TNF production was from CD11b+Gr1lo cells, indicative of Kupffer cells and splenic macrophages, respectively (Fig. 3A). Within this population, the majority (∼65%) of the TNF was produced in CD11b+Gr1hi cells (data not shown). Comparison of intracellular TNF production in these leukocyte subsets between the two strains revealed a significantly lower TNF production in 129S1/SvImJ mice compared with C57BL/6J mice (Fig. 3B). The diminished TNF production in 129S1/SvImJ mice was observed in the Gr1lo (∼33% decrease) and the Gr1hi (∼50–65% decrease) CD11b+F4/80+ subsets (Fig. 3C). We conclude that macrophage TNF production in response to LPS is impaired in 129S1/SvImJ mice after an in vivo challenge with LPS.

Equivalent cell-intrinsic macrophage responses to LPS in C57BL/6J and 129S1/SvImJ mice

It is well known that certain inbred strains possess mutations that impair their ability to respond to LPS (41, 42). In addition, it has been demonstrated that there are a number of genetic variants of TLR4 in inbred laboratory strains (43). Therefore, we asked whether the poor response observed in 129S1/SvImJ mice was due to an inability of innate immune cells to recognize or to respond to LPS. In vitro stimulation of thioglycollate-elicited peritoneal exudate cells (∼75% F4/80+) from C57BL/6J and 129S1/SvImJ mice with LPS or with LPS and IFN-γ revealed no difference in TNF, IL-6, or nitrite production (Fig. 4A). Similarly, stimulation of BMDMs from the two strains with LPS revealed no difference in TNF production (Fig. 4B). In support of these data, we detected no difference in the phosphorylation of IκBα in response to LPS between C57BL/6J and 129S1/SvImJ BMDMs, indicating equivalent downstream signaling through the NF-κB pathway in macrophages from both strains (Fig. 4C).

In addition to the similarities in macrophage responses, a comparison of TNF production in response to LPS by bone marrow-derived neutrophils (Fig. 4D) and IL-12 production by bone marrow-derived DCs and BMDMs (Fig. 4E, data not shown) revealed no strain-dependent differences. Therefore, we concluded that there is no obvious cell-intrinsic difference in the ability of C57BL/6J and 129S1/SvImJ macrophages, DCs, or neutrophils to produce these cytokines in response to LPS.

Slam locus polymorphisms control liver NKT cell number

We next investigated a means through which we could test the hypothesis that impaired NKT cell function and/or low numbers of liver NKT cells in the 129S1/SvImJ strain was responsible for the poor in vivo response to LPS. Jordan et al. (33) demonstrated that Slam polymorphisms affect NKT cell development in NOD mice. Because extensive polymorphisms distinguish the C57BL/6J and 129-derived strain Slam haplotypes (36), we examined whether the two major Slam haplotypes affected NKT cell number and function using C57BL/6 mice congenic for a 129 chromosome 1 interval that encompasses the Slam locus [B6.129c1 mice (36)]. Because the Slam locus in this congenic strain was derived from the 129X1/SvJ strain, this strain was used in all experiments using B6.129c1 mice.

A comparison of NKT cell number in the thymus, spleen, and liver among B6.129c1 and parental C57BL/6J and 129X1/SvJ strains revealed a striking decrease in liver NKT cell numbers in the B6.129c1 congenic and parental 129X1/SvJ strains (Fig. 5A). In contrast, similar numbers of NKT cells were observed in the thymus and spleen in both strains (Fig. 5A). Examination of developmental stage-specific markers CD44 and NK1.1 expression on thymic NKT cells revealed no differences in expression between C57BL/6J and B6.129c1 mice (Fig. 5B). These data suggested that one or more polymorphisms that differentiate the C57BL/6J and 129X1/SvJ Slam haplotypes control liver, but not...
spleen or thymus, NKT cell number and that the effect is not due to a defect in NKT cell development.

The majority of CXCR6+ cells in the liver are NKT cells (44). Because recent reports demonstrated a link between CXCR6 expression and NKT cell homeostasis in the liver (45, 46), we used quantitative PCR to examine CXCR6 expression in sorted liver NKT cells from C57BL/6J and B6.129c1 congenic mice. No significant difference in expression was observed (Fig. 5C), suggesting that the low numbers of liver NKT cells are not due to alterations in CXCR6 expression.
Contribution of Slam haplotypes to NKT cell function in vivo

To determine the contribution of the Slam haplotypes to the defective response of 129 NKT cells to a GalCer in vivo, C57BL/6J, 129X1/SvJ, and B6.129c1 congenic mice were injected with a GalCer, followed by the evaluation of serum cytokines and NKT cell cytokine production using intracellular cytokine staining. A comparison of serum cytokine production in response to a GalCer revealed significantly lower cytokine production in the B6.129c1 mice versus the C57BL/6J mice (Fig. 5D). Similarly, examination of intracellular cytokine production in response to a GalCer revealed significantly lower cytokine production in the B6.129c1 mice versus the C57BL/6J mice (Fig. 5E).

