

# Immunomodulatory activity of extracellular Hsp70 mediated via paired receptors Siglec-5 and Siglec-14

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## Abstract

The intracellular chaperone heat-shock protein 70 (Hsp70) can be secreted from cells, but its extracellular role is unclear, as the protein has been reported to both activate and suppress the innate immune response. Potential immunomodulatory receptors on myelomonocytic lineage cells that bind extracellular Hsp70 are not well defined. Siglecs are Ig-superfamily lectins on mammalian leukocytes that recognize sialic acid-bearing glycans and thereby modulate immune responses. Siglec-5 and Siglec-14, expressed on monocytes and neutrophils, share identical ligand-binding domains but have opposing signaling functions. Based on phylogenetic analyses of these receptors, we predicted that endogenous sialic acid-independent ligands should exist. An unbiased screen revealed Hsp70 as a ligand for Siglec-5 and Siglec-14. Hsp70 stimulation through Siglec-5 delivers an anti-inflammatory signal, while stimulation through Siglec-14 is pro-inflammatory. The functional consequences of this interaction are also addressed in relation to a *SIGLEC14* polymorphism found in humans. Our results demonstrate that an endogenous non-sialic acid-bearing molecule can be either a danger-associated or self-associated signal through paired Siglecs, and may explain seemingly contradictory prior reports on extracellular Hsp70 action.

**Keywords** glycobiology; Hsp70; innate immunity; Siglec

**Subject Categories** Immunology

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## Introduction

The innate immune system distinguishes between foreign invaders and the host “self” via a large array of intracellular and extracellular pathogen recognition receptors (PRRs). PRRs alert and activate the inflammatory response upon detection of exogenous pathogen-associated molecular patterns (PAMPs) or endogenous danger-associated molecular patterns (DAMPs) (Janeway & Medzhitov, 2002; Matzinger, 2002; Beutler, 2009). Less attention has been paid to how highly reactive innate immune leukocytes of the myelomonocytic lineage (neutrophils, monocytes and macrophages) identify “self” to minimize unwanted inflammatory responses.

Heat-shock protein 70 kDa (Hsp70) is classically known as an intracellular chaperone that binds to linear polypeptides of denatured proteins (Rudiger *et al*, 1997; Qi *et al*, 2013). However, Hsp70 is also secreted from cells through unknown, non-canonical mechanisms, and released from necrotic cells. In this context, Hsp70 has been described as an immunomodulatory protein of interstitial spaces and tissue fluids (Pockley *et al*, 1998; Mambula & Calderwood, 2006; De Maio, 2011). Extracellular Hsp70 gained further attention as a potential DAMP when it was found to bind human monocytes and activate secretion of pro-inflammatory cytokines such as TNF $\alpha$  (Asea *et al*, 2000). Toll-like receptor 4 (TLR4) and CD14 were initially proposed to bind extracellular Hsp70, but subsequent studies were unable to replicate the pro-inflammatory effects of Hsp70 in engineered murine macrophage cell lines; endotoxin contamination was instead suggested as an explanation (Gao & Tsan, 2003, 2004). Since then, modulatory effects of Hsp70 on innate immunity have been heavily debated, as neither the immunomodulatory receptors responsible for binding nor the downstream signaling events have been definitively

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established (Johnson & Fleshner, 2006; Theriault *et al.*, 2006; Tsan & Gao, 2009; Borges *et al.*, 2012; Lee & Repasky, 2012). Scavenger receptors such as LOX-1, SREC-1, and FEEL-1 have been observed to engage extracellular Hsp70, but the functional consequences on immune responses such as pro-inflammatory cytokine production are not well defined (Theriault *et al.*, 2006). Regardless, Hsp70 is undisputedly released into extracellular fluids and spaces such as serum and amniotic fluid (Pockley *et al.*, 1998; Jean-Pierre *et al.*, 2006), and elevated levels are found in the bronchoalveolar lavages (BAL) of individuals with lung inflammation (Pastor *et al.*, 2013).

