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14. ABSTRACT
This is the first annual report on the grant "CD24 as a Potential Therapeutic Target in Prostate Cancer". CD24 (heat-stable antigen) is a cell surface GPI-anchored mucin-like glycoprotein with broad expression on a variety of cell types, including hematopoietic cells, neuronal cells and various epithelial cells. There are accumulating evidence showing CD24 plays an important role in tumor development and tumor metastasis. We hypothesized that the expression of CD24 on both tumor cells and hematopoietic cells promotes tumor cell growth and metastasis. Therapeutic reagents target CD24 may block the tumor growth and metastasis. We proposed (1). To examine whether the intrinsic or extrinsic function of CD24 determine the prostate cancer incidence. (2). To examine whether CD24 expression affects the T cell priming and effector function to tumor antigen. (3) To examine whether CD24-IgG fusion protein can be used in immunotherapy of prostate cancer. The annual report summarized the results from specific aim 1 and 2.

15. SUBJECT TERMS
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(4) Introduction

This is the first annual report on the grant "**CD24 as a Potential Therapeutic Target in Prostate Cancer"**.

CD24 (heat-stable antigen) is a cell surface GPI-anchored mucin-like glycoprotein with broad expression on a variety of cell types, including hematopoietic cells, neuronal cells and various epithelial cells. There are accumulating evidence showing CD24 plays an important role in tumor development and tumor metastasis. CD24 expression has emerged as an important independent prognostic marker for epithelial ovarian cancer, breast cancer, pancreatic cancer, and prostate cancer. Our laboratory has been working on elucidating the role of CD24 in immune regulation and in autoimmune diseases for past 10 years. We also had extensive experience in mouse prostate cancer model TRAMP mice. Our preliminary data showed that prostate cancer incidence and tumor size were drastically reduced in TRAMP-CD24-deficient mice. CD24 deficient mice are more resistant to syngeneic tumor cell growth in comparison to wild type mice. These data intrigue us to further explore the role of CD24, whether its intrinsic or extrinsic, in tumor development. We have hypothesized that the expression of CD24 on both tumor cells and hematopoietic cells promotes tumor cell growth and metastasis. Therapeutic reagents target CD24 may block the tumor growth and metastasis.

In our proposal, we have proposed: (1). To examine whether the intrinsic or extrinsic function of CD24 determine the prostate cancer incidence. (2). To examine whether CD24 expression affects the T cell priming and effector function to tumor antigen. (3) To examine whether CD24-IgG fusion protein can be used in immunotherapy of prostate cancer.

In the past funding period, we have made significant progress in Specific Aim 2 and published a major milestone paper in Science that established CD24 as a molecule that binds to danger (or cell damage) signal molecules and dampens the immune response through signaling by Siglec 10. Although the paper does not link CD24 to cancer directly, it has tremendous implication for our future work. We have preliminary data in Specific Aim 1 suggesting that intrinsic function of CD24 may determine the prostate cancer incidence.

(5) Body of Annual Report

STATEMENT OF WORK (SOW)
PC073392. CD24 as a potential therapeutic target in prostate cancer
Pan Zheng

Task I. To examine whether the cancer-cell intrinsic or extrinsic function of CD24 determines the prostate cancer incidence and/or progression. (Month 1-36). (In Progress).

a. To generate TRAMP mice with CD24 deficient background. (Month 1-6). (Finished).

b. To generate four different groups of bone marrow chimera mice (Month 7-18). (In Progress)
6 week-old TRAMP WT or TRAMP CD24-/- mice will receive 1000 Rad of irradiation. $5x10^6$/mouse of T-depleted bone marrow cells from either WT or CD24-/- mice will be used to reconstitute the irradiated mice. The four groups of chimera mice WT>WT TRAMP (group I), WT>$CD24^{-/-}$TRAMP (group II), $CD24^{-/-}$>WT TRAMP (group III), $CD24^{-/-}$>$CD24^{-/-}$ TRAMP (group IV) mice will be generated. In group I mice, all cell types will have CD24 gene. In group II, the bone marrow derived cells will have CD24 gene, while all the other tissues and cells including prostate will be CD24 deficient. In group III, the bone marrow derived cells will be CD24 deficient, while other cell types will have CD24 gene. In group IV, all cell types will be CD24 deficient.

c. To monitor tumor growth in chimera mice by MRI (Month 9-36). (In progress)
We have established collaboration with Dr. Brian Ross in University of Michigan to use MRI to measure the growth of prostate cancer in the mouse TRAMP model. We will measure the prostate size determine prostate tumor size and to record the progression of prostate cancer. The tumor volume will be used to calculate the log transformation of tumor volume. The tumor growth over time was analyzed using the Stata^R XTGEE (cross-sectional generalized estimating equations) model. A significant difference will be based on a $P<0.05$.

For Specific Aim 1, we have generated data in group I and group III bone marrow chimera experiments. The preliminary results with small number of mice (group I, n=5, group III, n=4) showed that the tumor incidence in TRAMP mice didn't have significant difference (Fig. 1). We will continue this aim and finish the group II and IV, as well as prepare more mice for group I and group III bone marrow chimera to determine the role of CD24 expression in prostate cancer incidence.

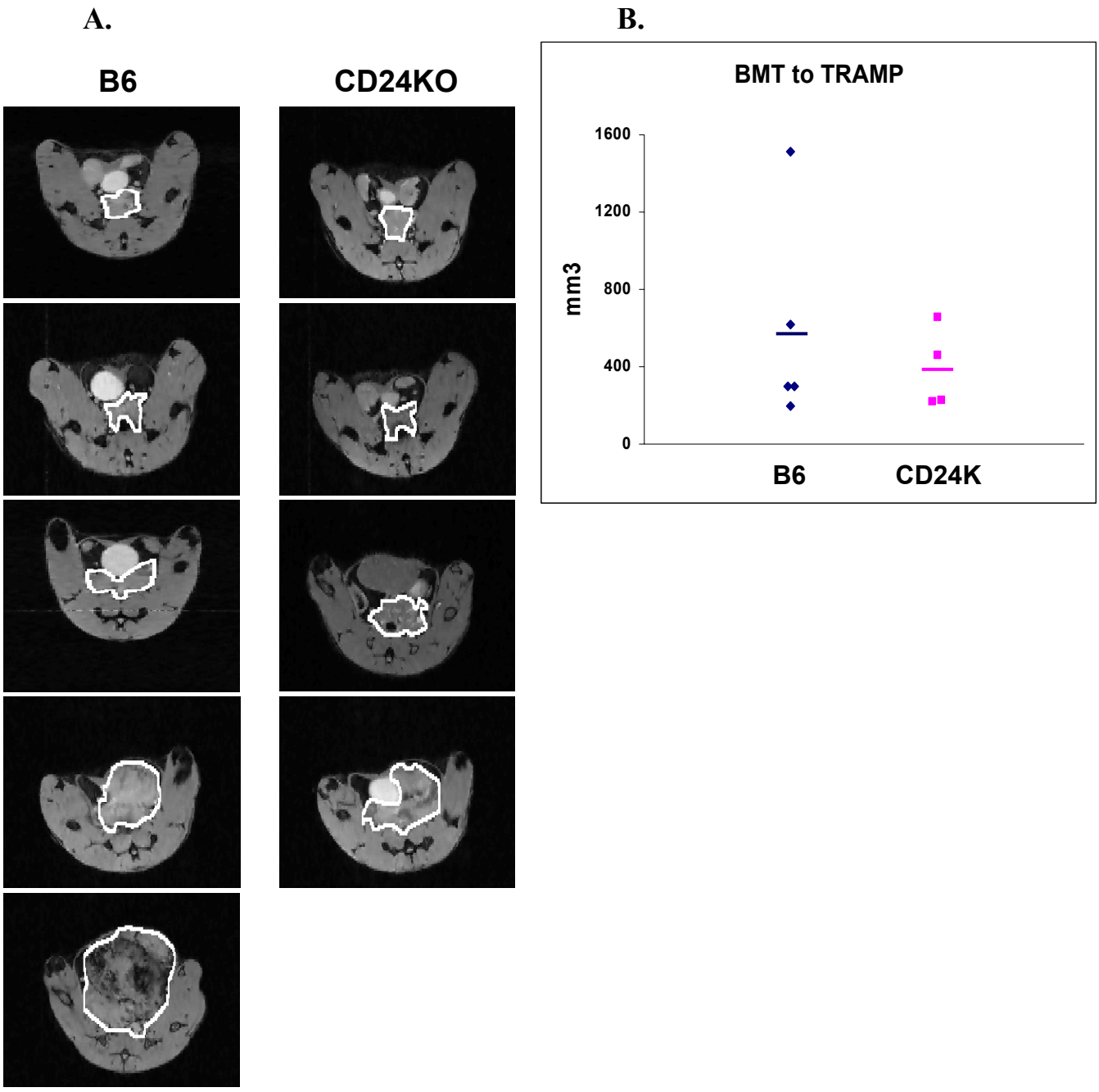

Fig. 1. CD24 expression in bone marrow derived cells does not significantly affect the prostate cancer incidence (Preliminary result). A. MRI images of 30 weeks old TRAMP mice. The TRAMP mice were lethally irradiated (1000 rad) at 8 weeks and reconstituted with $5x10^6$ T-depleted bone marrow cells from either WT or CD24-deficient mice. B. Prostate sizes as measured by MRI. P=0.48.