FIGURE 5. Slam-dependent control of liver NKT cell number and function. A, Comparison of NKT cell numbers in thymus, spleen, and liver cell suspensions among C57BL/6J, B6.129c1, and 129X1/SvJ strains. NKT cells were identified using anti-TCR and CD1dtetramer/PBS-57. CD45 was used in IHL preparations for gating purposes. NKT cell yield from each tissue (left panels) and representative contour plots of liver NKT cell percentages (right panels) are shown. Each circle represents data from a single mouse. The mean number of NKT cells is indicated by a line. B, Comparison of CD44 and NK1.1 expression on CD1dtetramer/PBS57-gated thymocytes from 7-wk-old C57BL/6J and B6.129c1 mice. Data are representative of two mice per strain. C, Comparison of CXCR6 gene expression in 129X1/SvJ and B6.129c1 liver NKT cells by quantitative real-time PCR. Results are the fold difference in B6.129c1 Slam gene expression compared with C57BL/6J mice. The dashed lines represent an arbitrarily assigned threshold of a 2-fold difference in gene expression. D, A comparison of serum cytokines in response to aGalCer among age- and sex-matched C57BL/6J, B6.129c1, and 129X1/SvJ strains. Data are mean ± SD (n = 3–4 mice per experiment) and are representative of two independent experiments. E, Impaired NKT cell cytokine production in B6.129c1 congenic mice. NKT cell cytokine production in response to aGalCer was assessed using intracellular cytokine staining. Splenocytes were stained with anti-TCR and CD1dtetramer/PBS57 and stained intracellularly with anti-cytokine mAbs or isotype-matched control mAb. Each circle represents data from a single mouse. The mean percentage of cytokine+ cells is indicated by a line. Data are the percentage of cytokine+ cells and are cumulative data from two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.

Contribution of Slam haplotypes to NKT cell function in vivo

To determine the contribution of the Slam haplotypes to the defective response of 129 NKT cells to aGalCer in vivo, C57BL/6J, 129X1/SvJ, and B6.129c1 congenic mice were injected with aGalCer, followed by the evaluation of serum cytokines and NKT cell cytokine production using intracellular cytokine staining. A comparison of serum cytokine production in response to aGalCer revealed significantly lower cytokine production in the B6.129c1 mice versus the C57BL/6J mice (Fig. 5D). Similarly, examination of intracellular cytokine production in response to aGalCer revealed a significant decrease in the percentage of B6.129c1 NKT cell IFN-γ, IL-4, and TNF production versus that of C57BL/6J mice (Fig. 5E). In both of these experiments, cytokine production in 129X1/SvJ mice was lower than that of B6.129c1 mice, suggesting the presence of one or more additional loci that control NKT cell cytokine production. We found no significant differences in CD154, CD69, or CD25 expression between B6 and B6.129c1 NKT cells 2 h after aGalCer administration, nor did we observe any significant differences in CD80, CD86, CD1d, or MHC class II expression between B6 and B6.129c1 DCs and macrophages 2 h after aGalCer administration (data not shown). Taken together, these data suggest that Slam haplotypes control the phenotypic difference in liver NKT cell number between C57BL/6J and 129X1/SvJ strains and that one or more genes at this locus modulate in vivo NKT cell cytokine production in response to aGalCer.

