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Blocking 4-1BB/4-1BB Ligand Interactions Prevents Herpetic Stromal Keratitis

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Herpetic stromal keratitis (HSK) is an inflammatory disorder induced by HSV-1 infection and characterized by T cell-dependent destruction of corneal tissues. Complications of HSK include corneal melting, glaucoma, and irreversable corneal scarring that often leads to blindness (1).

Several mechanisms have been proposed for the pathogenesis of HSK in mice. In corneas of BALB/c mice infected with the RE strain of HSV-1, Russell et al. (2) showed that HSK resulted from CD4+ T cells secreting IFN-γ, IL-12, and other lymphokines typically produced by Th1 cells. Deshpande et al. (3, 4) showed that activated CD4+ T cells were required for the development of HSK, which occurred as a non-TCR-mediated event. On the basis of these studies, the authors proposed a bystander activation model of HSK. Other investigators found that mice carrying the IgHb allele (i.e., C.B-17 and C57BL/6) were resistant (5). However, when soluble IgG2a was injected into C.AL-20 mice, they became tolerant and did not develop HSK. Zhao et al. (6) found that the IgG2a-specific T cell clones also recognized a peptide sequence embedded in the HSV-virion-associated protein UL-6. Infection with a mutant HSV-1 strain that was unable to express the UL-6 protein or had an altered UL-6 T cell epitope did not induce HSK, whereas wild-type control or gB-mutated KOS did produce the corneal disease (7). The results of these studies led to the proposal of an autoimmune mechanism in the pathogenesis of HSK.

HSK induced in mice by infection with the RE or KOS strain of HSV is marked by intense stromal inflammation that becomes evident on postinfection (PI) days 7–8, reaches a peak on PI days 14–21, and decreases thereafter (8). We demonstrate in this study that blocking 4-1BB/4-1BB ligand (4-1BBL) (CD137/CD137 ligand) interactions abrogates clinical symptoms, leukocyte infiltration, and induction of proinflammatory cytokines and chemokines in the HSV-infected cornea. These results suggest that the 4-1BB/4-1BBL pathway plays a critical role in the pathogenesis of HSK. Elucidation of the underlying mechanism that enhances T cell infiltration into the corneal stroma and the discovery of strategies to block T cell entry may lead to a new therapy for this leading infectious cause of human blindness.

Materials and Methods

Mice

4-1BB-deficient mice were established as previously described (9) and backcrossed with BALB/c mice for more than nine generations. Wild-type BALB/c mice were obtained from Hyochang Bioscience (Tae-Ku, Korea). All mice were maintained under specific pathogen-free conditions in the animal facility of the Immunomodulation Research Center (University of Ulsan) and were used at 7–10 wk of age.

Antibodies

Anti-m4-1BBL mAb was used for in vivo neutralization. The hybridoma cells (TKS-1) were a kind gift from Drs. H. Yagita and K. Okumura (Juntendo University, Tokyo, Japan). The mAb was produced from ascites, purified by protein G column (Sigma-Aldrich, St. Louis, MO), and then tested on P815-m4-1BBL transfectants. Anti-m4-1BB (3E1), a kind gift from Dr. R. Mittler (Emory University, Atlanta, GA), was conjugated with FITC for detection of 4-1BB. The following Abs were purchased from BD Pharmingen (San Diego, CA): PE-conjugated anti-mouse CD3 (145.2C11), PE-conjugated anti-mouse CD4 (RM4-5), PE-conjugated anti-mouse CD8 (53-6.7), PE-conjugated anti-CD25 (PC61), and PE-conjugated anti-CD62L (M5E1).

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3 Abbreviations used in this paper: HSK, herpetic stromal keratitis; PI, postinfection; 4-1BBL, 4-1BB ligand; DLN, draining cervical lymph node; RPA, RNase protection assay; MIP, macrophage-inflammatory protein; IP, IFN-γ-inducible protein.
that contained FITC-conjugated mAb against Vβ2, -3, -5, -5.1 and -5.2, -6, -7, -8.1 and -8.2, -8.3, -9, -10b, -11, -12, -13, -14, and -17a TCRs.