Task II. To examine the role of CD24 in the priming and effector function of cancer-reactive T cells (Month 1-36). (In Progress).

a. To breed the TCR-I transgenic mice with CD45.1 congenic mice (Month 1-12). We have order the TCR-I transgenic mice from Jackson Laboratory. We expect the mice will become available in three months as they are kept as cryopreserved embryos in Jackson Laboratory. We will amplify the colony and breed with CD45.1 congenic mice to facilitate the tracking of CD45.1 T cells after they are adoptive transferred to TRAMP mice which carry the CD45.2 marker. (Currently in breeding).

b. To perform the adoptive transfer experiments (Month 13-36). We will purify T cells from TCR-I transgenic mice and label the cells with CFSE. These T cells will adoptive transferred to the BM chimera mice generated in Task I in various time points correlated with tumor development.

c. To determine the T cell priming and effector functions (Month 14-36).

d. To examine the role of CD24 expression in tumor susceptibility to immunotherapy by CTL (Month 18-36).

We were hindered by prohibition of any breeding due to a serological test showing the rooms that housing our colonies were infected by MHV in August 2008. To effectively eliminate the viral infection, all breeding was stopped until the serological test showing two quarterly negative results.

However, we make significant progress in study the function and binding partners of CD24 molecule and published an important paper in Science. (Appendix 1).

Task 3. Immunotherapy targeting the CD24 molecule (Month 19-36). (Not started yet).

a. To generate B-cell deficient TRAMP mice (Month 1-12). We have obtained the μMT mice from Jackson Laboratories and have initiated the breeding. We expect that the mice will be available for use early 2008.

b. To use CD24Ig to prevent prostate cancer in TRAMP mice (Month 7-36).

c. To use CD24Ig to treat prostate cancer in TRAMP mice (Month 7-36).

(6) Key Research Accomplishments

- We published a major milestone paper in Science that established CD24 as a molecule that binds to danger (or cell damage) signal molecules and dampens the immune response through signaling by Siglec 10.
- We have preliminary data in Specific Aim 1 suggesting that intrinsic function of CD24 may determine the prostate cancer incidence.

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(7) Reportable Outcomes:

Manuscripts:

1. Chen GY, Tang J, Zheng P, Liu Y. 2009. CD24 and Siglect-10 selectively repress tissue damage induced immune responses. Science 323: 1722.

(8) Conclusions:

CD24 is a very interesting molecule. There are accumulating evidence showing CD24 plays an important role in tumor development and tumor metastasis. CD24 expression has emerged as an important independent prognostic marker for epithelial ovarian cancer, breast cancer, pancreatic cancer, and prostate cancer. Our laboratory has been working on the role of CD24 in immune regulation and in autoimmune diseases for past 10 years. We also had extensive experience in mouse prostate cancer model TRAMP mice. We found that if the TRAMP mice don't have any CD24, then the mice developed much less prostate cancer. These data intrigue us to further explore the role of CD24 in prostate cancer development.

Our working hypothesis is that the expression of CD24 on both tumor cells and white blood cells promotes tumor cell growth. We can develop therapeutic reagents target CD24 to block the tumor growth.

The initial preliminary study showed that CD24 expression in bone marrow derived cells did not appear to play a significant role in prostate cancer development. We are going to perform more experiment to confirm or to dispute this preliminary result.

We have made major breakthrough in identify CD24-Siglec 10 signaling pathway in regulating damage induced immune response. The abstract on the Science paper is presented here:

Patten recognition receptors, which recognize pathogens or components of injured cells (danger), trigger activation of the innate immune system. Whether and how the host distinguishes between danger- versus pathogen-associated molecular patterns remains unresolved. We report that CD24-deficient mice exhibit increased susceptibility to danger- but not pathogen-associated molecular patterns. CD24 associates with high mobility group box 1, heat shock protein 70, and heat shock protein 90; negatively regulates their stimulatory activity; and inhibits nuclear factor kB (NF-kB) activation. This occurs at least in part through CD24 association with Siglec-10 in humans or Siglec-G in mice. Our results reveal that the CD24–Siglec G pathway protects the host against a lethal response to pathological cell death and discriminates danger- versus pathogen-associated molecular patterns.

(9) References:

None.

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CD24 and Siglec-10 Selectively Repress Tissue Damage Induced Immune Responses

Guo-Yun Chen, *et al.*

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36. We thank D. C. Rees, I. Wilson, R. H. Spencer, M. B. Stowell, A. Senior, A. Frost, V. M. Unger, C. D. Stout, and P. Wright. Y. Weng was supported by a scholarship from P. R. China. We thank Stanford Synchrotron Radiation Lightsource, Advanced Light Source, and Advanced Photon Source. This work was supported by grants from the Army (W81XWH-05-1-0316), NIH (GM61905, GM078914, and GM073197), the Beckman Foundation, the Skaggs Chemical Biology Foundation, Jasper L. and Jack Denton Wilson Foundation, the Southwest Cancer and Treatment Center, and the Norton B. Gilula Fellowship. Coordinates and structure factors deposited to the Protein DataBank (PDB accession codes 3G5U, 3G60, and 3G61).

Supporting Online Material

www.sciencemag.org/cgi/content/full/323/5922/1718/DC1
Materials and Methods
Figs. S1 to S20
Tables S1 to S3
References

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CD24 and Siglec-10 Selectively Repress Tissue Damage–Induced Immune Responses

Guo-Yun Chen,[1] Jie Tang,[4] Pan Zheng,[1,2]* Yang Liu[1,3]*

Patten recognition receptors, which recognize pathogens or components of injured cells (danger), trigger activation of the innate immune system. Whether and how the host distinguishes between danger- versus pathogen-associated molecular patterns remains unresolved. We report that CD24-deficient mice exhibit increased susceptibility to danger- but not pathogen-associated molecular patterns. CD24 associates with high mobility group box 1, heat shock protein 70, and heat shock protein 90; negatively regulates their stimulatory activity; and inhibits nuclear factor κB (NF-κB) activation. This occurs at least in part through CD24 association with Siglec-10 in humans or Siglec-G in mice. Our results reveal that the CD24–Siglec G pathway protects the host against a lethal response to pathological cell death and discriminates danger- versus pathogen-associated molecular patterns.

Pathogen-associated molecular patterns (PAMPs) interact with Toll-like receptors (TLRs) on innate immune cells to initiate protective immune responses (*1*–*3*). Danger-associated molecular patterns (DAMPs) (*4*), which are intracellular components such as high mobility group box 1 (HMGB1), heat shock protein 70 (HSP70), heat shock protein 90 (HSP90), and cellular RNA released during cellular injury, also induce TLR-dependent inflammatory responses (*5*–*8*). Whether the host is able to discriminate between DAMPs and PAMPs is not clear.

We used an acetaminophen (AAP)-induced liver necrosis model (*9*) to identify genes that regulate the innate immune response resulting from tissue injury. A sublethal dose of AAP (10 mg/mouse), which is tolerated by wild-type (WT) mice, caused rapid death of *CD24*-deficient ($CD24^{-/-}$) mice within 20 hours (Fig. 1A). We then tested whether CD24 regulated the inflammatory response to AAP-induced liver injury because CD24 is expressed on liver oval cells and hematopoeitic cells, but not on hepatocytes (*10*). Indeed, we detected a massive increase in the inflammatory cytokines interleukin-6 (IL-6), monocyte chemotactic protein–1 (MCP-1), and tumor necrosis factor–α (TNF-α) after AAP treatment (Fig. 1B). This was accompanied by increased amounts of serum alanine transaminase (ALT), which is indicative of liver damage (Fig. 1C), and liver hemorrhage and necrosis (Fig. 1D). These observations revealed that CD24 protects against AAP-induced hepatoxicity, most likely by regulating the inflammatory response.

CD24 is a small glycosylphosphoinositol-anchored protein that is able to provide costimulatory signals to T cells and has been implicated in the development of autoimmune disease (*11*–*15*). We set out to identify proteins that associate with CD24 because none of the known CD24 ligands provided insight into its protective effect in our liver injury model. We focused on proteins whose interactions can be disrupted by the cation chelator EDTA, because more than 90% of the mass of CD24 is estimated to be derived from glycosylation (*12*) and because protein-polysaccharide interactions largely depend on cations. Briefly, we immunoprecipitated CD24 and its associated proteins from lysates of mouse splenocytes. The proteins eluted by EDTA were subjected to high-throughput mass spectrometry analysis and SDS–polyacrylamide gel electrophoresis (PAGE). HMGB1, a prototypical DAMP molecule that activates the immune response following tissue damage (*16*), was among the most prominent proteins that we identified (Fig. 2A and table S1). HMGB1 coimmunoprecipitated with CD24 and this interaction was specific (Fig. 2B and C). A recombinant CD24-Fc fusion protein specifically coimmunoprecipitated recombinant HMGB1, demonstrating that the interaction between CD24 and HMGB1 was direct (Fig. 2D).

