The role of the Siglec-G ITIM domain during bacterial infection

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abstract

Siglecs, membrane-bound lectins of the sialic acid-binding immunoglobulin superfamily, inhibit immune responses by recruiting tyrosine phosphatases (e.g., SHP-1 and SHP-2) through their cytoplasmic immunoreceptor tyrosine-based inhibition motif (ITIM) domain. The role of Siglecs in infection has been extensively studied, but downstream signaling through the ITIM domain remains unclear. Here, we used a GST pull-down assay to identify additional proteins associated with the ITIM domain during bacterial infection. Gdi2 bound to ITIM under normal homeostasis, but Rab1a was recruited to ITIM during bacterial infection. Western blot analysis confirmed the presence of SHP-1 and SHP-2 in eluted ITIM-associated proteins under normal homeostasis. We confirmed the association of ITIM with Gdi2 or Rab1a by transfection of corresponding expression vectors in 293T cells followed by immunoprecipitation-western blot assay. Thus, ITIM’s role in the inhibition of the immune response during bacterial infection may be regulated by interaction with Gdi2 and Rab1a in addition to SHP-1 and SHP-2.

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introduction

Immunoreceptor tyrosine-based inhibition motifs (ITIMs) share a consensus amino acid sequence in their cytoplasmic tail, namely (I/V/L/S)-X-Y-X-X-(L/V), where X denotes any amino acid (1). ITIM was first described in FcγRIIB and control lymphocyte activation by recruitment of Src homology 2 (SH2)-containing proteins (2). The role of ITIM in B cell receptor (BCR) signaling is well described. ITIMs are phosphorylated at the tyrosine residue by the BCR-associated kinase Lyn upon ligation of BCR. The ITIM domain then recruits and activates SH2-containing phosphatases such as SH2-containing protein tyrosine phosphatase 1 (SHP-1, also known as PTPN6), SHP-2 (also known as PTPN11), and SH2-domain-containing inositol polyphosphate 5-phosphatase 1 (SHP1, also known as INPP5D) (3). Additional ITIM-containing proteins such as Siglecs (4,5) have since been discovered (1,3).

Siglecs are membrane-bound lectins comprising the sialic acid-binding immunoglobulin superfamily that inhibit immune responses during infection through their cytoplasmic ITIM domains. Each Siglec varies in cellular distribution, glycan specificity and the number of ITIM domains (5). Previously, we found an interaction between CD24 and Siglec-G/10 selectively suppresses the inflammatory response to damage-associated molecular patterns (DAMPs) in tissue injury (6); this interaction is also a key regulator of polybacterial sepsis (7). The CD24/Siglec-10 signaling pathway protects cancer cells from the immune system, indicating a potential target for cancer immunotherapy (8). Another Siglec, Siglec-E, negatively regulates the inflammatory response in bacterial infection (9,10).

The role of Siglecs in pathogen infection has been extensively investigated (5,11-13), but downstream signaling through the ITIM domain remains unclear. Here, we explored potential ITIM-associated proteins by GST pull-down assay and mass spectrometry. We found GDP dissociation inhibitor 2 (Gdi2) and Ras-related protein Rab1a may regulate the role of the ITIM domain during bacterial infection.
Materials and methods

Reagents

The following supplies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA): anti-SHP-1, SHP-2, Myc and HRP-conjugated anti-mouse, anti-goat or anti-rabbit secondary antibodies. MONOCLONAL ANTI-V5, CLONE V5-10 (V8012) and anti-Flag M2 antibodies and LPS (from E. coli 0111:B4) were obtained from Sigma-Aldrich (St. Louis, MO). Anti-His tag HRP (MA121315HRP) was purchased from Thermo Fisher Scientific (Waltham, MA). 293T cells were obtained from ATCC (Manassas, VA) and cultured in Dulbecco’s Modified Eagle’s Medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 µg/ml penicillin and streptomycin.