**RT-PCR**

RNA was extracted from normal and inflamed corneas. RNA was reverse transcribed into cDNA using a PCR cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA). PCR was performed using the sense/antisense primers. PCR primer sequences were as follows: mouse 4-1BB, forward, 5′-TGTGCGCAGGCATTCCAGG-3′, and reverse, 5′-GAGCTGCTGTGAGTTCTCCTC-3′ (expected size, 504 bp); mouse 4-1BBL, forward, 5′-TTCTACAAACACAGGCCACA-3′, and reverse, 5′-GATAAAGGT CAGCACCACA-3′ (expected size, 203 bp); and mouse GAPDH, forward, 5′-TGAAGTGCTTGTAAGCGGTGAGC-3′, and reverse, 5′-CACCTGGGATACGGGTATGAC-3′ (expected size, 982 bp). PCR products were visualized using ethidium bromide after electrophoresis on 1% agarose gels.

**In vivo migration assay**

Cervical lymph node cells were isolated from 4-1BB−/− and 4-1BB+/+ mice on various days after HSV-1 infection. The CD4+ cells were suspended in PBS at a concentration of 1 × 10⁷ cells/ml and incubated with CFSE (Molecular Probes, Eugene, OR) at a final concentration of 5 μM for 5 min at 37°C, followed by two washes in PBS. Equal numbers (8 × 10⁵) of 4-1BB−/− and 4-1BB+/+ draining cervical lymph node (DLN) CD4+ T cells were reseeded in 400 μl of PBS, and injected i.v. into normal BALB/c mice. Three and one-half hours later, spleens and lymph nodes were removed, and the numbers of fluorescent cells were determined by FACs. At least 5 × 10⁵ events were counted.

**Statistical analysis**

Severity score data were analyzed using generalized estimating equations for non-normally distributed longitudinal data that had a repeated measures term to account for the within-subject variation of mice that were repeatedly scored over the weeks of the study period (10). The models also included main effect terms for treatment and time, as well as the interaction of treatment and time. When an overall significance for the model was found, post hoc testing of differences between the time mean score levels for all comparisons. For all analyses, the severity score or other scores were analyzed as a binary outcome, either 0 or ≥1. Data manipulation and statistical testing were conducted using SAS software (Statistical Analysis System, Cary, NC).

**Results**

Deletion of 4-1BB prevents development of HSK

To evaluate the effect of the elimination of the 4-1BB costimulatory receptor, we infected the corneas of 4-1BB−/− and 4-1BB+/+ littermates on BALB/c background with the RE strain of HSV-1 and scored the severity of HSK in each eye on a scale of 0 to 4.0 over a 25-day period (Fig. 1a). In the 4-1BB−/− mice (n = 19), HSK first appeared on PI days 7–8 (severity score, 0.32 ± 0.2; n = 6 of 38 eyes; mean ± SD). Severe inflammatory disease was evident by day 19 (severity score, 2.31 ± 0.4; n = 22 of 38 eyes) and persisted through PI day 22. Thereafter, the severity of HSK declined and the eyes were disease free by PI day 32. The incidence of HSK in the 4-1BB−/− eyes peaked at 57.9% (22 of 38 eyes) (Fig. 1b). However, in the 4-1BB+/+ mice (n = 25), only 8 of 50 eyes (16%) developed HSK, and the inflammation was mild (severity score, 0.21 ± 0.2) and lasted for just 1 or 2 days (Fig. 1b).

The differences in incidence (p < 0.0001) and severity (p = 0.013 for PI day 11 and p < 0.0001 for PI days 12–25) were significant.

Histological examination showed that the inflamed corneas of the 4-1BB−/− mice were swollen and heavily infiltrated with leukocytes (Fig. 1c), whereas the corneas of the 4-1BB+/+ mice appeared to be normal and showed no infiltration of leukocytes (Fig. 1c). The results indicate that 4-1BB expression may be required for the induction of HSK.