To determine whether the hypersensitivity to AAP observed in $CD24^{-/-}$ mice was the result of an enhanced immune response to HMGB1, we injected AAP-treated mice with antibodies to HMGB1 (fig. S1). In one representative experiment, blockade of HMGB1 rescued 87.5% of the mice that received AAP (Fig. 2E). Treated mice exhibited decreased ALT abundance, indicating reduced hepatocyte destruction (Fig. 2F). The production of IL-6, MCP-1, and TNF-α was also greatly reduced (Fig. 2G). Thus, CD24 protects against AAP-induced lethal hepatoxicity by dampening the immune response against HMGB1.

HMGB1 can be divided into two domains: an inhibitory A box and a stimulatory B box (*17*). To determine whether CD24 inhibits HMGB1 by binding to the inhibitory A box, we produced deletion mutants lacking either the A box or the B box. CD24-Fc immunoprecipitated full-length HMGB1 and the box B–containing mutant, but not the box A–containing mutant (fig. S2). Thus, inhibition of HMGB1 by CD24 does not require direct interaction with box A.

CD24 has no known mechanism for signal transduction. To understand how CD24 negatively regulates HMGB1, we searched for a potential CD24 receptor that may transduce signals downstream of CD24. We were particularly interested in sialic acid–binding immunoglobulin (Ig)-like lectins (Siglecs), which are cell surface receptors of the immunoglobulin superfamily that recognize sialic acid–containing proteins (*18*). Siglecs are primarily expressed by cells of hematopoietic origin (*18*). Most Siglecs are considered to be negative regulators of the immune system because they contain one or more cytosolic immune receptor tyrosine-based inhibitory motifs (ITIMs) (*18*). To determine whether CD24 interacts with Siglecs, we incubated splenocytes on plates coated with the recombinant extracellular domains of ITIM-containing Siglec-5, -7, -10 or -11. Siglec-10, but not Siglecs -5, -7, or -11, bound to CD24 (Fig. 3A). Flow cytometric analysis indicated that

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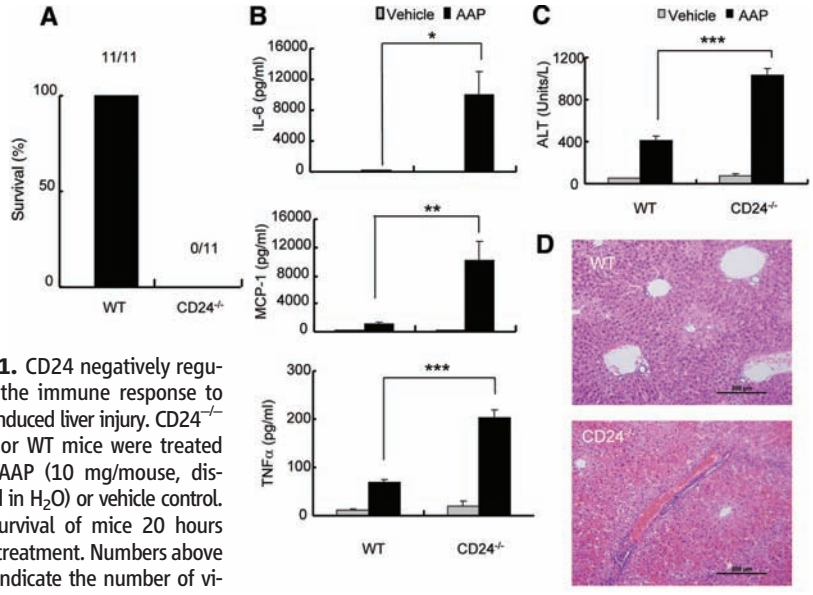

Fig. 1. CD24 negatively regulates the immune response to AAP-induced liver injury. $CD24^{-/-}$ mice or WT mice were treated with AAP (10 mg/mouse, dissolved in H_2O) or vehicle control. (**A**) Survival of mice 20 hours after treatment. Numbers above bars indicate the number of viable mice out of the total number of mice used per group. All WT mice remained healthy. (**B**) Serum concentrations of IL-6, MCP-1, and TNF-α at 6 hours after AAP injection (mean ± SD, $n = 5$; $^{*}P < 0.02$, $^{**}P < 0.009$; $^{***}P < 0.002$, Student's t test) . (**C**) ALT concentrations measured at 6 hours after treatment (mean ± SD, $n = 5$; $^{***}P < 0.00004$, Student's t test). Data shown in (B) and (C) were repeated two times. (**D**) Livers were isolated at 9 hours after treatment. Representative images (magnification, ×20) of hematoxylin and eosin (H&E) staining are shown ($n = 3$).

CD24 is the primary receptor for Siglec-10 because WT but not $CD24^{-/-}$ splenocytes showed detectable binding to soluble Siglec-10-Fc (Fig. 3B). Furthermore, in COS cells, FLAG-tagged Siglec-10 coimmunoprecipitated with CD24-Fc, whereas the inactivating R119A mutation (in which Arg^{119} is replaced with Ala) of Siglec-10 (analogous to the R97A in sialoadhesin (*19*)) abrogated the interaction (Fig. 3C).

We hypothesized that CD24, Siglec-10, and HMGB1 might form a trimolecular complex because CD24 can interact with both HMGB1 and Siglec-10. Indeed, Siglec-10-Fc was able to immunoprecipitate HMGB1 from lysates of WT but not $CD24^{-/-}$ splenocytes (Fig. 3D), indicating that their interaction was strictly dependent on CD24 expression.

The likely murine homolog of Siglec-10 is Siglec-G (*18*). We prepared antibodies to Siglec-G by immunizing $Siglecg^{-/-}$ mice (*20*) with WT spleen cells (fig. S3). With the use of this antisera, Siglec-G coimmunoprecipitated CD24 (Fig. 3E). CD24-Fc showed stronger binding to WT splenocytes in comparison to $Siglecg^{-/-}$ splenocytes, indicating that Siglec-G contributed to CD24-Fc binding; however, consistent with previous reports of multiple CD24 receptors (*12*), Siglec-G deficiency did not abrogate CD24-Fc splenocyte binding (fig. S4). We next determined if the absence of Siglec-G would also convey hypersensitivity to AAP. Indeed, only 25% of $Siglecg^{-/-}$ mice