Slam haplotypes modulate the response to LPS in vivo

To test the hypothesis that impaired liver NKT cell number and function in 129X1/SvJ mice was responsible for the poor in vivo response to LPS in this strain, serum proinflammatory cytokines were measured after i.p. LPS administration to C57BL/6J, 129X1/SvJ, and B6.129c1 congenic mice. Although the mean serum TNF concentration in C57BL/6J mice was 7.3 ± 1.5 ng/ml, B6.129c1 and 129X1/SvJ mice had mean serum TNF concentrations of 2.2 ± 1.2 ng/ml and 2.3 ± 0.48 ng/ml, respectively (Fig. 6A). Therefore, introgression of the 129X1/SvJ Slam locus onto the
C57BL/6J background resulted in a 70% decrease in serum TNF production in response to LPS. Ex vivo analysis using intracellular cytokine staining revealed significant decreases in TNF production by CD11b^F4/80^Gr1^lo^ and CD11b^F4/80^Gr1^hi^ leukocytes in response to LPS in the B6.129c1 congenic strain (Fig. 6B). These data indicated that, in addition to controlling NKT cell number and function, Slam haplotypes modulate in vivo macrophage TNF production in response to LPS. Analysis of CD86, MHC class II, and CD1d on B6 and B6.129c1 DCs and macrophages revealed no differences in expression after LPS stimulation (data not shown).

Because we did not observe any difference in the ability of myeloid-derived cells to respond to LPS in vitro between C57BL/6J and 129 mice, and because TNF production in response to LPS is impaired in NKT-deficient C57BL/6J mice (21) (Fig. 2B), these data suggested that the impaired in vivo response to LPS in both 129-derived strains was due to a Slam haplotype-dependent impairment in NKT cell number and function. However, we could not formally exclude the possibility that the phenotypic differences in macrophage TNF production in response to LPS and the phenotypic differences in the NKT cell compartment were mutually exclusive outcomes dependent on polymorphisms at the Slam locus. To investigate whether NKT cells modulated the macrophage response to LPS, we used adoptive transfer to assess whether C57BL/6J NKT cells could rescue the poor response of 129 macrophages to LPS in vivo. Adoptive transfer of C57BL/6J (H-2^b^) NKT cells (~87% purity) or control CD8^+^ T cells (~88% purity) to 129X1/SvJ (H-2^b^) recipients was followed by evaluation of macrophage TNF production in response to an LPS challenge in vivo. A significantly higher level of liver macrophage TNF production was observed in 129X1/SvJ mice that received C57BL/6J NKT cells (209 ± 62 median fluorescence intensity [MFI] [48 ± 6%]) versus those that received control C57BL/6J CD8^+^ T cells (137 ± 42 MFI [35 ± 11%]) (Fig. 6C). Similarly, a significantly higher level of spleen macrophage production was observed upon transfer of NKT cells (262 ± 65 MFI [50 ± 2.8%]) versus control CD8^+^ T cells (148 ± 58 MFI [37 ± 8%]). Despite the increase in intracellular macrophage TNF, a significant increase in serum TNF was not observed (data not shown). We concluded that increased numbers and/or enhanced function of NKT cells results in an increase in vivo TNF production by macrophages and that divergent Slam haplotypes modulate the response to LPS in vivo through their effect on NKT cell number and function.

**FIGURE 6.** Modulation of in vivo macrophage TNF production through Slam haplotype-dependent control of NKT cells. A. Serum TNF production in age- and sex-matched C57BL/6J, B6.129c1, and 129X1/SvJ mice 1 h after LPS administration. Data are mean ± SD and are representative of three independent experiments. B. A comparison of CD11b^F4/80^Gr1^lo^ and CD11b^F4/80^Gr1^hi^ TNF production in response to in vivo LPS challenge among the three strains. Data are mean ± SD (n = 4 mice per strain) and are representative of two independent experiments. C. Increased 129X1/SvJ macrophage TNF production in response to LPS after adoptive transfer of C57BL/6J NKT cells. NKT cells or CD8^+^ T cells were sorted from the livers of C57BL/6J mice and adoptively transferred to 129X1/SvJ recipients. The following day, all recipient mice were challenged with LPS, after which macrophage TNF production was assessed ex vivo using intracellular cytokine staining. Top panels, Representative intracellular TNF staining of CD11b^F4/80^Gr1^lo^ liver IHLs. Shaded graphs indicate isotype control mAb staining. Open graphs indicate TNF staining. Bottom panels, Cumulative MFI of TNF staining in liver CD11b^F4/80^Gr1^lo^ leukocytes. C57BL/6 and 129 parental strains that received no cells are included for comparison. Data are mean percentage ± SD (n = 5–6 mice per strain) and are the combined data from two independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001.
Differential NKT SLAM receptor expression among divergent Slam haplotypes

