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*J Immunol* 2006; 177:2258-2264; doi: 10.4049/jimmunol.177.4.2258

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Macrophages are a prominent component of the effector cell compartment in a number of CD4+ T cell-mediated organ-specific autoimmune diseases. In this study, we investigated the role of the sialic acid binding Ig-like lectin sialoadhesin (Sn, Siglec-1) in a model of interphotoreceptor retinal binding protein peptide-induced experimental autoimmune uveoretinitis in mice with targeted deletion of Sn. Our data show that compared with wild-type mice, experimental autoimmune uveoretinitis is reduced in severity in the initial stages in the Sn knockout (KO) mice. In addition, there is a reduction in the proliferative capacity of T cells from the KO mice draining lymph nodes after immunization with interphotoreceptor retinal binding protein peptides, which is manifest some days before disease onset and persists for the duration of disease. Furthermore, activated T cells from the draining lymph nodes of Sn KO mice secrete lower levels of IFN-γ. The data suggest a role for Sn in “fine tuning” the immune response to autoantigens by modulating T cell priming. The Journal of Immunology, 2006, 177: 2258–2264.

Macrophages are recognized as major effectors of tissue damage in several models of autoimmune disease, including CNS disease (1–3). Total depletion of macrophages leads to suppression of experimental autoimmune encephalomyelitis (4) and experimental autoimmune uveoretinitis (EAU) (5). Macrophages are now known (6, 7) to be a markedly heterogeneous cell subtype and may regulate as well as promote inflammation (8, 9). In a study of EAU, we previously (10) showed that different macrophage surface markers were expressed during different stages of the disease, with sialoadhesin (Sn) expression occurring at the peak and later stages of disease, rather than during initiation of EAU. The precise role of Sn in EAU, however, was unclear.

Molecular characterization of Sn from spleen macrophages was described in 1991 (11), following an earlier demonstration that stromal macrophages express a receptor for unopsonized sheep erythrocytes via sialylated glycoconjugates (12), Sn, also known as Siglec-1 or CD169, is one of a subset of sialic acid binding lectin-like molecules (Siglecs) (13). Each Siglec exhibits a unique specificity for sialylated glycans, and Sn prefers α2,3-linked sialic acids of the Neu5Ac rather than the Neu5,9(Ac)2 or Neu5Gc types (see Ref. 14). Sn has been shown to bind several membrane receptors via both sialic acid-dependent and -independent mechanisms; for instance, the sialomucins leukosialin (CD43) on T cells and MUC-1 on breast cancer cells are putative sialic acid-dependent counterreceptors (15), whereas the macrophage mannose receptor, which is present on several types of myeloid cells (15), and the mouse macrophage galactose-type C-type lectin 1 (16) binds Sn in a sialic acid-independent manner.

Under steady-state conditions, highest levels of Sn expression are found on subsets of macrophages located in the marginal zone of the spleen and the subcapsular sinus of the lymph node (17, 18). Sn can also be induced on macrophages present at sites of inflammation in both humans and rodents (10, 19–21). Although its function is not known, it is involved in the attachment and internalization of certain viruses (22, 23), has the potential to endocytose sialylated bacteria such as Neisseria meningitidis (24), and it has long been known to promote adhesion of macrophages to T cells, as well as to other cell types such as neutrophils and macrophages (20, 25). Because Sn is expressed on subsets of inflammatory macrophages, it could serve as a marker of activation in models in which macrophages are considered to be the pathogenic cell. Anti-inflammatory treatments, for instance with IL-11, are associated with reduction in overall tissue damage with a selective decrease in the number of Sn+ macrophages (19).

In this study, we have induced EAU in Sn-deficient (Sn knockout (KO)) mice using uvetoegenic peptides from interphotoreceptor retinal binding protein (IRBP) (10, 26, 27) and show that compared with wild-type (WT) control mice, the disease is reduced in severity in the initial stages. In addition, there is a reduction in the proliferative capacity of T cells from the draining lymph nodes (DLNs) after immunization with IRBP peptides which is manifest some days before disease onset and persists for the duration of disease. Furthermore, activated T cells from Sn KO mice secrete lower levels of IFN-γ. Macrophages from Sn KO mice housed in conventional clean (not specific-pathogen-free) conditions also constitutively display lower levels of NO production. The data suggest a role for Sn in fine tuning the immune response to autoantigens by modulating T cell priming.