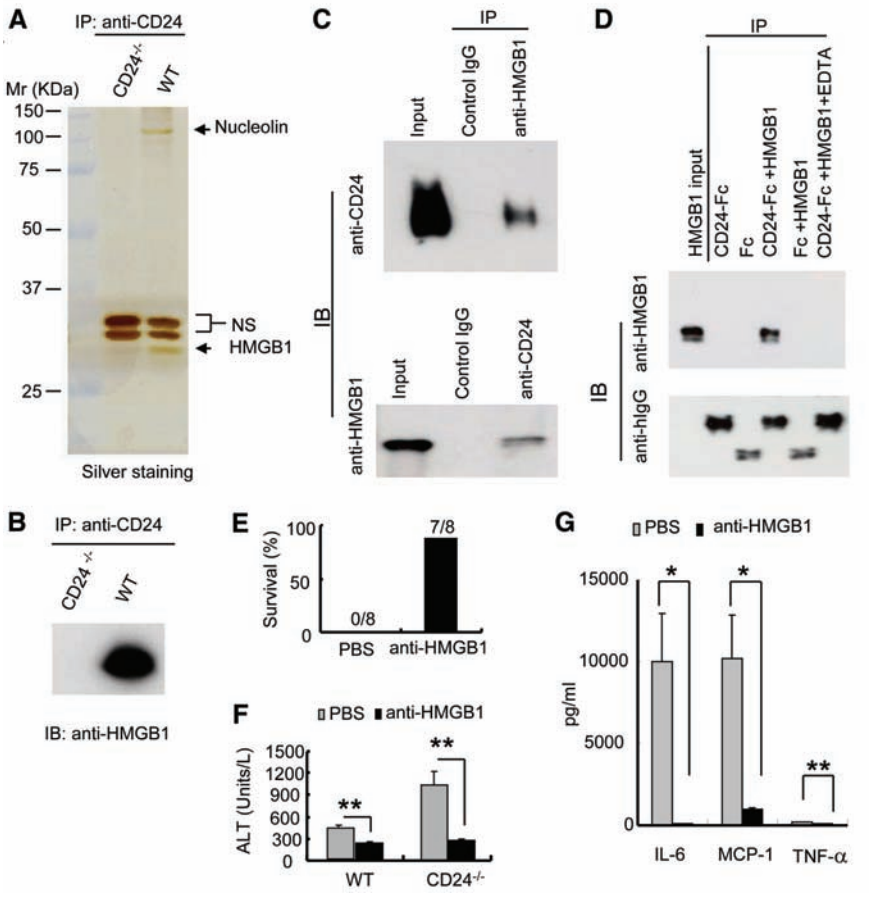

Fig. 2. CD24 associates with, and negatively regulates, the immune response to HMGB1. (**A**) Identification of CD24-associated proteins by coimmunoprecipitation. Silver staining of the SDS-PAGE gel is shown. Arrows indicate the positions of HMGB1 and nucleolin, two abundant CD24-associated DAMP molecules. NS: proteins that coimmunoprecipitated with anti-CD24 nonspecifically. (**B**) Confirmation of CD24-HMGB1 association by Western blot of EDTA-disassociated proteins. (**C**) Reciprocal immunoprecipitations of CD24 and HMGB1 were performed with splenocyte lysates isolated from WT mice. (**D**) Direct, cation-dependent interaction between CD24 and HMGB1. Coimmunoprecipitation of recombinant HMGB1 protein with CD24-Fc fusion protein or control IgG-Fc. The requirement for cations was confirmed by disruption of the complex with EDTA. This experiment was repeated three times. (**E**) Mice received intravenous injections with either vehicle (phosphate-buffered saline) or mouse HMGB1 monoclonal antibody (mAb) (clone 3B1, 150 μg/mouse) 30 min before intraperitoneal (ip) injection of AAP. Composite data from two independent experiments are shown ($n = 8$). (**F**) Serum ALT at 6 hours after treatment with AAP- and HMGB1-specific antibodies (mean ± SD, $n = 5$, $^{**}P < 0.005$). (**G**) Serum cytokine concentrations at 6 hours after treatment with AAP- and HMGB1-specific antibodies (mean ± SD, $n = 5$, $^{*}P$, 0.03, $^{**}P < 0.004$). Samples in (F) and (G) represent two independent experiments; the statistical significance was determined by Student's t test.

survived a sublethal dose of AAP (Fig. 3F). The enhanced susceptibility was accompanied by increased release of ALT (Fig. 3G), liver necrosis, and hemorrhage (Fig. 3H), as well as increased amounts of inflammatory cytokines in the blood (Fig. 3I). To test whether the enhanced liver toxicity was mediated by HMGB1, we treated *Siglecg*$^{-/-}$ mice with antibodies to HMGB1. Inhibition of HMGB1 prevented mortality in 90% of AAP-treated *Siglecg*$^{-/-}$ mice (Fig. 3J). Serum ALT and inflammatory cytokines were also largely diminished (Fig. 3, K and L).

CD24 and Siglec-10 are unlikely to function by acting directly on hepatocytes because they are not expressed by these cells (*10*, *18*). Dendritic cells (DCs), however, respond to HMGB1 (*21*) and express both CD24 (*22*) and Siglec-G (*20*). To test whether DCs can respond to HMGB1, we cultured bone marrow–derived DCs isolated from WT, *CD24*$^{-/-}$, or *Siglecg*$^{-/-}$ mice and stimulated them with HMGB1 or the TLR ligands lipopolysaccharide (LPS) or poly(I:C). HMGB1 stimulation resulted in significantly greater production of IL-6 and TNF-α by *CD24*$^{-/-}$ or *Siglecg*$^{-/-}$ DCs than by WT DCs (Fig. 4A). In contrast, CD24 or Siglec-G deficiency did not affect the production of inflammatory cytokines by DCs in response to LPS or poly(I:C) (Fig. 4A).

Siglec-10 associates with the tyrosine phosphatase SHP-1, a known negative regulator of nuclear factor κB (NF-κB) activation (*23*). In a subpopulation of B cells that reside in the peritoneum (*20*), the absence of Siglec-G results in the constitutive activation of NF-κB. To test whether activation of NF-κB by HMGB1 or LPS is affected by the absence of CD24 or Siglec-G, we assayed the nuclear translocation of the NF-κB subunit p65 in WT, *CD24*$^{-/-}$, and *Siglecg*$^{-/-}$ DCs. Both LPS and, to a much lesser extent, HMGB1, induced nuclear translocation of p65 in WT DCs; however, in *CD24* or *Siglecg*-deficient DCs, HMGB1 caused even greater increases in nuclear translocation of p65 than did LPS (Fig. 4B). These data suggest that the CD24-Siglec-G pathway may serve to decrease the host response to DAMPs, such as HMGB1, but not to TLR ligands of microbial origin (PAMPs), by selective repression of NF-κB.

To substantiate this hypothesis, we administered a lethal dose of LPS to WT, *CD24*$^{-/-}$, or *Siglecg*$^{-/-}$ mice. Neither the absence of Siglec-G nor the absence of CD24 affected the kinetics of LPS-induced lethality (Fig. 4C) or production of inflammatory cytokines (Fig. 4D). Despite an established contribution of HMGB1 to the late stage of sepsis (*24*), potential amplification of HMGB1 signaling by mutation of *CD24* or *Siglecg* did not affect host survival in response to LPS. Therefore, CD24 and Siglec-G are selective modulators of the host response to HMGB1, but not to TLR ligands such as LPS, despite their potential to induce release of HMGB1 (*24*, *25*).

In addition to nuclear DAMPs, such as HMGB1, DCs also respond to cytoplasmic DAMPs such as