Siglecs are single-pass transmembrane sialic acid-binding immunoglobulin-like lectins found mainly on leukocytes (Varki & Angata, 2006; Crocker *et al.*, 2007; Pillai *et al.*, 2012; Macauley *et al.*, 2014; Schwarz *et al.*, 2015). Siglecs are divided into two subcategories: a highly conserved group, and the rapidly evolving CD33-related Siglecs (CD33rSiglecs), which include Siglec-5 and Siglec-14 (Angata *et al.*, 2006; Ali *et al.*, 2014). Likely due to their rapidly evolving nature, Siglec-5 and Siglec-14 do not have direct murine homologs.

The primary and eponymous ligands of CD33rSiglecs are sialic acids (Sias), the most common terminal glycans covalently linked to vertebrate cell surface and secreted glycoconjugates (Varki & Gagneux, 2012). Concentrations of Sias within the glycocalyx of mammalian cells reach up to 100 mM (Collins *et al.*, 2004). Sias can be modified at several positions and have various linkages to underlying glycan structures (Varki & Schauer, 2009); these combinations create a diverse sialoglycan repertoire that defines the specificity of many binding proteins. While Siglecs have multiple extracellular Ig-like domains, they typically engage Sias through an essential arginine residue in their outermost amino-terminal ligand-binding V-set domain (Varki & Angata, 2006; Crocker *et al.*, 2007; Pillai *et al.*, 2012; Macauley *et al.*, 2014). Most CD33rSiglecs harbor one or more intracellular immunoreceptor tyrosine inhibitory motifs (ITIM) or ITIM-like motifs that can serve to dampen the immune response. These ITIM domains are phosphorylated after ligand engagement, facilitating recruitment of SHP-1/2 phosphatases, events that counteract intracellular kinase signaling cascades to suppress immune activation. Given their ubiquitous presentation on the mammalian cell surface, Sias are ideal self-associated molecular patterns (“SAMPs”) that innate immune leukocytes use to distinguish self from non-self (Varki, 2011). The recent finding of direct interaction between TLRs and Siglecs and their regulation by the sialidase Neu1 have further emphasized the key immunomodulatory roles played by Siglecs (Chen *et al.*, 2014).

Microbial pathogens such as group B *Streptococcus* (GBS), *Neisseria* spp., *Haemophilus influenzae*, and others take advantage of this self-recognition mechanism by decorating their extracellular glycans with Sias (Carlin *et al.*, 2007; Chang & Nizet, 2014). By engaging inhibitory Siglecs through their Sia “cloak,” these pathogens downregulate the host innate immune response (Carlin *et al.*, 2009b). Such pathogen exploitation of immunosuppressive Siglecs challenges the host to evolve novel methods of counteracting the microbial mimicry (Padler-Karavani *et al.*, 2014). One mechanism employed by primates is the emergence of immunostimulating Siglecs, such as Siglec-14, that recognize identical ligands as a direct counterpart to an immunosuppressive

Siglec (Angata *et al.*, 2006). Immunostimulating Siglecs contain a transmembrane domain that binds to immunoreceptor tyrosine activating motif (ITAM) containing adaptors such as DAP12. These complexes initiate a pro-inflammatory signaling cascade after ligand engagement.

Expressed prominently on primate neutrophils and monocytes, immunosuppressive Siglec-5 and immunostimulating Siglec-14 are paired receptors entwined in the evolutionary balance between self-recognition, pathogen exploitation, and microbial defense (Ali *et al.*, 2014). Because of ongoing gene conversions between genomic sequences encoding the first two domains, these two receptors recognize identical ligands yet send opposing intracellular signals to regulate inflammation (Angata *et al.*, 2006). In addition to its Sia capsule, GBS engages human Siglec-5 in a Sia-independent manner through its cell wall-anchored  $\beta$ -protein to further suppress the innate immune response (Carlin *et al.*, 2009a; Nordstrom *et al.*, 2011; Ali *et al.*, 2014).