Materials and Methods

Animals

Generation of Sn-deficient mice was described in detail elsewhere (28). In brief, the Sn gene was targeted in embryonic stem cells by homologous
recombination, involving insertion of a neomycin resistance gene expression cassette in exon III, encoding domain 2 of Sn. The resulting chimeric mice were backcrossed to C57BL/6 mice and B10.RIII mice for eight generations. Because EAU in C57BL/6 background mice is very mild, we also generated Sn KO mice on the EAU-susceptible B10.RIII background to confirm the results. Genotyping of mice was performed by PCR using the following primers: forward, CACCAGGTCACTGGTGAAC; reverse, GCCCATATGAGGTTGCTCT. This resulted in a 468-bp product for the WT allele and a 1729-bp product for the mutated allele. Sn-deficient mice were characterized as true nulls, based on complete absence of detectable protein using Abs to the N and C termini of Sn (28).

Inbred Sn WT, heterozygous (HET), and KO mice at the age of 6 to 10 wk old were used for the experiments. The procedures adopted conformed to the regulations of the Animals License Act (U.K.) and to the Association for Research in Vision and Ophthalmology Statement for the use of Animals in Ophthalmic and Vision Research.

Antigens

IRBP peptide 1–20 (GPHTLQPSSLVLMANKVLLD) and IRBP peptide 161–180 (SGIPYISLYHPGNTILHVD) (purity >95%) were used to induce EAU in C57BL/6 and B10.RIII background mice, respectively (both synthesized by Sigma Genosys).

EAU induction and evaluation

Sn WT, HET, and KO mice on the C57BL/6 background were immunized s.c. with 300 μg of IRBP peptide 1–20 emulsified with an equal volume of CFA (H37Ra; Difco Labs) in a total volume of 100 μl. An additional i.p. injection of 0.5 μg of purified *Bordetella pertussis* toxin (strain Wellcome-28; Speywood Pharmaceuticals) in 100 μl was also given to each animal. Similar experiments were performed on Sn WT and KO mice bred onto an EAU-susceptible B10.RIII background; mice were immunized s.c. with 100 μg of IRBP peptide 161–180 mixed with an equal volume of CFA and an additional i.p. injection of 0.5 μg of *Bordetella pertussis* toxin as described previously (10).

The animals were killed at different time points after immunization as designed, and the eyes were removed and immediately fixed in 2.5% buffered glutaraldehyde and embedded in resin for standard H&E staining. The intensity of uveoretinitis disease was evaluated histologically and graded using the modified version of the customised histological grading system (29) by independent observers as reported previously (30). This grading system permits a semiquantitative assessment of the severity and extent of both inflammatory cell infiltrative (lymphocyte invasion) and structural changes (tissue thickening, hemorrhage, and rod outer segment loss) of the anterior and posterior segments of the mouse eye including the iris, ciliary body, retina, and choroid at the time of the experiment.

Lymphocyte proliferation

Two sets of experiments were performed to evaluate lymphocyte proliferation in Sn WT and KO mice. In the first set, both WT and KO mice on a C57BL/6 background were immunized with IRBP peptide 1–20, as described in Materials and Methods. At days 12 and 26 after immunization, both WT and KO mice were sacrificed and the inguinal DLNs of the immunization site and spleens were collected and pooled within each group. Tissues were mashed through a sieve and spleen cells were depleted of RBC using ammonium chloride lysis buffer (BD Pharmingen), then single cells were collected and counted for culture. For lymphocyte proliferation, triplicate cultures of 1 × 10^5 cells/well were incubated in 96-well round-bottom tissue culture plates in 200 μl of complete RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS (Sigma-Aldrich), 2 mM t-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 5 × 10^-2 mM 2-ME. Cells were stimulated with IRBP peptide 1–20 at the final concentrations of 0, 2, and 20 μg/ml. The cultures were incubated for 90 h at 37°C in 5% CO_2 in air and were pulsed with 0.5 μCi [3H]thymidine/well during the last 16 h of incubation.

A second set of experiments was performed on similarly immunized mice, as a time course study beginning 4 days after immunization, and samples were collected at days 4, 6, 8, 10, 12, 15, and 17 after immunization. In this set of experiments, DLNs and spleens were collected and prepared separately from individual mice to reflect more accurately the individual variability of the assay, and a minimum of six mice was evaluated at each time point. Because fewer cells were harvested from individual mice DLNs, 1 × 10^5 cells/well and 5% FCS were used for the assay with the peptide concentration at 20 μg/ml, since this was found in preliminary experiments to permit optimal cell growth. Otherwise conditions were as those above.