Construction of plasmids

To generate a construct expressing mouse Siglec-G-ITIM, cDNA for Siglec-G-ITIM was amplified by RT-PCR with the primers (5'-AGGCAGA TCTCAG AAGAAAGGAACCCAGGAGG-3', 5'-AAGCTTGTGGACT CTGACCTCTGGTA-3') and subcloned into expression vector pCMVTag2B (Agilent, Santa Clara, CA), yielding the plasmid pCMV-Tag2B-Flag-ITIM. To generate a construct expressing mouse Rab1a, cDNA for Rab1a was amplified by RT-PCR with the primers (5'-AGTGACGGATCCGCCACCATGTCCAGCATG-3', 5'-AGGCAGAATGAGGAATAC TCCCG-3') and subcloned into expression vector pCDNA6/myc-His (Life Technologies, Carlsbad, CA), yielding the plasmid pCDNA6/myc-His-Rab1a. To generate a construct expressing mouse Gdi2, cDNA for Gdi2 was amplified by RT-PCR with the primers (5'-AGGATCCGCCACCATGGAATGAGGAATAC GCCG-3', 5'-CAGATTCTCGAGCCAGCAGCTCCAC CTGAC-3') and subcloned into expression vector pCDNA6/myc-His (Life Technologies, Carlsbad, CA), yielding the plasmid pCDNA6/myc-His-Gdi2. To generate a construct expressing mouse Gdi2, cDNA for Gdi2 was amplified by RT-PCR with the primers (5'-AGGATCCGCCACCATGGAATGAGGAATAC GCCG-3', 5'-CAGATTCTCGAGCCAGCAGCTCCAC CTGAC-3') and subcloned into expression vector pCDNA6/myc-His-Rab1a. To generate a construct expressing mouse Gdi2, cDNA for Gdi2 was amplified by RT-PCR with the primers (5'-AGGATCCGCCACCATGGAATGAGGAATAC GCCG-3', 5'-CAGATTCTCGAGCCAGCAGCTCCAC CTGAC-3') and subcloned into expression vector pCDNA6/myc-His-Gdi2.

GST fusion proteins

The GST-ITIM fusion protein (GST protein with the fused ITIM domain of Siglec-G) was prepared as described previously (14). Briefly, the coding region of the Siglec-G ITIM domain was generated by PCR with specific primers and cloned into the pGEX-KG vector to create the pGEX-KG-ITIM fusion protein expression vector. The recombinant plasmid pGEX-KG-ITIM and empty pGEX-KG were transferred into E. coli BL21 (DE3). The production of fusion proteins was achieved by incubation with protein inducer 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 37°C for 5 hours.

The resuspended bacterial pellet was sonicated in a buffer (HEPES, pH 8.0, 100 mM NaCl, 5% glycerol, 0.25 mM ZnCl2, 0.1 mM EDTA, 1% Triton X-100, 0.01% Nonidet P-40, 1 mM dithiothreitol and a protease inhibitor phenylmethylsulfonyl fluoride at 1 mM). The supernatant was applied to a glutathione-agarose resin column (Sigma). After washing, the protein was eluted with 5 mM glutathione in the same buffer and then dialyzed against 10 mM Tris–HCl buffer, pH 7.9, containing 0.1 M NaCl, 10 AM ZnCl2 and 10% glycerol.

Experimental animal models of bacterial infection

Wild-type C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and all mice used were 6-8 weeks of age. All animal procedures were approved by the Animal Care and Use Committee of the University of Tennessee Health Science Center. To induce inflammation in mice, we injected (i.p.) C57BL/6J wild-type mice with LPS (100 µg/mouse) and collected spleens 16 hours after LPS injection. We also used CLP as a bacterial infection model. The procedure for lethal CLP was performed as previously described (7). Briefly, C57BL/6J wild-type mice were anesthetized with isoflurane anesthesia. Through a midline incision, the cecum was exteriorized and tightly ligated 1 cm from its base with 3-O silk. The cecum was then punctured through-and-through once with a 23G3/4 needle to ensure that the majority of mice survived the procedure. A small amount of stool was expelled from the puncture before the cecum was returned to the
peritoneal cavity and the abdominal incision was closed. Spleens were also collected from the mice 16 hours post-CLP. Mice injected (i.p.) with PBS were used as controls.