One explanatory model for paired receptor evolution is that Siglec-14 evolved as an activating receptor to neutralize microbes expressing ligands that exploit the immunosuppressive Siglec-5. In this scenario, *SIGLEC14* would be expected to be unidirectionally gene converted by *SIGLEC5* throughout primate evolution. However, genetic analysis favors a model in which *SIGLEC5* and *SIGLEC14* are undergoing bidirectional rather than unidirectional gene conversion (Angata *et al.*, 2006). This finding implies that it is equally significant for both Siglecs to recognize identical ligands. Pathogen-derived ligands are unlikely candidates for driving such bidirectional gene conversions, since Siglec-5 should then evolve away from pathogen exploitation. Another intriguing feature of these paired Siglecs is that the essential arginine residue for Sia recognition was independently mutated to other amino acids in multiple non-human primate species, with the consequence that these Siglecs can no longer bind Sias (Angata *et al.*, 2006). Given all these observations, we hypothesized that the evolution of Siglec-5 and Siglec-14 to balance the innate immune response may also be influenced by engagement of one or more endogenous Sia-independent ligands. Further complexities arise from the fact that humans have a unique *SIGLEC14* deletion polymorphism that occurs at varying frequencies, in different geographic populations (Yamanaka *et al.*, 2009; Ali *et al.*, 2014). The deletion allele (*SIGLEC14*<sup>-/-</sup>) appears to be the product of a gene fusion event between the adjacent placements of the *SIGLEC5* and *SIGLEC14* genes in the genome. This fusion event effectively removes *SIGLEC14* from the genome, and simultaneously creates a new gene encoding a functionally Siglec-5-like (immunosuppressive) protein driven under the endogenous *SIGLEC14* promoter. Recent work indicates that the *SIGLEC14* genotype directly influences inflammatory disease phenotypes such as GBS-dependent pre-term labor and exacerbation of chronic obstructive pulmonary disease (COPD) (Angata *et al.*, 2013; Ali *et al.*, 2014).

Here, we report an unexpected connection between two otherwise disparate fields of immunobiology, showing the Siglec-5 and Siglec-14 specifically recognize Hsp70 as an endogenous ligand that thereby modulates the innate immune response. This discovery is likely also relevant to current debates about the roles of extracellular human Hsp70, which directly interacts with the human immunoreceptors Siglec-5 and Siglec-14.

## Results

### An unbiased approach identifies Hsp70 as an endogenous ligand of Siglec-5 and Siglec-14

To search for endogenous Sia-independent ligands of Siglec-5 and Siglec-14, we first performed an immunohistochemical screen on human tissue sections using chimeric Siglec-Fc probes that contain the entire extracellular portion of the respective Siglec ligated to human IgG-Fc. To distinguish Sia-independent ligands, we also mutated the arginine residue essential for Sia recognition (R119A). Both wild-type (WT) and R119A mutant Siglec-5-Fc fusion proteins (hereafter called Sig5-Fc and Sig5RA-Fc) were used to probe for ligands (Sig14-Fc was not used in the screening because its V-set Sia-binding domain is 100% identical to Sig5-Fc). Putative Sia-independent ligands for Sig5-Fc were identified by immunohistochemistry in multiple tissues, including prostate, pancreas, liver, and lung (Fig 1A). Notably, strong staining occurred within the mucus lining the epithelia of lung bronchioles and prostate, suggesting a soluble secreted molecule. In the pancreas, a potential Sig5-Fc ligand appeared to localize within the Islets of Langerhans.