Blocking 4-1BBL inhibits the development of HSK

Because of the dramatic differences in the incidence and severity of HSK in the 4-1BB−/− and 4-1BB+/+ littermates, we considered the possibility that the 4-1BB−/− mice might have some other,
Various cytokines and chemokines have been implicated in the pathogenesis of HSK (11, 12). We analyzed the expression of cytokines and chemokines in HSK by RPA using total corneal RNA from infected and uninfected mice. Infected corneas with HSK had IL-15, IL-6, and IFN-γ mRNA, whereas uninfected corneas showed only IL-15 mRNA (Fig. 3a). Similar results were obtained in three independent experiments. In other studies (S. Seo, H. Park, Y. Kim, J. Choi, and B. Kwon, manuscript in preparation), we have found that cultured stromal keratocytes produce IL-15 constitutively; thus, some of the IL-15 mRNA detected in these corneas may have been produced by stromal keratocytes rather than T cells.

Expression of 4-1BB on infiltrating T cells

4-1BB is inducible on T cells that are activated through TCR signaling (9), and 4-1BB+ T cells presumably recognize Ags in corneal stroma, including herpes Ags. Therefore, we examined the unknown defects that contributed to the results. To rule out this possibility and examine another means of inhibiting the 4-1BB/4-1BBL interactions, we injected normal BALB/c mice with anti-4-1BBL mAb and compared the incidence and severity of HSK with that seen in control mice treated only with IgG (Fig. 2). The results were similar to those obtained with 4-1BB+ and 4-1BB− littermates; the control mice developed severe disease (PI day 15: incidence, 55.0%; mean severity score, 2.42 ± 0.7; n = 19 of 40 eyes), and the mice treated with anti-4-1BBL mAb did not (incidence, <5% at all times; peak mean severity score, 0.35 ± 0.7 on day 17; n = 2 of 40 eyes). These differences were also significant (p < 0.0001). The results suggest that blocking 4-1BB/4-1BBL interaction can prevent the development of HSK.

Profile of cytokines and chemokines in HSK

Various cytokines and chemokines have been implicated in the pathogenesis of HSK (11, 12). We analyzed the expression of cytokines and chemokines by RPA using total corneal RNA from normal infected and uninfected mice. Infected corneas with HSK of 1.0+ severity demonstrated mRNA for RANTES, macrophage-inflammatory protein (MIP)-1β, MIP-1α, MIP-2, INF-γ-inducible protein (IP)-10, monocyte chemoattractant protein-1, and T cell activation 3, whereas uninfected corneas showed only a small amount of RANTES (Fig. 3a). Production of these chemokines is characteristic of activated T cells and monocytes. Corneas with HSK also had IL-15, IL-6, and IFN-γ mRNA, whereas uninfected corneas showed only IL-15 mRNA (Fig. 3a).

Similarly, herpes-infected 4-1BB+ corneas with severe HSK (2.0 severity score) demonstrated high levels of chemokines, whereas herpes-infected but relatively disease-free corneas from 4-1BB− mice (severity score, 0.45) showed low to undetectable levels (Fig. 3b). Also, IL-6 and IFN-γ mRNA were detected in the 4-1BB+ corneas, but not in the 4-1BB− corneas. IL-15 was present in both, but at a reduced level in the 4-1BB− corneas (Fig. 3b). In other studies (S. Seo, H. Park, Y. Kim, J. Choi, and B. Kwon, manuscript in preparation), we have found that cultured stromal keratocytes produce IL-15 constitutively; thus, some of the IL-15 mRNA detected in these corneas may have been produced by stromal keratocytes rather than T cells.

Expression of 4-1BB on infiltrating T cells

4-1BB is inducible on T cells that are activated through TCR signaling (9), and 4-1BB+ T cells presumably recognize Ags in corneal stroma, including herpes Ags. Therefore, we examined the
time course of T cell infiltration and 4-1BB expression during the development of HSK. Infiltrating leukocytes were extracted from infected corneas at various time points (Fig. 4). CD3+ T cells constituted ~3% of the cells on PI day 8, when the mean severity score was 0.5; ~17% on PI day 13, when the mean score increased to 1.0; and ~23% on day 15, when the mean score peaked at 2.0 (Fig. 4, a and b). Immediately thereafter, the proportion of T cells decreased sharply (~7% on PI day 17), although the ocular disease remained severe (Fig. 4, a and b).