Cytokine measurement

Sn WT and KO C57BL/6 mice were immunized with peptide 1–20 and the mice were killed at days 12 and 26 after immunization. The DLNs and spleens were collected and pooled within each group and cells were cultured in complete RPMI 1640 with 50 μg/ml IRBP peptide 1–20, which was the optimal dose for T cell cytokine production shown by our preliminary experiment. After 72 h, supernatants were collected and stored at −20°C. IFN-γ in the supernatant was measured using the optELISA kits from BD Pharmingen. Brieﬂy, ﬂat-bottom 96-well plates were coated with anti-IFN-γ capture Ab at 4°C overnight. The plates were then blocked with 10% FCS for 1 h and incubated for another 2 h with sample supernatants or different concentrations of IFN-γ standard, followed by a 1-h coating of biotin-conjugated IFN-γ Ab. Finally, HRP-conjugated streptavidin was added before the plates were developed with tetramethylbenzidine substrate (BD Pharmingen). The ELISA procedures were performed at room temperature except where stated.

Isolation and cytokine stimulation of macrophages

Cold RPMI 1640 medium (4°C) was injected into the peritoneal space of freshly culled mice. Five minutes later, the medium was collected and centrifuged to provide a pellet containing resting peritoneal macrophages. Cell purity was conﬁrmed by using ﬂow cytometry and a series of mouse macrophage Abs (F4/80, CD11b, and MOMA-2). Then the cells were
plated in 24-well plates at a concentration of 1 × 10^6 cells/ml per well with or without the following stimuli: LPS (1 μg/ml), IFN-γ (20 U/ml) plus TNF-α (5 ng/ml), or IL-4 (10 ng/ml).

**Measurement of NO synthesis**

After a 48-h stimulation, macrophage supernatant was collected and used to detect nitrite as an indirect measure of NO production using Griess reagent assay as described previously (31). Briefly, 100 μl of the cell supernatant was incubated with 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride; Sigma-Aldrich) in 96-well flat-bottom plate for 10 min at room temperature, then the ODs were measured at 540 and 690 nm to account for background. Nitrite concentration was determined by comparison to a sodium nitrite standard (Sigma-Aldrich) on the same plate.

**Reproducibility and statistical analysis**

Experiments were repeated at least twice. Figures show data from one representative experiment. EAU grades (nonparametric) were analyzed by the Mann-Whitney U test. Analysis of lymphocyte proliferation responses, cytokine production, and NO production was performed by independent t test. Probability values of <0.05 were considered statistically significant.

**Results**

**The onset and severity of EAU are delayed and reduced in Sn-deficient mice**

EAU was induced with IRBP peptide 1–20 in Sn KO mice on a C57BL/6 background and compared with their littermate heterozygote. Our result shows that EAU onset was significantly delayed and severity reduced in Sn KO with an incidence of 22% and disease severity grade of 0.17) in the initial stages day 12 after immunization compared with the control HET mice (with an incidence of 71% and disease severity of 0.74) (Fig. 1A). In the later stages of EAU (days 16 and 26), there was little or no difference in the level of retinal damage. These results were further confirmed when we used EAU-susceptible mice on a B10.RIII background immunized with IRBP peptide 161–180, as shown in Fig. 1B in which EAU in the Sn KO mice was delayed in onset for a short period. At day 10, only 30% of the B10.RIII Sn KO mice developed EAU with an average grade of the disease of 0.25 compared with the WT group in which ~70% of the mice developed EAU with a disease severity of 1.01. Again, no difference was seen at a later stage of 14 days after immunization. These data suggest that the Sn molecule contributes to the development of EAU in early stages of the disease, but the deletion of Sn cannot protect mice from developing autoimmune uveitis. The experiments were repeated at least twice in each strain with consistent results on each occasion. Histologically (Fig. 2), the delay in onset was manifest by a reduction in cellular infiltration and overall retinal damage in the KO mice at day 10, but not day 14 after immunization. At day 10, WT mice developed anterior ciliary body inflammation (cyclitis) with infiltrating cells in the ciliary body and vitreous (Fig. 2B) compared with the unaffected normal iris and ciliary body in the KO mice (Fig. 2F). In the posterior segment, many of the WT mice had developed signs of severe disease with granuloma formation and retinal detachment (Fig. 2C); in contrast, the majority of KO mice at this time still had early signs of disease, mostly mild intraocular cellular infiltration and retinal vasculitis (Fig. 2G). By day 14, EAU had reached a peak in both WT (D) and KO (H) mice with extensive infiltration throughout the retinal layers, retinal folds and severe structural damage.