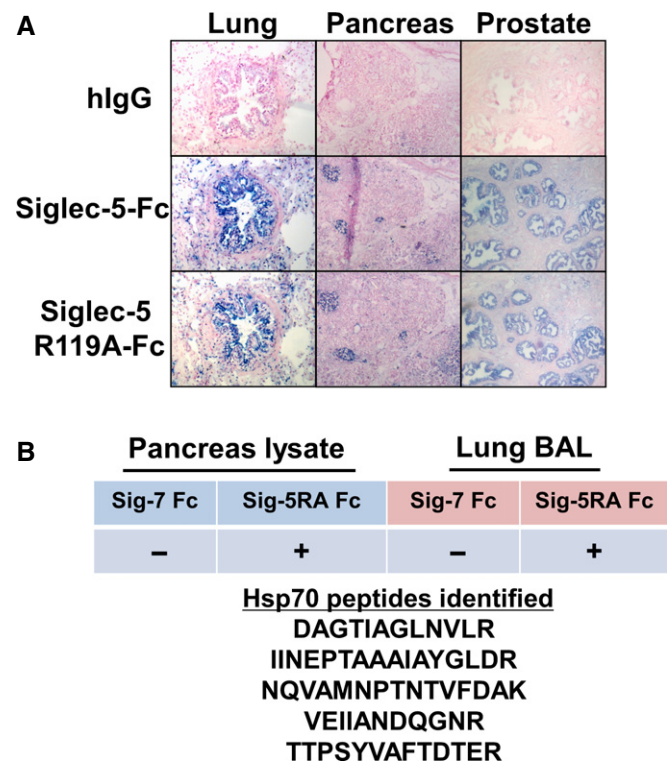
To identify the ligands corresponding to the strong immunohistochemical signals, homogenized human pancreas lysates and human bronchoalveolar lavage samples were incubated with Sig5RA-Fc conjugated to Protein G beads. Siglec-7-Fc (Sig7-Fc) was selected as a negative control as there is no evidence of gene conversion between Siglec-7 and Siglec-5 or Siglec-14. The mixture of proteins captured by the Siglec-Fc-Protein G complexes was submitted to the UCSD Proteomics and Mass Spectrometric Facility for identification. Remarkably, the only protein captured by Sig5RA-Fc in both BAL and pancreas homogenate (but not by Sig7-Fc) was the stress response protein Hsp70 (Fig 1B). Although extracellular Hsp70 has previously been detected in extracellular fluids and spaces, we asked whether the critical Sig5RA-Fc ligand detected in our immunohistochemical screen could indeed be Hsp70 by employing dual-color immunofluorescence to stain lung bronchioles and prostate tissues. Indeed, we found that signals for a sialic acid-independent ligand of Siglec-5 and Siglec-14, identified using Sig5RA-Fc and Sig14RA-Fc, co-localized with signals for an anti-Hsp70 antibody in both tissues (Fig 2). Although this result supports the role of Hsp70 as a sialic acid-independent ligand of these paired Siglecs, it does not negate the possibility of additional sialic acid-independent ligands of Siglec-5 and Siglec-14 in the lung bronchioles.

### Hsp70 binds to Siglec-5 and Siglec-14

Since Hsp70 is not shuttled through the canonical ER–Golgi secretory pathway, it is not modified by Sia-containing glycans (Mambula & Calderwood, 2006; De Maio, 2011). Therefore, the native sequences of the extracellular domains of Siglec-5 and Siglec-14 (rather than the corresponding arginine mutants) were used to study specific interactions between these proteins (unless otherwise noted). Since Hsp70 readily exhibits non-specific binding to other proteins through hydrophobic patches in its substrate-binding domain (Rudiger *et al*, 1997; Qi *et al*, 2013), we sought to validate this newly identified ligand–receptor interaction through various complementary methods. In addition, all subsequent binding interactions were validated with both Siglec-14 and Siglec-5. Purified

recombinant human Hsp70 was probed with Sig5-Fc and Sig14-Fc by Western blot (Fig 3A) and ELISA (Fig 3B). Positive interactions were clearly detected in both assays with appropriate negative controls. B6N, a Siglec-5 and Siglec-14 binding peptide fragment derived from GBS  $\beta$ -protein, was used as a positive control (Nordstrom *et al*, 2011). Clusterin, an unrelated secretory protein, was used as a negative control for non-specific protein–protein interaction with the Siglec-Fc.