**GST pull-down assay**

Spleens collected from the two bacterial infection models were lysed and used in the GST pull-down assay. Spleens collected from PBS-treated mice were used as controls. Purified GST-ITIM fusion protein was used to immunoprecipitate ITIM-associated proteins from lysates of mouse PBS-, LPS- or CLP-treated splenocytes. After washing, the proteins eluted by 500 mM NaCl were subjected to SDS-PAGE and high-throughput mass spectrometry analysis.

**Mass spectrometry**

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

**Immunoblotting**

293T cells were transfected with the three plasmids (pCMV-Tag2B-Flag-ITIM, pEF1/V5-His-Gdi2 and pCDNA6/myc-His-Rab1a) using Lipofectamine 3000 for 48 hours and then lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 % Triton X-100, pH 7.6, including protease inhibitors 1 μg ml-1 leupeptin, 1 μg ml-1 aprotinin and 1 mM phenylmethylsulfonyl fluoride), sonicated, and centrifuged at 13,000 rpm for 5 min. Next, the samples were subjected to Western blot analysis or immunoprecipitation with the appropriate primary antibodies. The concentration of running gel was 15%. After blocking, the blots were incubated with primary antibodies (1:1,000 dilution). After incubation with the second antibody (HRP-conjugated goat anti-rat IgG, rabbit anti-goat IgG, or goat anti-mouse IgG) (1:5,000 dilution), the signal was detected with an ECL kit (Santa Cruz Biotechnologies).

**Results and discussion**

**Generation of GST-ITIM fusion protein**

Negative regulatory signaling by most Siglec proteins can be attributed to their ITIM domain, which recruits either SH2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2 or inositol phosphatases SHIP1 and SHIP2 to mediate negative signaling (15). To explore the potential role of ITIM during bacterial infection, we prepared a GST-ITIM fusion protein. The plasmids pGEX-KG and pGEX-KG-ITIM expressed recombinant proteins of approximately 26 kDa and 39 kDa, respectively (Figure 1). The size of the proteins matched the calculated molecular weight and the purity was >90%.

**Interaction of ITIM with Gdi2 and Rab1a in mouse splenocytes**

GST pull-down assay was used to identify potential novel proteins that associate with the ITIM domain during bacterial infection. Since the ITIM domain is associated with tyrosine phosphatases SHP-1 and SHP-2, known negative regulators of NF-κB activation, we first tested whether SHP-1 and SHP-2 were eluted by 500 mM NaCl. The GST-ITIM fusion protein was able to immunoprecipitate both SHP-1 and SHP-2 from lysates of PBS-treated but not LPS- or CLP-treated splenocytes (Figure 2).

Interestingly, Gdi2 was among the most prominent proteins identified in lysates of PBS-treated splenocytes (Table 1). Gdi2 is a critical regulator of signal transduction cascades mediated by a subset of Rho GTPases (16) and can suppress cancer metastasis (17,18). We did not detect GDI2 in the control GST protein, indicating that GDI2 coimmunoprecipitated...
with the ITIM domain and this interaction was specific.

Rab1a was among the most prominent proteins identified in lysates of LPS-treated splenocytes (Table 2). Rab1a is a regulator of early endosome sorting for multiple cargo species (19) but can also activate the inflammasome (20) and promote cancer metastasis (21). We did not detect Rab1a in the control GST protein, indicating that Rab1a coimmunoprecipitated with the ITIM domain and this interaction was specific. Furthermore, Rab1a was among the most prominent proteins identified in lysates of CLP-treated splenocytes (Table 2).