**Sn-deficient mice show a reduced proliferative response to IRBP peptide in the DLNs after immunization**

Because EAU is a CD4 T cell-mediated disease in which tissue damage is mediated by activated macrophages (32–35), it was important to determine whether the effect Sn deletion was mediated via suppression of T cell activation and/or by inhibition of macrophage function. T cell proliferation assays were therefore performed on Sn KO and control WT mice immunized with IRBP peptide. In the first set of experiments, standard proliferation assays were performed at the same time as EAU evaluation using pooled lymphocyte preparations from spleens and DLNs from KO and WT groups of mice. Our results showed that proliferative responses to IRBP peptide at two different concentrations were significantly reduced in DLN T cells from Sn KO mice compared with controls, but the difference was less obvious in spleen T cell cultures (Fig. 3, A and B).

These data suggested that there might be a difference in the initial T cell proliferative responses which are estimated to occur within the first 50 h after immunization and to continue for several days (36, 37). Accordingly, a second set of experiments was performed to examine the earliest stage of T cell proliferation in a time course study. Mice were immunized with IRBP peptide and DLN and spleen T cells harvested from individual mice and assayed for proliferation from day 4 after immunization. In WT mice, a peak of mean T cell proliferation was observed 6 days after immunization in the DLNs which declined by 8 days and was followed by a second smaller peak by 12 days (Fig. 3C). In contrast, DLN T cells from Sn KO had a significantly reduced initial
T cell response at 6 days and no response at 12 days. Examination of individual spleen T cell responses revealed that this early effect was lost and that in fact spleen mean T cell responses in Sn KO mice were slightly higher in Sn KO mice compared with the WT controls in the initial stages (Fig. 3). Activated T cells from Sn-deficient mice have reduced IFN-γ responses to IRBP peptide

Additional evidence for altered T cell responses in Sn KO mice was sought using cytokine assays. C57BL/6 mice were immunized with IRBP peptide 1–20 and T cell cultures were prepared from DLNs and spleens harvested at the time of EAU evaluation (12 and 26 days after immunization) and incubated with 50 μg/ml IRBP peptide for 72 h. IFN-γ levels were measured in the supernatant as described in Materials and Methods. Pooled T cell cultures from the DLNs of IRBP peptide immunized Sn KO mice demonstrated markedly reduced levels of IFN-γ secretion in response to Ag stimulation in vitro (Fig. 4). Reduced IFN-γ production was evident in both DLN and spleen T cells at 12 days when EAU was in evolution as active disease. By 26 days, the difference in IFN-γ levels persisted in the DLN T cells but spleen T cells from both Sn KO and WT mice produced low levels.

Sn-deficient mice constitutively release low levels of NO

Macrophage effector function is frequently linked to NO production as an agent of free radical damage. We wished to determine whether there was evidence of impaired effector function in Sn KO mice. Peritoneal macrophages were collected from naive or immunized C57BL/6 WT and Sn KO mice and cultured with various stimuli; the supernatant was then collected and assayed using the Greiss reaction which is an indirect measure of NO production. We found macrophages stimulated with LPS and a combination of IFN-γ plus TNF-α cytokines induced high levels of NO production, which was significantly reduced in naive Sn KO compared with the WT mice (Fig. 5). IRBP peptide-immunized Sn KO mice also produced reduced levels of NO compared with WT mice but to a lesser extent (data not shown).

Discussion

The data in this study demonstrate that Sn, a macrophage-specific marker expressed at high levels by marginal zone metallophils in the spleen, and the subcapsular sinus and medullary macrophages of the lymph node, is required for full expression of the immune response.
response to the autoantigen IRBP peptide in mice. The observations are based on experiments in Sn-deficient mice which normally display minimal changes in immunological phenotype when housed under conventional clean conditions, i.e., untreated Sn KO mice have no alteration in myeloid cells, have a small increase in total CD8 T cells, have no change in CD4 T cells, and a reduced circulating IgM, all of which support a primary role of Sn in lymphocyte interactions (28). However, when immunized with Ag, Sn-deficient mice display reduced inflammatory responses to IRBP peptide in the initial stages of EAU, have impaired T cell proliferative responses in the DLNs, produce markedly less IFN-γ from DLN T cells after IRBP peptide stimulation, and constitutively elaborate lower levels of macrophage NO after stimulation with LPS or inflammatory cytokines, all experiments being performed on mice housed in conventional conditions.