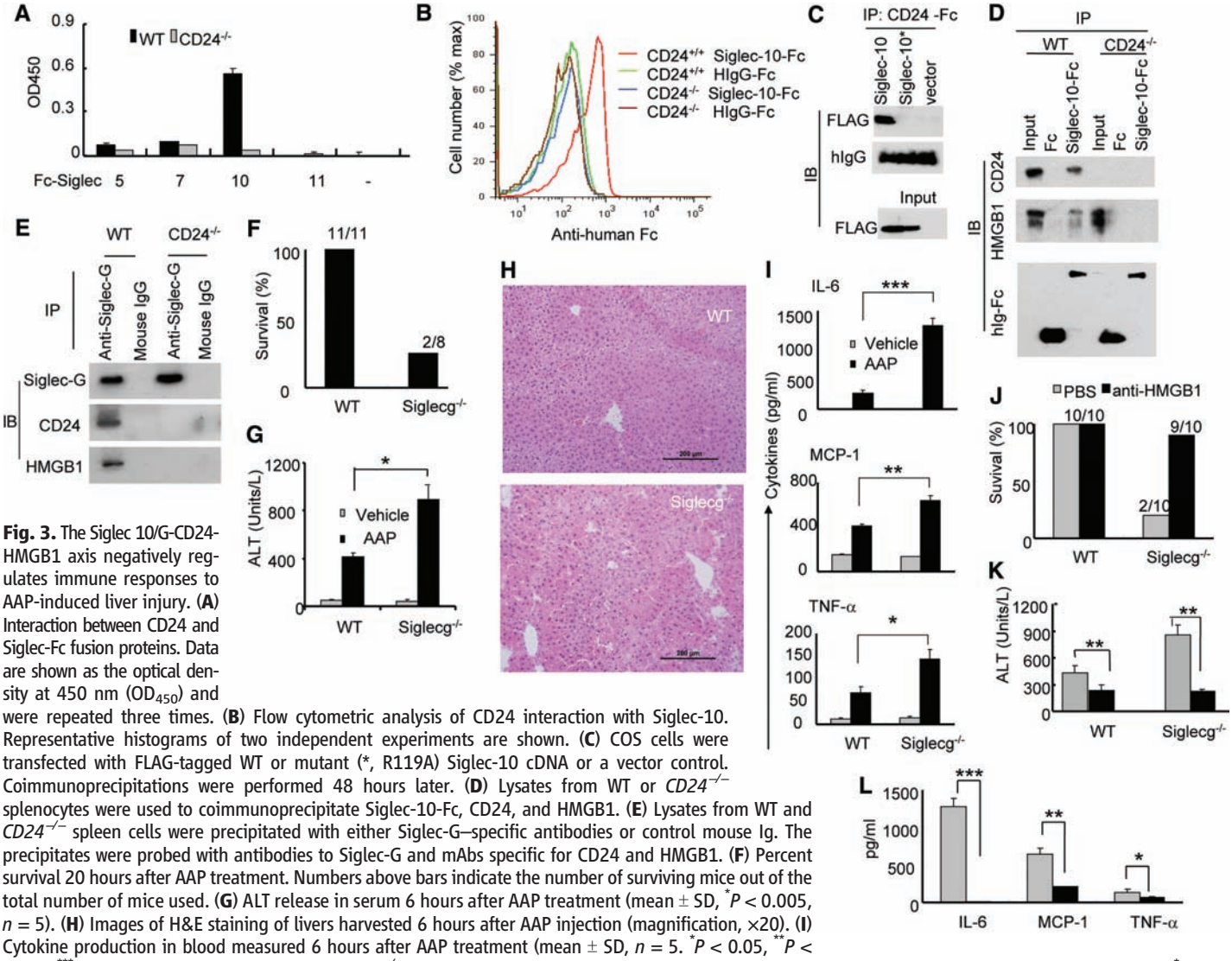

Fig. 3. The Siglec 10/G-CD24-HMGB1 axis negatively regulates immune responses to AAP-induced liver injury. (**A**) Interaction between CD24 and Siglec-Fc fusion proteins. Data are shown as the optical density at 450 nm (OD_{450}) and were repeated three times. (**B**) Flow cytometric analysis of CD24 interaction with Siglec-10. Representative histograms of two independent experiments are shown. (**C**) COS cells were transfected with FLAG-tagged WT or mutant (*, R119A) Siglec-10 cDNA or a vector control. Coimmunoprecipitations were performed 48 hours later. (**D**) Lysates from WT or *CD24*$^{-/-}$ splenocytes were used to coimmunoprecipitate Siglec-10-Fc, CD24, and HMGB1. (**E**) Lysates from WT and *CD24*$^{-/-}$ spleen cells were precipitated with either Siglec-G–specific antibodies or control mouse Ig. The precipitates were probed with antibodies to Siglec-G and mAbs specific for CD24 and HMGB1. (**F**) Percent survival 20 hours after AAP treatment. Numbers above bars indicate the number of surviving mice out of the total number of mice used. (**G**) ALT release in serum 6 hours after AAP treatment (mean ± SD, $^{*}P < 0.005$, $n = 5$). (**H**) Images of H&E staining of livers harvested 6 hours after AAP injection (magnification, ×20). (**I**) Cytokine production in blood measured 6 hours after AAP treatment (mean ± SD, $n = 5$. $^{*}P < 0.05$, $^{**}P < 0.009$, $^{***}P < 0.002$). (**J**) Survival of WT and *Siglecg*$^{-/-}$ mice 20 hours after treatment. (**K**) ALT release in the blood 6 hours after treatment (mean ± SD, $n = 5$, $^{*}P < 0.006$). (**L**) Cytokine release in the blood 6 hours after treatment (mean ± SD, $n = 5$, $^{*}P < 0.03$, $^{**}P < 0.0006$, $^{***}P < 0.0004$). (K) and (L) are representative of two independent experiments. Statistical significance was determined by the Student's *t* test.

HSP70 and HSP90 by TLR-dependent mechanisms (*6*). To determine if the CD24-Siglec-G pathway also regulates host responses to HSP70 and HSP90, we first evaluated whether HSP70 and HSP90 associate with CD24 and Siglec-G. Coimmunoprecipitations revealed that CD24 associates with both HSP70 and HSP90 (Fig. 4E). Similar to HMGB1, Siglec-G association with HSP70 and HSP90 was CD24 dependent (Fig. 4F), and *CD24*$^{-/-}$ and *Siglecg*$^{-/-}$ DCs produced significantly more IL-6 and TNF-α in response to recombinant HSP70 and HSP90 (Fig. 4G) compared to WT DCs. These data reveal a critical role for CD24 and Siglec-G in the negative regulation of DC response to multiple DAMPs.

Our results suggest that CD24 partners with Siglec-10 in humans or Siglec-G in mice to negatively regulate the immune response to proteins released by damaged cells, but not to ligands of microbial origin. Pattern recognition receptors such as TLRs and the receptor of advanced glycation end products (RAGE) mediate activation induced by DAMP (*7*, *8*). Our data indicate that repression of response to HMGB1 may be achieved by inhibition of NF-κB activation. Inhibition may be mediated by SHP-1. SHP-1 associates with Siglec-10 via its ITIM motif (*26*), and deficiency of either Siglec-G or SHP-1 enhances NF-κB activation (*20*, *23*). Given the role of HMGB1 in the pathogenesis of a number of diseases, including drug toxicity (*9*) and liver and cardiac ischemia and reperfusion (*27*, *28*), this pathway may uncover new targets for disease intervention.

Although it is well established that the host can recognize "danger" induced by damaged tissue (*4*), it is unclear whether or how an immune responses triggered by tissue damage is regulated. By identifying the CD24-Siglec-G pathway that selectively suppresses the immune response to DAMPs, our data demonstrate a mechanism by which tissue injury and infection are distinguished, even though they both use the evolutionarily conserved TLRs (*5*–*8*).

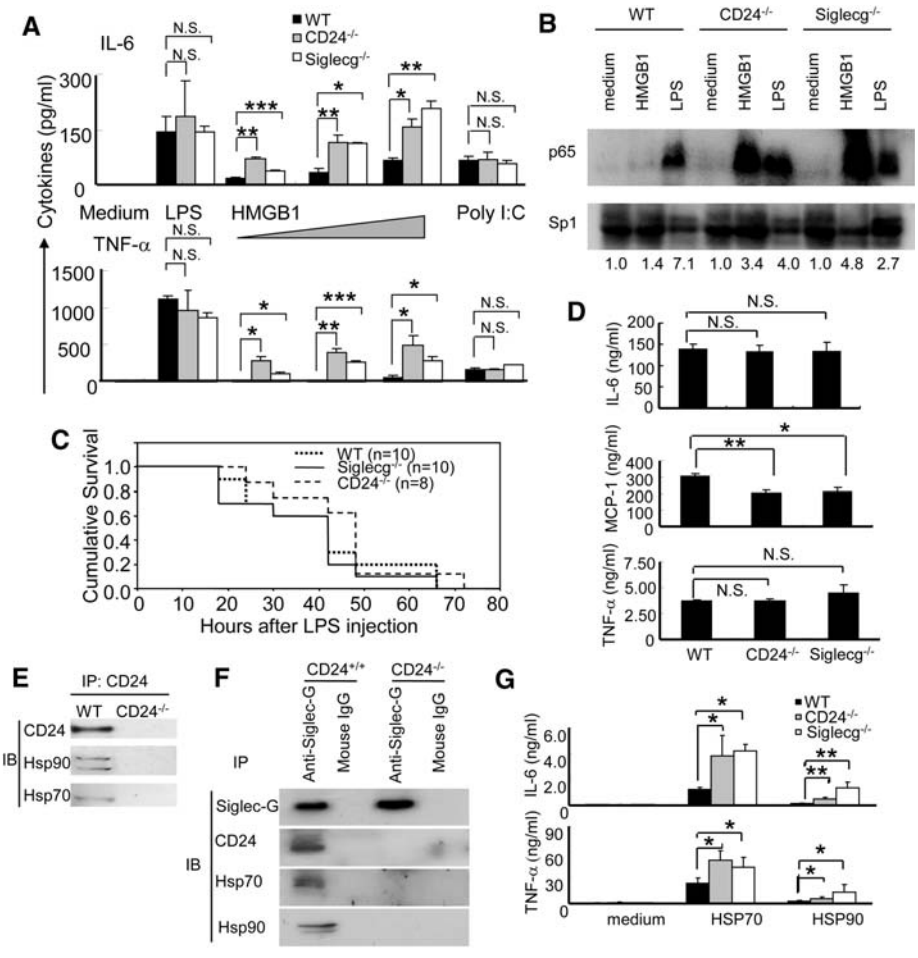

Fig. 4. CD24 and Siglec-G negatively regulate immune responses to HMGB1, HSP70, and HSP90, but not to LPS and poly(I:C). (**A**) Production of cytokines by DCs. DCs cultured from WT, *CD24*$^{-/-}$, or *Siglecg*$^{-/-}$ bone marrow were stimulated with LPS (100 ng/ml), poly(I:C) (10 μg/ml), or increasing doses (5, 10, and 20 μg/ml) of HMGB1 for 6 hours, and then the supernatants were analyzed for the presence of inflammatory cytokines with cytokine beads array. Data represent the mean ± SD for three independent cultures of DCs in each genotype and were repeated at least three times. (**B**) Bone marrow DCs isolated from WT, *CD24*$^{-/-}$, or *Siglecg*$^{-/-}$ mice were stimulated under the indicated conditions for 6 hours. The nuclear lysates were prepared and the activation of NF-κB was assessed by blotting for the p65 subunit of NF-κB. The loading of nuclear protein was determined by amounts of Sp1 protein. Fold induction over medium control is shown below the immunoblots. Data are representative of two independent experiments. (**C**) Age-matched male mice received ip injections of LPS (450 μg/mouse). Kaplan Meier survival plots are shown. No statistical significance was found by log-rank tests. (**D**) Cytokine production in the serum 4 hours after LPS injection (mean ± SD; the statistical significance of the differences between the control and one of the treated groups was determined by Student's *t* test. $^{*}P < 0.03$, $^{**}P < 0.002$). The numbers of mice used were the same as in (C). (**E**) Coimmunoprecipitation of CD24 and Hsp70 and Hsp90. (**F**) Siglec-G associates with Hsp70 and Hsp90 through CD24. The same precipitates used in Fig. 3E were analyzed for Hsp70 and Hsp90 by immunoblot. (**G**) Deficiencies in CD24 and Siglec-G enhanced production of IL-6 and TNF-α at 6 hours after stimulation with HSP70 and HSP90. Data shown represent the mean ± SD of cytokines from four independent isolates of DCs from each genotype and were repeated twice.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1168988/DC1
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Supporting Online Material for

CD24 and Siglec-10 Selectively Repress Tissue Damage–Induced Immune Responses

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This PDF file includes:

Supplemental Information.