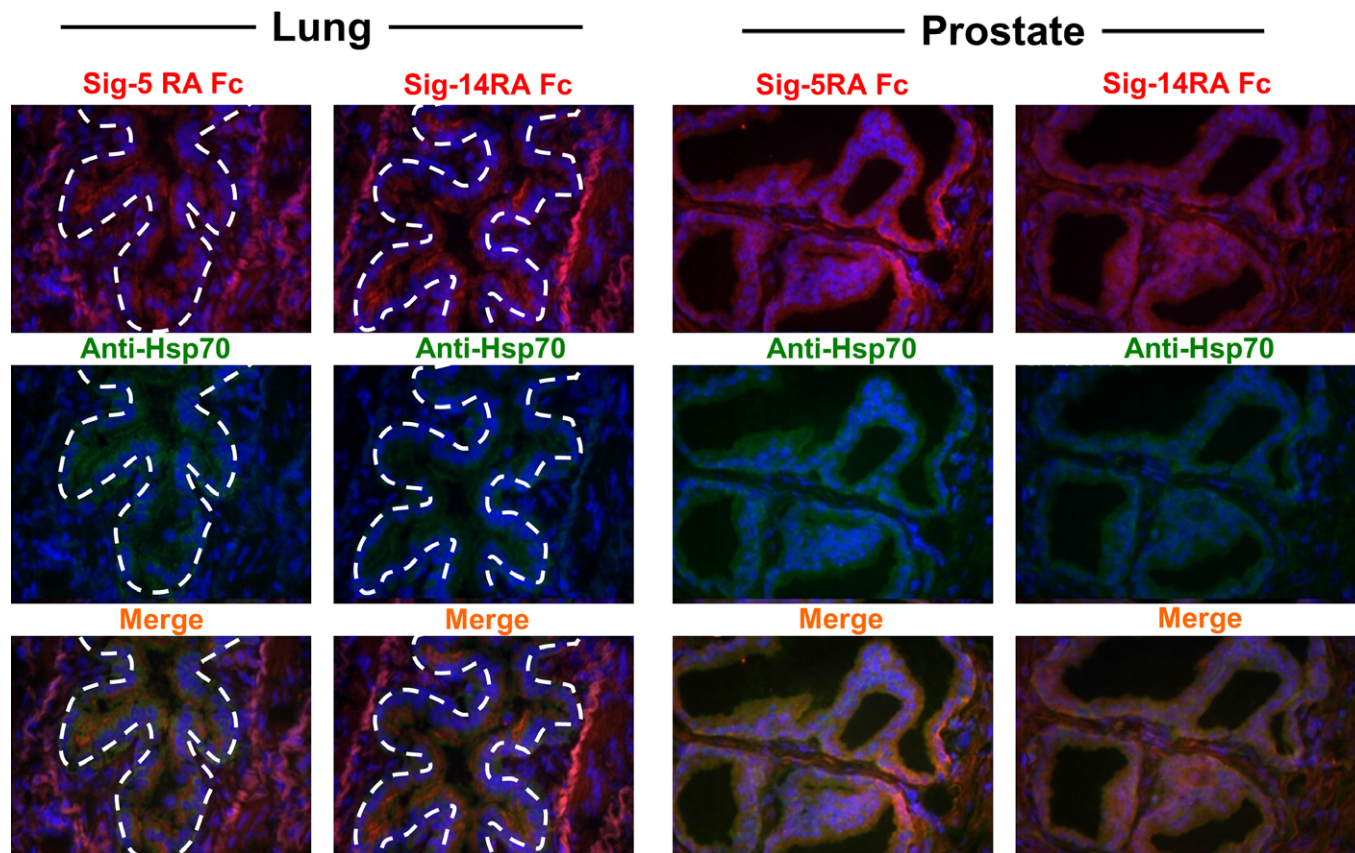
To determine whether Siglec-5 and Siglec-14 recognize endogenous human Hsp70, lysates of heat-shocked HEK293A cells were incubated with Protein G beads pre-conjugated with Sig5-Fc or Sig14-Fc. Proteins captured by the Siglec-Fc-Protein G complex were eluted, separated by SDS–PAGE, and subjected to Western blot analysis with an anti-Hsp70 antibody (Fig 3C). Hsp70 was detected in complexes captured by Sig5-Fc and Sig14-Fc incubated with heat-shock lysates, but not in the negative controls. Next, we asked whether the full-length membrane-bound Siglec-5 and Siglec-14 receptors recognize Hsp70. HEK293A cells, which do not endogenously express any Siglecs, were transiently transfected with cDNAs encoding full-length Siglec-5, -14, or Siglec-7. After incubation with