Clearly, a principal question is how Sn promotes the inflammatory response in EAU. Macrophages are best recognized as effectors of inflammatory cell damage and this is particularly so in EAU (1, 5, 29, 31). Sn is regarded as an activation marker in macrophages (21, 38, 39) and thus deletion of this gene might be expected to be associated with impaired effector function. To some extent, evidence for this is presented by the reduced production of NO by Sn KO macrophages after challenge with LPS and inflammatory cytokines IFN-γ plus TNF-α (Fig. 5) since NO mediates some of the tissue damage produced by macrophages in EAU (31). However, this constitutive defect could have been an indirect consequence of altered T cell functions in these mice.

Macrophages are also considered to be APCs, although in this regard they are considerably less potent than dendritic cells (DCs) (36, 40–43). And unlike DCs, they do not function as initiators of T cell responses since they do not activate naive T cells (41).

Considerable new information is now available on the kinetics of the early stages of initial T cell activation in DLNs by APCs which have trafficked from a site of inflammation (36, 37). Ag deposited in tissue sites such as the skin is delivered to the DLNs via the lymphatics in two forms, soluble and cell-associated. Soluble Ag reaches the T cell area of the DLN within a few hours via the afferent lymphatics and the subcapsular sinus, through reticular stromal trabeculae (conduits (44, 45)). Here in the DLN, the soluble Ag is taken up by resident lymph node DCs which line the conduits and is presented to T cells, which arrive at the same site from the blood stream via the high endothelial venules (46, 47).

Cell-associated Ag is taken up in situ (e.g., the skin) by immature tissue-resident DCs and processed for presentation by the DCs to T cells, DCs become active and mature when they reach DLN. Mature DCs arrive at the lymph node via the same afferent lymphatics and the subcapsular sinus, and continue their migration onward into the T cell area where they either present MHC class II-peptide complexes directly to naive T cells or are themselves phagocytosed by resident DCs for cross-presentation of Ag to T cells (48). In this process, DCs traverse through the Sn+ macrophage-rich subcapsular sinus where they almost certainly come into intimate contact with the macrophages.

Some studies have investigated outcomes of this potential cellular interaction. Sn is potentially capable of promoting adhesion between lymphocytes and macrophages (21). Sn is also a counter-receptor for other molecules that are expressed on macrophages and DCs such as the mannose receptor (49) and MGL1 (16), and thus probably engage DCs in such clusters. If DC-macrophage clusters occur in the vicinity of naive T cells and cognate Ag, then conditions are optimized for T cell activation, facilitated by Sn. Indeed, because Sn binds to CD43, it may be able to remove this barrier molecule from the site of the immunological synapse to the uropod of the T cells (50–53), to promote initial T cell activation in a three-cell-cluster model.
Alternatively, whether Sn is expressed on DCs themselves, then they might more directly involve Sn in T cell activation by acting on CD43 intransmembrane translocation, at the same time, simultaneously presenting MHC class II peptide to the exposed TCR in the immunological synapse. In previous studies (15), it was suggested that there may be two populations of Sn+ macrophages, one of which is MR+ (15). Although definitive dual staining studies for DC markers were not performed in this study, it is possible that the smaller subpopulation of Sn+ MR+ cells represent DCs. Their behavior after immunization (altered phenotype to dendritic-like shape, migration into the T cells area) is consistent with this possibility (15). Other studies (54) in humans have provided direct evidence that at least in humans, DC subtypes may express Sn. The question of whether Sn+ DCs occur and are functional in murine models of autoimmune inflammation remains to be resolved.

The data in the present study, however, clearly show that Sn+-myeloid cells are involved in the initial stages of T cell priming and proliferation and are supported by the more marked effect of Sn deletion on DLN T cell proliferation than on spleen T cell proliferation. Sn is a large transmembrane molecule with 17 Ig-like domains protruding beyond the cell surface glycoconjugates, presumably to allow cellular interactions of the type suggested above. Thus, in the LN, terminal binding sites would be available for interaction with molecules such as CD43 and MR. Macrophage heterogeneity is currently an intense area of investigation and discrete functional roles are being described for macrophages expressing different receptors (9). For instance, F4/80, a long recognized macrophage marker, has now been linked to tolerance induction (55, 56). Similarly the MR and other scavenger receptors such as CR3 and the Fc receptor family are more involved in homeostasis, whereas other macrophage receptors are associated with inflammation. Macrophage glycosylation is also important for the expression of adhesion molecules (57). The data in the present study, however, clearly show that Sn+ cells at the subcapsular sinus, as well as having a fine-tuning role in promoting an optimal immune response. How this is achieved remains to be determined and may provide a new direction toward therapeutic means for modulating the inflammatory response.

Disclosures

The authors have no financial conflict of interest.

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