The introgressed extended Slam locus consists of a number of immunologically relevant genes that could account for the phenotypic differences between the two parental strains (Table I). Given previous reports implicating Slam genes in NKT development (33, 34), as well as reports demonstrating numerous polymorphisms at Slam family loci (36, 37), we evaluated the differential expression of members of this gene family on macrophages and NKT cells between the parental C57BL/6J mice and B6.129c1 congenic mice. We used a panel of mAbs to compare the expression of SLAMF1 (CD150), SLAMF2 (CD48), SLAMF3 (CD229), SLAMF5 (CD84), and SLAMF6 (Ly108) between C57BL/6J and B6.129c1 CD11b+Gr1hi and CD11b+Gr1lo monocytes/macrophages. Although all SLAM receptors evaluated were expressed on both subsets, we noted no significant difference in their expression between strains, with the exception of SLAMF3, which exhibited a small, but significant, decrease in expression on CD11b+Gr1hi cells in C57BL/6J mice (Fig. 7A).

The B6.129c1 interval also contains two genes encoding the SLAM family adapter proteins Sh2d1b1 and Sh2d1b2 (EAT-2 and ERT, respectively). Because these genes are homologous to the well-studied Sh2d1a gene that encodes the SLAM-associated protein and are known to be expressed in myeloid lineage cells as well as NK cells, they were possible candidate genes that could account for strain-dependent differences in macrophage TNF production. However, a comparison of Sh2d1b1 and Sh2d1b2 expression between C57BL/6J and B6.129c1 macrophages and DCs by quantitative PCR revealed no difference in expression (Fig. 7B).

In contrast, a comparison of SLAM family gene expression on NKT cells between C57BL/6J and B6.129c1 mice revealed significant differences in expression (Fig. 8A). Quantitative PCR analysis revealed that compared with B6.129c1 NKT cells, C57BL/6J NKT cells exhibited a >2-fold overexpression of Slamf2, Slamf4, Slamf5, and Slamf8, whereas B6.129c1 overexpressed Slamf3 (Fig. 8A). Slamf6 was demonstrated to undergo alternative splicing, which results in two expressed isoforms differing in their cytoplasmic tails (48). Expression of each isoform is characteristic of the divergent Slam haplotypes, with Slam haplotype-1 strains (e.g., C57BL/6J) preferentially expressing the long isoform (Ly108-2; herein termed Slamf6.2) and Slam haplotype-2 strains (e.g., 129X1/SvJ) expressing the short isoform (Ly108-1; herein termed Slamf6.1) (36). Quantitative PCR using primers designed to specifically identify each alternative splice product revealed an ~8-fold overexpression of the Slamf6.1 isoform in the B6.129c1 versus C57BL/6J NKT cells and a corresponding ~5-fold overexpression of the Slamf6.2 isoform in C57BL/6J versus B6.129c1 NKT cells (Fig. 8A).

We next compared SLAM family receptor expression on NKT cells and CD1dtet T cells using a panel of mAbs. Of the receptors analyzed, there was an overexpression of SLAMF3, SLAMF5, and SLAMF6 on B6.129c1 NKT cells, whereas SLAMF2 expression was decreased, and SLAMF1 expression was unchanged (Fig. 8B). The expression of SLAMF2 on B6.129c1 NKT cells was 35% lower than that on C57BL/6J NKT cells, whereas the expression of SLAMF6 on C57BL/6J NKT cells was 48% lower than that on its B6.129c1 counterparts. Therefore, with the exception of SLAMF5, there was general agreement between SLAM family receptor expression and Slam gene-expression data. Interestingly, although SLAMF3 and SLAMF5 exhibited similar strain-dependent differences in their expression in CD1dtet T cells, altered expression of SLAMF2 and SLAMF6 seemed to be NKT cell specific (Fig. 8B). Taken together, these data indicated that significant differences exist in SLAM receptor expression on NKT cells, but not macrophages, between the two divergent Slam haplotypes.

Discussion

The extensive polymorphism that distinguishes multiple Slam haplotypes in commonly used inbred strains of mice has previously been linked to the development of autoimmunity (36, 37), B cell tolerance (49), and NKT cell development (33). In this study, we demonstrated that divergent Slam haplotypes significantly modulate the in vivo host response to TLR ligands, such as LPS. Furthermore, our results indicated that Slam haplotype-mediated control of the in vivo response to LPS is not due to a cell-intrinsic effect on innate immune cells; rather, it is due to the control of NKT cell number and function.