**Figure 1. Identification of sialic acid-independent Siglec-5 ligands.**

**A** Human tissue sections were probed with Siglec-5 Fc and Siglec-5 R119A Fc to determine the presence of potential sialic acid-independent ligands. Positive signals (in blue) for such ligands were identified in the lumen of the lung bronchioles and prostate ducts, as well as in pancreatic tissues.

**B** Pancreatic tissue lysate or lung BAL were incubated with Siglec-5 R119A Fc (Sig5-RA Fc) or Siglec-7 Fc (Sig7Fc) immobilized onto Protein G beads. The proteins captured by these complexes were identified by mass spectrometric analysis. Hsp70 was the only protein that was captured by the Sig5-RA Fc bead complex from pancreas lysate and bronchoalveolar lavage, but not captured by Sig7Fc bead complex.



**Figure 2. Co-localization of Hsp70 with Siglec-5 and Siglec-14 ligands in lung bronchioles.**

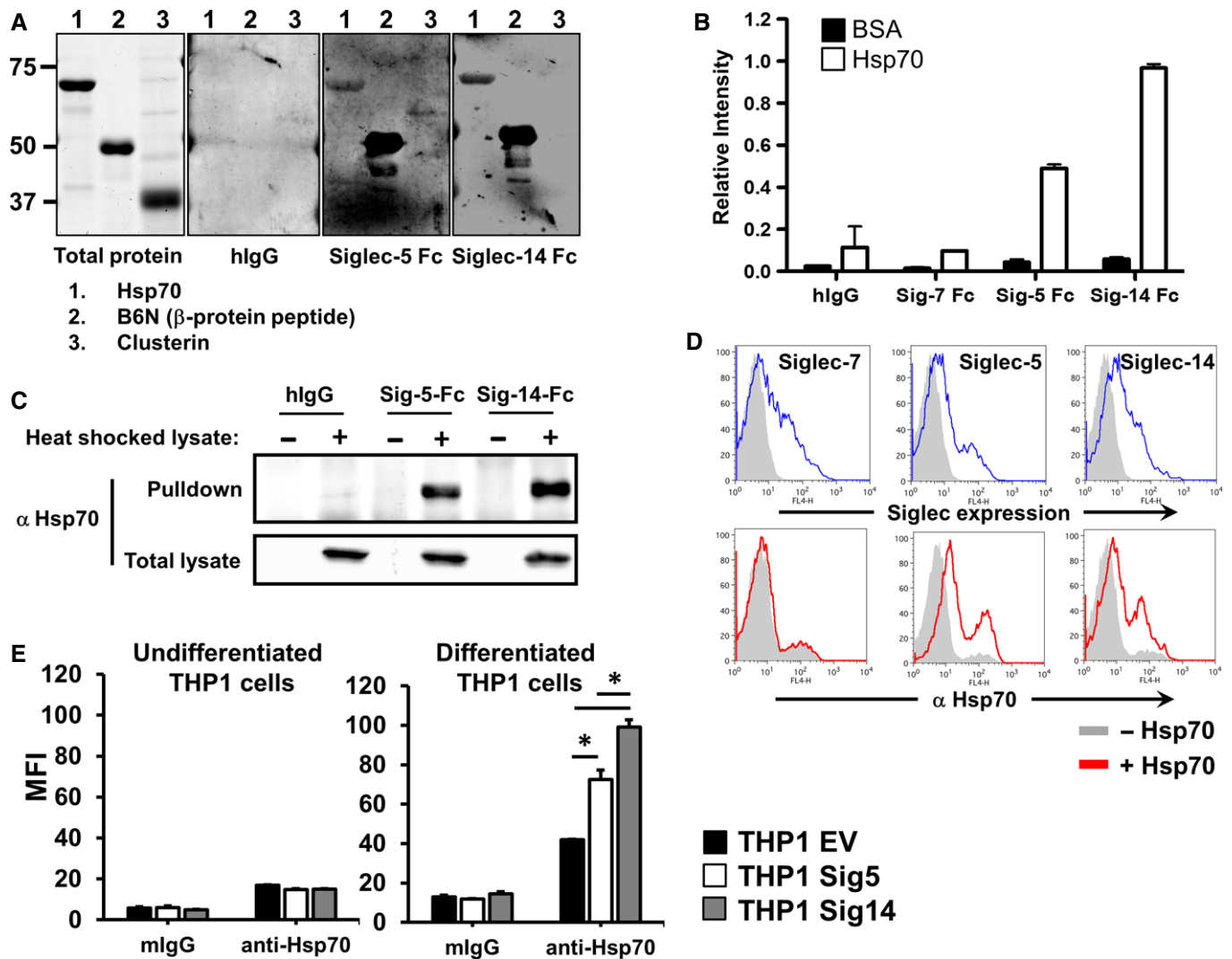
Immunofluorescence of human lung and prostate sections probed with Siglec-5 R119A Fc or Siglec-14 R119A Fc (red) with an anti-Hsp70 antibody (green). The co-localized signals for the Siglec-Fc probes with an anti-Hsp70 antibody suggest that Hsp70 is one of the possible sialic acid-independent ligands of Siglec-5 and Siglec-14 in the lung bronchiole. White dashes mark the lung bronchiole epithelium. All images are representative of at least 2–3 replicates.