![Image](image-url)

**Figure 2.** Immunoblot analysis of SHP-1 ad SHP-2 in eluted samples.
1, 2: PBS treated; 3, 4: LPS treated; 5, 6: CLP treated. GST: 1, 3, 5; GST-SG-ITIM: 2, 4, 6

**Table 1.** Confirmation of SiglecG-ITIM-Gdi2 interaction by mass spectrometry

| Peptide matches | Position | Sequence          |
|-----------------|----------|-------------------|
|                 | 90-98    | MLLFTEVTR         |
|                 | 104-112  | VIEGSFYVK         |
|                 | 116-137  | IYKVPEAALASSIMGLFEK |
|                 | 119-137  | VPSTAEALASSIMGLFEK |
|                 | 157-164  | TFEGVDPK          |
|                 | 222-240  | LSAIYGGTYMLNKPIEEIVQNGK |
|                 | 241-264  | SPYLYPLGLPLPGFAR  |
|                 | 365-379  | EIRPALELEIEEQK    |
|                 | 380-390  | FVSISDLFVFK       |
|                 | 391-402  | DLGTDSQIFISR      |

**Table 2.** Confirmation of SiglecG-ITIM-Rab1a interaction by mass spectrometry

| Peptide matches | Position | Sequence          |
|-----------------|----------|-------------------|
|                 | 14-24    | LLLLLDSGVGK       |
|                 | 141-156  | EFADSLGIPFLETSAK  |

**Interaction of ITIM with Gdi2 and Rab1a in HEK293T cells**

To confirm the physical interaction between ITIM and Gdi2 and Rab1a identified by high-throughput mass spectrometry analysis, we performed immunoprecipitation with the cell lysates of HEK293T cells co-transfected with expression vectors for pCMV-Tag2B-Flag-ITIM, pEF1/V5-His-Gdi2 and pCDNA6/myc-His-Rab1a. At 48 hours post-transfection, the cells were infected with or without bacteria for 4 hours and then lysed. Lysates were immunoprecipitated using antibodies against anti-Flag
antibodies, followed by immunoblotting with antibodies against the V5 or Myc epitope.

ITIM was able to immunoprecipitate Gdi2 from uninfected HEK293T cell lysates but not from bacteria-infected HEK293T cells lysates (Figure 3). In contrast, ITIM was able to immunoprecipitate Rab1a from bacteria-infected HEK293T cell lysates but not from uninfected HEK293T cells lysates (Figure 3). These results suggest that Gdi2 binds to the ITIM domain under normal homeostasis but is removed during bacterial infection, whereas Rab1a is recruited to the ITIM domain during bacterial infection (Figure 4).

The ITIM domain is an immunoreceptor tyrosine-based inhibitory motif, which is phosphorylated by Src family tyrosine kinase upon ligand engagement. The phosphorylated ITIMs bind several phosphatases, including the tyrosine phosphatases, SHP-1, SHP-2, and SHIP. In this study, we have identified novel ITIM associate proteins by mass spectrometry analysis, we found Gdi2 bound to ITIM under normal homeostasis, but Rab1a was recruited to ITIM during bacterial infection. Given that ITIM containing proteins are involved in multiple cellular responses, including the engagement of BCR triggers both B-cell activation and B cell proliferation(22,23), ligation of CD22 relieves a strong inhibitory signal for B-cell stimulation(24), ligation of T-lymphocyte antigen-4 (CTLA-4) negatively regulates T-cell activation(25), the engagement of FcεRI by IgE antibodies and a specific antigen induces mast cell activation but not proliferation(26), KIR-Ls negatively regulates of NK
cell cytotoxicity induced by the recognition of MHC class I molecules on target cells (27), understanding the interaction between ITIM domains and novel identified GDI2 and RAB1a will help us appreciate the complex regulation of these cellular responses (28, 29).

Moreover, we previously found Siglec-E controls reactive oxygen species (ROS) production during bacterial infection through an interaction between the ITIM domain in Siglec-E and p47phox (10). It would be interesting to determine whether the ITIM domain from other Siglecs interacts with Gdi2 and Rab1a and whether this interaction also controls ROS production or other functions during bacterial infection.

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Author contributions
G.C. designed the experiments. Y.W., D.Y., and G.C. conducted the experiments. G.C. wrote the paper.

Declaration of interests
The authors declare no conflicts of interest or financial interests.

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