In contrast to the well-described interactions of NKT cells and DCs, less is known about the interactions between NKT cells and macrophages. Increasing evidence suggests that the interplay of NKT cells and macrophages may play important roles in infectious disease models (24, 50–52), tumor immunology (53), and inflammation (54). Macrophages can activate NKT cells in vitro indirectly via TLR ligands or directly through CD1d-mediated presentation of αGalCer (55). However, reports conflict on their efficiency in presenting αGalCer in vivo (56–58).

The ability of NKT cells to influence macrophage function has previously been demonstrated (21, 59). In JA18-deficient mice lacking the semi-invariant NKT cell population, impaired macrophage TNF production and serum TNF levels in response to LPS were associated with reduced NKT cell IFN-γ production (21). This study was consistent with earlier reports demonstrating that NKT cells play a critical role in a model of LPS-induced septic shock (25, 60), which is dependent on macrophage-derived TNF (61). Taken together, the data suggest a model in which NKT cells may serve as a regulatory checkpoint in the sequence of events leading from macrophage TLR4 signaling to TNF production. Our data extend this model by demonstrating that genetic polymorphisms that confer phenotypic variation in the NKT cell compartment have the potential to modulate the macrophage response to TLR ligands in vivo.

The influence of Slam haplotypes on liver NKT cell number in the 129X1/SvJ strain is consistent with previous work identifying the Slam locus as one of two major loci controlling NKT cell numbers in NOD mice (33, 62), which are known to have decreased numbers of NKT cells (27, 28, 31, 32, 63). However, in

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Table I. Immunologically relevant genes in the B6.129c1 interval

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chr 1 Position (Mb)</th>
<th>Gene</th>
<th>Chr 1 Position (Mb)</th>
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<tbody>
<tr>
<td>Sh2d1b2</td>
<td>172162880</td>
<td>Slamf6 (Ly108)</td>
<td>173847668</td>
</tr>
<tr>
<td>Sh2d1b1</td>
<td>172207507</td>
<td>Pex19</td>
<td>174056903</td>
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<td>Fcrlb</td>
<td>172837404</td>
<td>Slam9</td>
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<td>Fcrla</td>
<td>172847707</td>
<td>Slam8</td>
<td>174511508</td>
</tr>
<tr>
<td>Fcrlb2</td>
<td>172890689</td>
<td>Fcrlb</td>
<td>174528329</td>
</tr>
<tr>
<td>Fcrlb4</td>
<td>172949051</td>
<td>Cpr</td>
<td>174628197</td>
</tr>
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<td>Fcrl3</td>
<td>172981301</td>
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<td>174620903</td>
</tr>
<tr>
<td>Fcrl1g</td>
<td>173159703</td>
<td>Fcrl1a</td>
<td>175151415</td>
</tr>
<tr>
<td>Dedd</td>
<td>173259276</td>
<td>Darc</td>
<td>175262017</td>
</tr>
<tr>
<td>Slamf4 (CD244)</td>
<td>173489324</td>
<td>Aim2</td>
<td>175392278</td>
</tr>
<tr>
<td>Slamf3 (Ly9)</td>
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<td>Ifi204</td>
<td>175677424</td>
</tr>
<tr>
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<td>175562534</td>
<td>Mnda</td>
<td>175826486</td>
</tr>
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<td>Ifi202b</td>
<td>175892706</td>
</tr>
<tr>
<td>Slamf5 (CD84)</td>
<td>175769828</td>
<td>Ifi205</td>
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</table>

Positions based on National Center for Biotechnology Information Build 37/mm9 (47).
contrast to the C57BL/6 and NOD strains, we detected no significant difference in thymic or spleen NKT cell numbers among the parental C57BL/6J, B6.129c1, or the two 129 strains, nor could we find any evidence of developmental arrest in the B6.129c1 thymocytes. These differences could be due to the different congenic intervals or to strain-specific differences in interactions between Slam haplotype genes and C57BL/6J and NOD background genes, as seen in B6.Sle1b mice (36).

It is unclear why Slam haplotypes preferentially affect liver NKT cell number in B6.129c1 congenic mice. However, the Slam haplotype is not unique in this regard. Decreased liver NKT cell number was also observed in B6.NOD-Idd4 mice (32). In addition, it was recently reported that deletion of the Id2 transcriptional regulator results in a specific depletion of NKT cells from the lung and liver (46). Our analysis of the B6.129c1 mice indicated no difference in liver NKT CXCR6 expression and no difference in lung NKT cell number (data not shown). Thus, multiple pathways seem to be able to affect liver NKT cell numbers, suggesting that this population may be particularly sensitive to a variety of signals affecting homeostasis.