CD11b+ cells constituted ~30% of the population on PI day 8, increased steadily as the disease worsened (67.4 ± 1.84% on PI day 15), remained abundant through day 17 when the disease scores were at their maximum, and then decreased as the disease resolved (27.7 ± 4.24% on PI day 25, when the severity score was 0.5). Thus, the peak population of CD11b+ cells coincided with the sharp decline in T cells (Fig. 4, a and b), suggesting that T cells played a role in the induction of HSK and CD11b+ cells in the maintenance of the disease.
Approximately 65% (65.6 ± 6.0% on PI day 15) of the infiltrating CD3+ T cells expressed 4-1BB (Fig. 4, b–d). In contrast, expression of 4-1BB and 4-1BBL was not detected in the peripheral blood T cells in mice with HSK (data not shown). The data again indicate that interaction between 4-1BB and 4-1BBL in the corneal stroma contributes to the pathogenesis of HSK.

**T cell apoptosis in the inflamed corneal stroma**

We sought to identify the mechanism that leads to the disappearance of T cells immediately after the peak of HSK severity (Fig. 5). Using the TUNEL assay, we observed massive T cell apoptosis on PI day 16 (Fig. 5, c–e). Sections were stained with PE-anti-CD3 (145.2C11) and terminal transferase. Red, CD3+ T cells (c); green, apoptotic cells (f); and yellow, apoptotic CD3+ T cells (g).

**Phenotype of 4-1BB+ T cells**

The majority of infiltrating 4-1BB+ T cells also expressed CD44, CD25, and CD62L on their surfaces (Fig. 6, a and b). On day 15, ~12% of the total cells isolated from the HSK corneas were 4-1BB+ T cells (Fig. 6a). Of the 4-1BB+ T cells, 68.1 ± 7.1% were CD44+, 67 ± 2.1% were CD25+, and 76 ± 15.6% were CD62L+ (Fig. 6b). Taken together, the results indicate that the infiltrating T cells, especially the ones expressing 4-1BB, were effector T cells.

Using RPA, we found that the infiltrating leukocytes produced mRNA for the chemokine receptors CCR1, CCR2, and CCR5 (Fig. 3). High levels of CCR5 expression were detected in the T cells (Fig. 6). Of the 4-1BB+ T cells, 6% were CD25+, 7.1% were CD44+, and 76% were CD62L+ (Fig. 6c). Taken together, the results indicate that the infiltrating T cells, especially the ones expressing 4-1BB, were effector T cells.

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We then determined the TCR Vβ expression profile for the infiltrating T cells. We found that 14.5 ± 3.0% of the T cells carried Vβ8.1/8.2, 10.8 ± 2.7% carried Vβ8.3, 9.8 ± 1.5% carried Vβ10b, and 8.9 ± 2.1% carried Vβ5.1/5.2 (Fig. 7). No population expressed γδ TCR chains. This result indicates that the infiltrating T cells in HSK display a limited heterogeneity.

Reduced expression of CD62L in 4-1BB−/− T lymphocytes associated with the absence of HSK

To further elucidate the mechanisms involved in the diminished T cell infiltration into the corneas of 4-1BB−/− mice, the abundance of various T cell surface markers was determined from infection through PI day 8, when the T cells begin to migrate into the corneal stroma from the cervical lymph nodes. Among the markers tested, only CD62L showed a significant difference in expression in herpes-infected 4-1BB+/+ and 4-1BB−/− mice.

The expression levels of CD62L were first measured in CD4+ T cells from the spleens and DLNs in normal HSV-1-infected BALB/c mice. We found that the expression of CD62L was reduced in the cells from both organs early in the period after infection. Specifically, CD62Llow− was seen on ~3% of DLN CD4+ T cells on PI day 0, 35% on PI day 6, and 14% by PI days 8–12 (Fig. 8a). In splenic CD4+ T cells, the CD62Llow− phenotype increased even more dramatically, from 3% on PI day 0 to 60% on days 6–12 (Fig. 8a).