Materials and Methods

Reagents Recombinant proteins consisting of human IgG Fc and extracellular domains of SIglec 5, 7, 10 and 11 were purchased from R&D Systems. Horse-radish perioxidase conjugated anti-mouse, or anti-rabbit secondary-step reagents, as well as anti-p65 and anti-sp1 were purchased from Santa Cruz Biotechnology. Anti-FLAG M2 affinity gel, anti-FLAG mAb, acetaminophen (AAP) and lipopolysaccharide (LPS, from E. coli 055:B5) were purchased from Sigma (St Louis, MO). The composition CD24Fc have been described [1], the product is obtained from OncoImmune, Inc. (Columbus, OH). Human HSP70, HSP90 and anti-mouse Hsp70, Hsp90 antibodies were purchased from Biovision, Inc. (Mountain View, CA). The anti-HMGB-1 antibodies 3E8 and 3B1 were described in supplemental information.

cDNAs encoding either full-length or specifically truncated human HMGB-1 and N-FLAG-tagged WT or mutant (119R>A) Siglec10 were cloned into expression vector pCMV-Tag 2B (Sigma). All constructs were verified by DNA sequencing. For purification of FLAG-tagged HMGB-1, the full-length HMGB-1 expression vector was transfected into TSA cells, the lysates were used as source to purify recombinant HMGB-1 according to a reported procedure [2].

Experimental animals Mice with targeted mutations of *CD24* and *Siglecg* were produced from ES cells of C57BL/6 origin as have been described [3, 4]. Age- and sex-matched wild type C57BL/6 mice were used as controls. All mice were used at 6-8 weeks of age. All procedures involving mice have been approved by the University of Michigan Animal Care and Use Committee.

Mouse pathology For ALT measurements, blood was collected at given time points. Serum was isolated by centrifugation of clotted blood at 12,000 x g for 10 min at room temperature and then sent to Animal Diagnostic Laboratory of Animal Research Facility, University of Michigan (Ann Arbor, USA) for determining ALT activity. For histology, liver was removed and immediately fixed in 4% formaldehyde-PBS solution, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Serum cytokines were determined using mouse cytokine bead array designed for inflammatory cytokines (Cat. No 552364, BD Biosciences).

Flow cytometric analysis for Slglec10 ligands Spleen cells from WT or $CD24^{-/-}$ mice were washed in buffer A (150 mM NaCl, 3 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25mM Tris, pH 7.6, 2% BSA), and incubated for 1 hour at 37 °C with 1 μg of Siglec-10-Fc or Fc control. The bound receptors were detected with PE conjugated anti-human IgG-Fc and analyzed on a BD LSII.

Immunoprecipitation and immunoblotting Cell lysates were prepared in the buffer B (1 % Triton X-100, 150 mM NaCl, 3 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 25 mM Tris, pH 7.6) and protease inhibitors (1 μg/ml leupeptin, 1 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride). Samples were pre-cleared with 60 μl of protein A-conjugated agarose beads (Upstate, Lake Placid, NY) for 2 h at 4°C or 37°C with rotation, and then incubated with corresponding antibodies (anti-CD24 mAbs M1/69 and 20C9, 10 μg/ml; anti-HMGB-1, 2 μg/ml; anti-HSP70 and HSP90 antibodies, 3 μg/ml). The beads were washed four times with buffer B and re-suspended in SDS sample buffer for Western blot

analyses with given antibodies (0.5 μg/ml). The anti-Siglec-G antisera were used at 1:100 dilution.

Mass spectrometry After gel concentration, the protein samples were submitted to Taplin Spectrometry Facility at Harvard Medical School for high throughput analysis.

Statistics The differences in cytokine proteins and ALT activities were analyzed by Student's t test. The differences in survival rates were analyzed by Kaplan-Meier survival analysis with log-rank test.

2. Characterization of HMGB-1 antibodies used for the study. HMGB-1 is highly conserved (98% identity between mouse and human). In order to break immune tolerance, we introduced a universal T cell epitope from a mycobacterium tuberculosis Ag [5] into C-terminus of HMGB-1 and the resulting recombinant protein was used as an antigen for immunization. With the help of the T cell epitope and autoimmune NZB/W mice, we were able to obtain a panel of mouse anti-HMGB-1 antibodies that cross-react with mouse and human HMGB-1. Two of them, 3E8 and 3B1 were used in this study. As shown in Fig. S1a, both antibodies react with recombinant HMGB-1 in Western blot. In pilot studies, we have found 3E8 to be a more efficient in immunoprecipitation and Western blot than 3B1 (data not shown). Moreover, 3B1 completely blocked production of TNFα by DC after stimulation of recombinant HMGB-1 (Fig S1b).

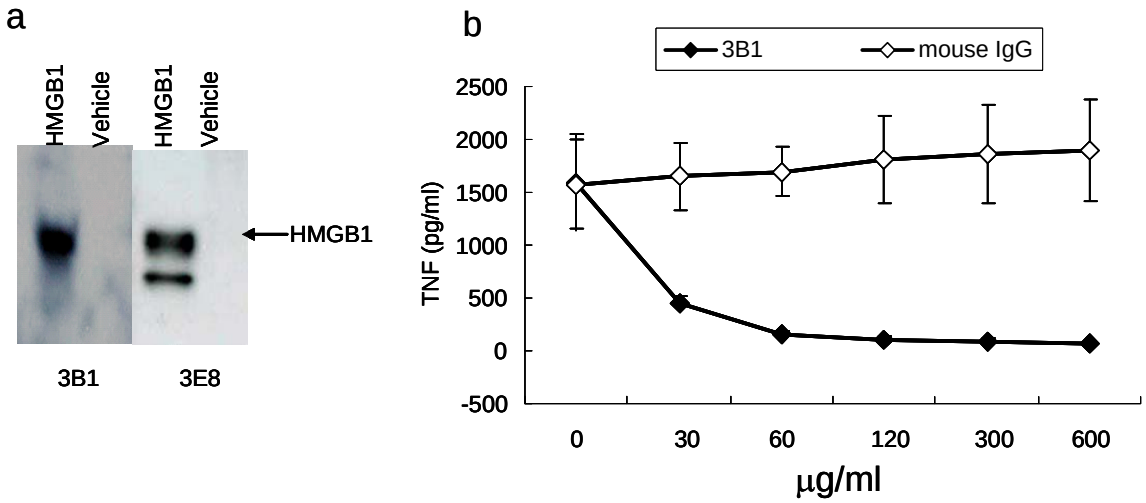


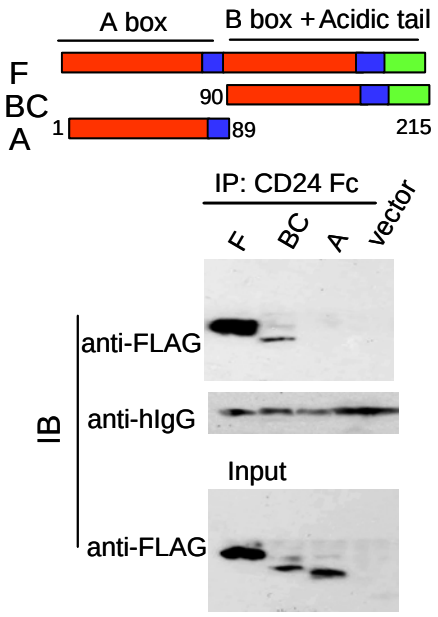
Fig. S1. Characterization of anti-HMGB-1 mAbs used in the study. a. Immunoblot showing the interaction of 3E8 and 3B1 with recombinant HMGB-1 obtained from R&D system. Note that while 3E8 also binds to a truncated HMGB-1 in the preparation, 3B1 only recognize the full length form from the same preparation. Surface Plasmon Resonance (SPR) using the BIACORE3000 revealed that Kd for 3B1-HMGB-1 interaction is 7.8 nM, while that for 3E8-HMGB-1 interaction is 1.3 nM. b. Inhibition of TNFα production from $CD24^{-/-}$ dendritic cells, stimulated with HMGB-1 (20 μg/ml) in the presence of given amounts of 3B1 or mouse IgG control. Supernatants were harvested after 6 hours of culture and measured by cytokine beads array.

Table S1. Confirmation of CD24-HMGB-1 interaction by mass-spectrometry.

The lysates from WT and CD24-deficient hosts were incubated with anti-CD24 mAbs (a mixture of 20C9 and M1/69 and precipitated with protein G beads. The precipitates were incubated with the EDTA to release cation-dependent binding. The eluted proteins were subject to trysinization followed by mass-spectrometry analysis. The data shown are peptides identified from WT spleen cells, and no HMGB-1 peptides were identified from the immunoprecipitates of the *CD24*$^{-/-}$ spleen cells.