In addition to the effect on liver NKT cell number, Slam haplotypes significantly affected the NKT cell cytokine production in response to aGalCer. In contrast to NOD NKT cells, which exhibit a defect in IL-4 production, NKT cells from 129 mice, as well as other Slam haplotype-2+ inbred mouse strains, exhibit diminished production of IFN-γ, IL-4, and TNF (30). Moreover, our data suggest that Slam haplotypes control a significant fraction of the phenotypic variation in NKT cytokine production between the C57BL/6J and 129X1/SvJ parental strains. In this context, it is interesting to note that impaired SLAMF1 (CD150) signaling was recently suggested to be the defect underlying impaired IL-4 production in NOD NKT cells (64).

Although a number of immunologically relevant genes are contained within the 129 interval in the B6.129c1 mouse, the previous reports describing a role for the Slam family of receptors and their adapter proteins in NKT cell development and in NKT and T cell function (33, 34, 64–66) suggest they are likely candidate genes that control the phenotypes reported in this study. In
this context, the differential expression of the *Slamf6.1* and *Slamf6.2* isoforms and the decreased cell surface expression of SLAMF6 on C57BL/6J liver NKT cells is intriguing. The C57BL/6J and 129X1/SvJ Slam haplotypes are distinguished by their differential expression of *Slamf6* isoforms that result from alternative splicing of the exons encoding the cytoplasmic domains (36). Interestingly, the *Slamf6.1* isoform (associated with 129X1/SvJ) was demonstrated to signal more efficiently than its *Slamf6.2* counterpart (C57BL/6J) (36, 67), raising the possibility that liver-specific variation in NKT cell number may be due to altered NKT cell homeostasis as a result of SLAM family receptor signaling. We speculate that similar alternative splicing of *Slamf5* could explain the discordance between the transcript levels measured by quantitative PCR and the SLAMF5 protein expression on liver NKT cells.

SLAM family receptors have also been implicated in innate immune function. *Slamfl* (SLAM, CD150)- and *Slamf6* (Ly108)-deficient mice exhibit defects in the recognition of LPS by macrophages (65) and neutrophils (68), respectively, raising the possibility that differential expression of Slam family alleles in C57BL/6J and 129 parental strains revealed no differences in NF-κB signaling or cytokine production. The fact that the variation in response to LPS was observed in vivo, but not in vitro, supports our conclusion that the poor in vivo response by macrophages to LPS is not the result of an intrinsic macrophage defect but is instead the result of in vivo regulation of macrophage function through another leukocyte subset.

It is important to stress that the extended Slam haplotype includes numerous additional candidate genes that could control the phenotypes reported in this study (Table I) and that additional analysis of subcongenic lines is needed to identify the gene(s) involved in modulating the macrophage and NKT cell function. Nevertheless, we note that the altered function in the NKT cell and macrophage compartments in 129-derived strains has important implications regarding examination of the role of Slam family genes using genetically engineered mice. Because seven of the nine Slam family genes are located in a ~400 kb segment of chromosome 1, analysis of NKT cell and macrophage function using Slam-deficient mice on a mixed B6.129 background is problematic due to the tight linkage of the Slam genes, and should be interpreted cautiously (69).
Divergent *Slam* haplotypes were implicated in alteration of T cell function and in B cell tolerance (36, 49). In this study, we demonstrated for the first time that divergent *Slam* haplotypes significantly modulate the in vivo response of macrophages to TLR ligands such as LPS as well as the innate-like NKT cell lymphocyte subset. The ability of NKT cells to respond rapidly to agonist stimulation and to modulate the function of a wide variety of leukocytes, including macrophages, suggests that they may be uniquely positioned to influence the developing immune response. Our data provide evidence to support the notion that the influence of host genetic background on NKT cells can be propagated through the innate arm of the immune response.

Acknowledgments

We thank Colette Charland for flow cytometry and Karen Spach, Isaac Hoehn, and Ben Schott for technical assistance. We thank Mercedes Rincon and Juan Anguita for helpful discussion. The real-time PCR was performed at the Vermont Cancer Center DNA Analysis Facility. Single nucleotide polymorphism genotyping was performed by DartMouse, Norris Cotton Cancer Center, Dartmouth-Hitchcock Medical Center, Lebanon, NH. The CD1d tetramer/PBS57 was obtained from the NIH Tetramer Core Facility.

Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The middle initials of the sixth author’s name were published incorrectly. The correct name is Graham W. J. Lilley.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1190052