Next, we compared the level of CD62L expression on T cells from spleens and DLNs from herpes-infected 4-1BB+/+ and 4-1BB−/− mice. In the DLN samples obtained on PI day 3, there was no difference: ~85% of the T cells were CD62L+ and 15% were CD62Llow− in both groups of mice (Fig. 8b). However, on PI days 6 and 8, T cells expressing CD62L were less numerous in the 4-1BB+/+ mice relative to the 4-1BB−/− mice: on day 6, 44.3% of the T cells from the 4-1BB+/+ mice were CD62Llow−, compared with 34.4% in the 4-1BB−/− mice (Fig. 8b). By day 8, the corresponding numbers were 37.5% in the 4-1BB+/+ mice and 14.4% in the 4-1BB−/− mice (p = 0.008) (Fig. 8b). No differences in levels of CD62L were observed in the splenic CD4+ T cells from 4-1BB+/+ and 4-1BB−/− mice (Fig. 8b).

Finally, to evaluate migratory potential, DLN CD4+ T cells purified from 4-1BB+/+ and 4-1BB−/− littermates on PI days 3 and 8 were labeled with CFSE, injected i.v. into normal mice, and counted in specimens from cervical lymph nodes and spleens. When the T cells obtained on PI day 3 were used, the numbers of labeled cells in the two groups of mice were similar for both spleens and lymph nodes (Fig. 8c). However, when the PI day 8 T cells were injected, the 4-1BB−/− mice showed significantly fewer labeled T cells in the DLN and spleen, compared with the 4-1BB+/+ littermates (spleen, p = 0.013; DLN, p = 0.0094) (Fig. 8d).

In summary, CD62L expression in 4-1BB+/+ and 4-1BB−/− littermates was similar during the acute phase of infection (PI day 3). By PI day 6, when the acute infection had usually resolved, somewhat higher numbers of T cells expressing CD62Llow− were seen in the 4-1BB−/− mice, compared with their 4-1BB+/+ littermates, although the difference was not significant. However, by day 8, when T cells were beginning to infiltrate the corneas, CD62L expression was significantly less in the 4-1BB−/− mice, compared with their 4-1BB+/+ littermates. These results suggest the possibility that reduced infiltration of T cells in herpes-infected 4-1BB−/− mice is related to the lower expression of CD62L in a subset of T cells, potentially those T cells that would have been expected to migrate into the corneal stroma. This notion is supported by the finding that CD4+ T cells isolated from DLNs of 4-1BB−/− mice on PI day 8, but not those isolated on PI day 3, had reduced migratory potential. If so, CD62L expression may be regulated by signals through 4-1BB. The absence of 4-1BB/4-1BBL interactions may result in reduced expression of CD62L on a subset of T cells. If the 4-1BB−/−CD62Llow− T cells have a reduced migratory potential as a result of the absence of CD62L, the ultimate result could be fewer T cells available to stimulate inflammation and the development of HSK in the cornea.

Discussion

A variety of costimulatory receptors on T cells and their ligands on dendritic cells are required for full activation of T cells, which ultimately leads to the generation of effector T cells. Generally, costimulatory receptors can be classified into two families: 1) the
CD28/inducible costimulator family and 2) the TNFR family, to which 4-1BB belongs.

4-1BB is expressed on activated CD4+ T cells, CD8+ T cells, NK cells, and NK T cells (reviewed in Ref. 12). CD4+CD25+ regulatory T cells appear to express 4-1BB constitutively (13, 14). Recent studies have shown that 4-1BB expression is not restricted to subpopulations of lymphoid cells, but rather is distributed across a variety of blood cells. For example, myeloid cells, including monocytes, neutrophils, and dendritic cells, express 4-1BB constitutively (15, 16). 4-1BBL is expressed on activated APCs, such as dendritic cells, B cells, and macrophages (12), and on keratinocytes (8). This expression pattern raises the possibility that 4-1BB/4-1BBL interactions may be involved in multiple steps in various innate and adaptive immune responses. Studies that block or stimulate the 4-1BB costimulatory pathway demonstrate the involvement of 4-1BB in a variety of CD4+ T cell-mediated responses in vivo. These responses include induction of a Th1 cell anergy (17), an alloimmune response (18), an acute inflammation (19), an autoimmune disease (20), and a Th1 cell response to tumor cells (21). A critical role for 4-1BB in the Th1-mediated immune response suggests that intervening in the 4-1BB costimulatory pathway could provide an immunotherapeutic approach to the treatment of inflammatory diseases.