Peptide matches

Position	Sequence
57-64	GKFEDMAK
154-162	YEKDIAAYR
76-85	TYIPPKGETK
30-42	HPDASVNFSEFSK
114-126	GEHPGLSIGDVAK
29-42	KHPDASVNFSEFSK
112-126	IKGEHPGLSIGDVAK
128-145	LGEMWNNTAADDKQPYEK
127-145	KLGEMWNNTAADDKQPYEK



Supplemental Fig. S2. CD24 does not bind to inhibitory Box A of HMGB-1. cDNA encoding FLAG-tagged full-length (F), inhibitory Box A (A) or Box B plus acidic tail (BC) were transfected into COS7 cells. The cells were lysed and precipitated with recombinant CD24Fc. The precipitates were blotted with either anti-FLAG or anti-IgG Fc. The relative amounts of truncated proteins expressed were measured by anti-FLAG. The positions of the truncated products were diagrammed on the top.

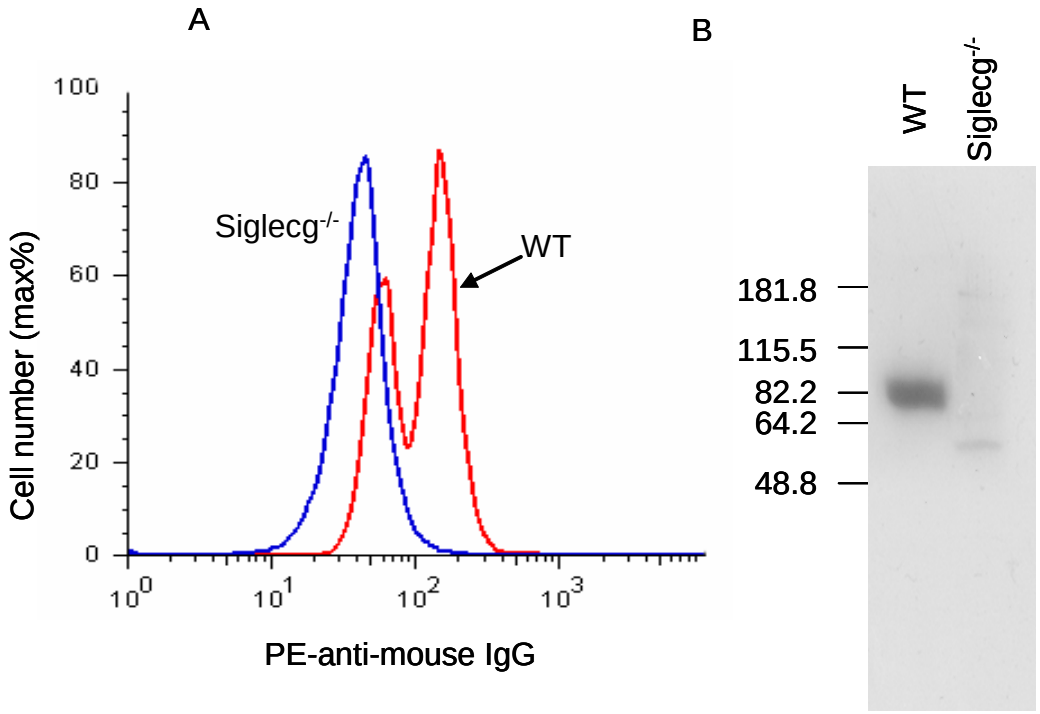


Fig. S3. Characterization of anti-Siglec-G antisera. Siglecg$^{-/-}$ mice were immunized with WT spleen cells (approximately 10^7/mouse/injection) that have been stimulated with LPS (10 μg/ml) for 24 hours. After three immunizations, the sera were collected. (A) Specific binding to WT but not Siglecg$^{-/-}$ spleen cells. Spleen cells were stained with 1:100 dilution of the mouse anti-serum, and the IgG bound to the cells were determined by phycoerythorin-conjugated goat-anti-mouse IgG-Fc. The size of the positive subset roughly matches what was revealed by the GFP markers (*3*). (B) Western blot reveals a specific band that reacts to anti-Siglec-G anti-sera (1:100).

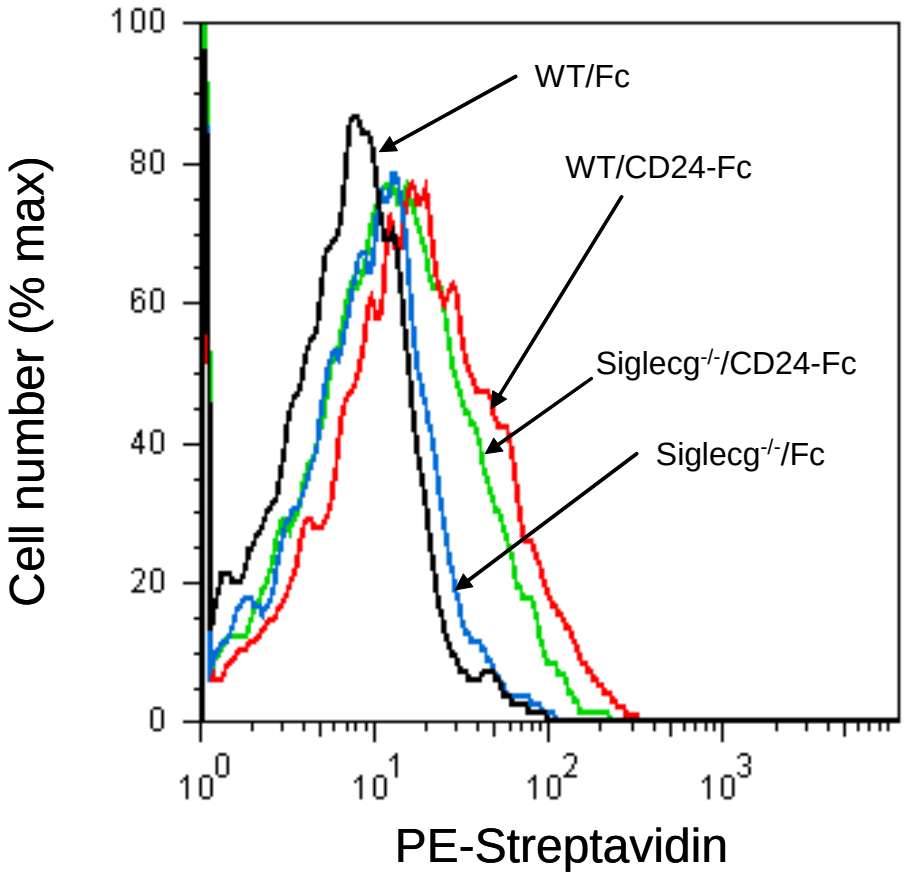


Fig. S4. Siglec-10Fc reacts with both *Siglecg*$^{+/+}$ and *Siglecg*$^{-/-}$ spleen cells. Spleen cells from *Siglecg* $^{+/+}$ and *Siglecg* $^{-/-}$ mice were incubated with biotinylated CD24-Fc or Fc control (2 μg/ml). After washing away the unbound proteins, the cell-associated proteins were detected by phycoerythorin-conjungated streptavidin. The FACS stainings have been repeated twice.

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delivers its genome by ejection/injection (rather than by disassembly of its capsid). Most of these pressurized viruses are likely to be bacteriophages, which generally do not enter their host cell but rather inject their genome upon binding to the outer cell membrane (see the figure, panel A). The genome involved must be dsDNA, because single-stranded nucleic acid is easily compressible and hence does not get sufficiently pressurized upon being confined.

It is thus no coincidence that most bacterial viruses have dsDNA genomes. In contrast, plant and animal viruses, whose capsids generally enter the cytoplasm of their host cells and then disassemble, mostly have ssRNA genomes. On the other hand, a mammalian dsDNA virus such as herpes, whose capsid enters the cytoplasm of its host cell by passing through the outer cell membrane, must still inject its genome into the nucleus upon binding to a nuclear pore complex (see the figure, panel B). The pressure in its capsid should be comparable to those in bacteriophages. The motor protein that packages its genome is thus expected to exert forces as high as tens of piconewtons.

In many phage life cycles, the freshly replicated, not-yet-packaged DNA genomes are linked together in a polymer. High-resolution cryoelectron microscopy studies on phage P22 (*11*) have revealed the configuration of the packaged DNA and shown how the motor protein complex acts as a pressure sensor when a certain density of DNA is achieved. At this point, packaging stops and the DNA is cut.

As always, new understanding raises new questions. For example, because the host cell cytoplasm has an osmotic pressure of several atmospheres, phage ejection stops when the capsid pressure drops to a few atmospheres; what drives delivery of the rest of the genome? In some cases, it is transcription of the genes that have already been delivered; in others, it may involve the influx of water through the phage to accommodate the growth of the host bacterium (*12*).

State-of-the-art biophysical studies will help to elucidate these and other issues, such as how capsids can withstand pressures on the order of 50 atmospheres. Notable among these studies are the reconstitution of bacteriophage λ "from scratch" (*13*), the probing of elastic properties of individual viruses by atomic force microscopy (*14*), the observation of genome ejection from single viruses by fluorescence microscopy (*15*), and the simulation of protein capsid assembly, as well as single-molecule manipulations and high-resolution cryoelectron microscopy. Much as phages played key roles in the development of molecular biology and the genetic engineering revolution, pressurized viruses are likely to be central to the emerging field of physical virology.