Present studies demonstrate that interference in the interactions between 4-1BB and 4-1BBL can abolish the development of HSK in the murine model. 4-1BB/4-1BBL interactions can be blocked by deletion of 4-1BB or by mAbs to 4-1BBL (Figs. 1 and 2). Absence of the 4-1BB signal appears to produce a dual effect: blocking T cell migration from the regional lymph nodes to the corneal stroma and inhibiting the inflammatory responses in the corneal stroma. Blocking B7-1/CD28 interactions, as well as CD154/CD40 interactions, was also observed to reduce the incidence of HSK in mice (22).

The most intriguing aspect of the present investigation is the potential effect of the 4-1BB signal on CD62L expression (Fig. 8). CD62L (L-selectin) is expressed constitutively on all classes of leukocytes. It mediates the binding of lymphocyte subpopulations to specialized high endothelial cells present in postcapillary venules of peripheral lymph nodes, and also facilitates the rolling of leukocytes on the endothelium at sites of tissue injury or inflammation (23, 24). The migration of lymphocytes into peripheral lymph nodes and the recruitment of leukocytes to sites of inflammation are reduced dramatically in CD62L-deficient mice (25, 26). In fact, Grewal et al. (27) showed that CD62L-deficient mice did not develop experimental allergic encephalomyelitis. These studies indicate a key role for CD62L in inflammation and leukocyte trafficking.

As shown in the present investigation (Fig. 8), it is possible that 4-1BB signaling regulates the expression of CD62L. We have previously noticed that 4-1BB stimulation induces ICAM-1, a molecule that is involved in cell adhesion in monocyte cell lines (H. Lee, W. Kang, and B. Kwon, manuscript in preparation). It may be important to determine the role of 4-1BB-mediated signals in CD62L expression. Additionally, a defect in the APCs in 4-1BB-deficient mice cannot be ruled out.

The phenotypes of the stroma-infiltrating T cells were analyzed. As shown in Fig. 3, the infiltrating T cells produced chemokines that are normally induced when T cells are activated. In the absence of 4-1BB, levels of CCR5 (Fig. 3) and IP-10 (Fig. 1) were reduced.
markedly reduced, suggesting that without 4-1BB, Th1 responses may be diminished. Although the abundance of chemokines and IL-6 mRNA may reflect the severity of HSK, the markedly elevated levels of MIP-2, IP-10, monocyte chemotactic protein-1, and IL-6 are also noteworthy. Lausch and colleagues (28, 29) reported that MIP-2 and IL-6 are important in the development of HSK. How much the cytokine network contributes to the initiation and maintenance of HSK is not known, and the significance of the unusually high expression of the chemokines noted above, as well as IL-6, remains to be determined.

The majority of the infiltrating T cells (CD44^hi, CD25^hi, and CD62L^lo) were effector cells, with possibly some memory T cells, and most appeared to express 4-1BB on their cell surfaces (Fig. 6). It appeared that HSK was induced by 4-1BB^+ T cells, and then, at the peak of disease, the T cells were suddenly eliminated by apoptosis (Fig. 5). The mechanism controlling this phenomenon is unknown, although it is possible that CD95 is induced at this stage of inflammation and interacts with CD95 ligand on stromal tissue (30). The course of HSK was prolonged, probably by the presence of CD11b^+ monocytes, but the disease eventually resolved without treatment. The disappearance of the T cells might be a precursor to resolution of the disease, but how these two phenomena are related remains to be determined.

In summary, blocking 4-1BB/4-1BBL interactions can abolish the effector level of the immune response. Ultimately, it may be possible to develop an agent to block 4-1BB/4-1BBL activity for treatment of HSK in the clinical setting.

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