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IMMUNOLOGY

Dangers In and Out

The immune system relies on specific signaling molecules to dampen its response to injury while maintaining the capacity to fight infection.

Marco E. Bianchi and Angelo A. Manfredi

Every organism faces a bewildering array of threats, pathogens foremost among them. But how does the immune system distinguish between an infection and trauma? Both elicit similar inflammatory immune responses. On page 1722 of this issue, Chen *et al*. (*1*) explain why and how the immune system responds appropriately in either scenario.

All mammals have an impressive arsenal of molecules and cells specialized to fight pathogens. Adaptive immunity, in the form of antibody production by B cells and the instruction of "killer" or cytotoxic T cells, is a critical component of the body's defenses. However, this is only a second line of defense that selectively recognizes microbes attacking for a second time. Without a first line of defense—innate immunity—mammals would succumb to pathogens still unrecognized by B and T cells.

The broad outlines of our current understanding were first sketched 20 years ago by Charles Janeway (*2*), starting from the idea that the immune system cannot recognize pathogens individually, because the information required is huge and would rapidly become obsolete. Pathogens continually evolve, confounding the ability of the immune system to recognize them. Instead, immune cells recognize broad molecular patterns rather than detailed features of specific pathogens. Such pathogen-associated molecular patterns (PAMPs) comprise molecular structures that are found in microbes but not in host tissues. Moreover, PAMPs are essential for the survival or the pathogenicity of microbes; thus, they cannot simply do away with PAMPs to evade recognition by the immune system.

Toll-like receptor 4 (TLR4) was the first receptor to be identified that recognizes PAMPs (*3*). It recognizes lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria. As the name implies, it is related to Toll, a receptor involved in pathogen recognition in the fly *Drosophila melanogaster*. Thus, Toll-like receptors are evolutionarily ancient, although their number in mammals has grown to about one dozen.

When cells of the innate immune system—such as macrophages, mast cells, natural killer cells, and dendritic cells—encounter a PAMP, they secrete cytokines and chemokines, soluble molecules that signal "danger" to other cells. The most immediate response is inflammation at the site of infection, and the recruitment of additional immune cells, including neutrophils. Importantly, neutrophils "shoot at first sight," releasing reactive oxygen species and proteases, thereby causing extensive collateral damage to the host tissue. Usually, the intruding pathogens are eliminated, and the adaptive immune system "remembers" their identity in case the organism is reinfected by

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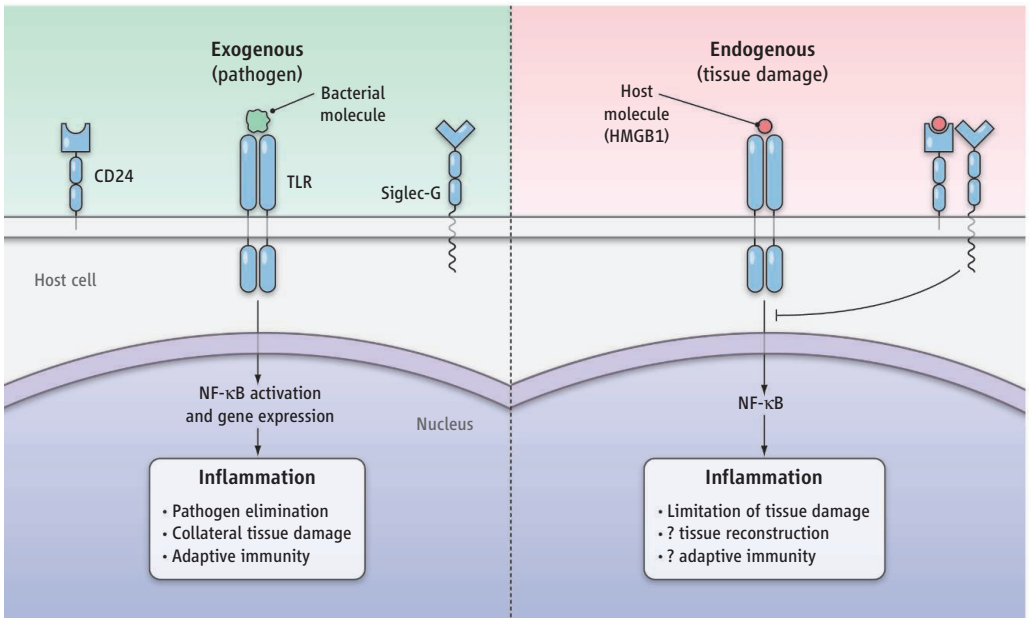
the same pathogens; eventually, the tissue is reconstructed and healed. Inflammation, therefore, is a damaging but essential response, and becomes a problem only when it is excessive, or persists (chronic inflammation).

This picture of pathogen infection is complicated by the fact that physical trauma (such as a wound or a broken bone) causes many of the same effects as invading pathogens, including inflammation. Indeed, pathogens are thought to be initially recognized by the immune system precisely because they cause tissue damage (*4*). How, then, does the immune system recognize tissue damage? Damage-associated molecular patterns (DAMPs) were postulated as the counterparts to PAMPs, with the important distinction that DAMPs should be endogenous—the body's own molecules—just as the PAMPs should be pathogen-borne and thus exogenous. For example, the molecule high mobility group box 1 (HMGB1) fits the hypothetical description of an endogenous danger signal (*5*) and instructs adaptive immunity in ways similar to those elicited by exogenous danger signals (*6*). HMGB1 is a component of chromatin, the DNA-protein complex that makes up chromosomes, and thus normally resides in the cell nucleus. The release of HMGB1 by cells that have died as a result of tissue trauma signals "danger" to neighboring cells and to the immune system. Importantly, HMGB1 is also recognized by the pattern recognition receptors TLR2, TLR4, and TLR9, as well as by the receptor of advanced glycation endproducts, another "danger" receptor (*7*–*9*). Thus, trauma and pathogens (DAMPs and PAMPs) engage the same immune cell receptors, neatly explaining why they elicit the same inflammatory responses, although the molecular details are still largely unknown.

Yet, some outcomes must be different between the two scenarios. Chen *et al.* explored how these partly different outcomes occur by inducing liver necrosis in mice with an excess of acetaminophen. This treatment causes the release of HMGB1, and thus leads to inflammation in the absence of any pathogen. The authors found that mice lacking CD24, a membrane protein expressed by immune and stem cells, developed an inflammatory response that was more powerful and lethal than that in wild-type mice. In fact, HMGB1 was found to directly associate with CD24 and Siglec-G, a member of the sialic acid–binding immunoglobulin-like lectin family. Mice lacking Siglec-G also were sensitive to inflammation due to acetaminophen-induced liver necrosis. CD24 does not contain a cytosolic domain, and signals through Siglec-G, which contains an immune receptor tyrosine-based inhibitory motif (ITIM). ITIMs are cytosolic domains that reduce activation of nuclear factor κB (NF-κB), which is a transcription factor activated by both DAMPs (*10*) and PAMPs (*11*) and is essential for many aspects of the inflammatory response, including the secretion of cytokines and chemokines. The CD24-Siglec complex also recognizes heat shock proteins, another class of endogenous danger signals, but does not respond to lipopolysaccharide or poly-(dI:dC), two exogenous danger signals.

Signaling pathways negatively regulate Toll-like receptor responses to PAMPs as a control for excessive inflammation during infection. It now appears that endogenous danger signals activate a different "braking circuit" that is specific for DAMPs (see the figure). This dampens the immune response to injury and limits collateral damage to the tissue. Interestingly, both HMGB1 and Toll-like receptors appeared early in evolution, but only more modern vertebrates have CD24 and Siglecs. It appears that this particular braking circuit is an add-on to the ancient activating system.

Can the braking circuit also moderate adaptive immunity (which first appeared with fishes), so as to avoid autoimmune responses? CD24 has already been implicated in autoimmunity, and genetic variations in CD24 influence the susceptibility to autoimmune diseases, including multiple sclerosis and lupus (*12*). The CD24-Siglec system might also respond to the various complexes that HMGB1 forms with lipopolysaccahride, interleukin-1β, single-stranded DNA, and nucleosomes. At least one of these complexes, nucleosome-bound HMGB1, is implicated in dendritic cell activation and the production of autoantibodies (*13*). Can the HMGB1-CD24-Siglec system limit the more severe forms of sterile inflammation, such as sepsis? The growing insight into how the immune system distinguishes between internal and external danger is likely to have a substantial impact on therapeutic approaches.



Danger signals. Exogenous and endogenous signals, such as bacterial and host cell molecules, respectively, elicit the inflammatory response through the same Toll-like receptors on immune cells. However, a specific signaling pathway limits the response to endogenous signals. This may prevent a runaway immune response to injuries.

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