recombinant human Hsp70, an anti-Hsp70 antibody was used to detect extracellularly bound Hsp70 by flow cytometry. Only HEK293A cells transfected with Siglec-5 and Siglec-14, but not Siglec-7, displayed Hsp70 binding to the extracellular surface (Fig 3D).

Recent studies have demonstrated Hsp70 secretion from THP1 cells after stimulation with a pro-inflammatory agonist (Tulapurkar *et al*, 2015). We asked whether Hsp70 endogenously secreted from these cells engaged cell surfaces in a Siglec-5- and Siglec-14-dependent manner. To answer this question, we used THP1 monocytic cells overexpressing Siglec-5 or Siglec-14 (THP1+Sig5 or THP1+Sig14) (Yamanaka *et al*, 2009), or its equivalent empty vector transfected control THP1+EV. After stimulating THP1 cells with phorbol 12-myristate 13-acetate (PMA) for 24 h, we used an anti-Hsp70 antibody to detect Hsp70 on the cell surface by flow cytometry. PMA-stimulated THP1+Sig5 or THP1+Sig14 cells displayed greater levels of Hsp70 bound on their surface compared to control THP1+EV cells (Fig 3E). However, a baseline level of Hsp70 bound to the surface of PMA-stimulated THP1+EV cells supported observations and conclusions of previous studies that monocytes and macrophages have multiple Hsp70 receptors (Borges *et al*, 2012). Non-stimulated THP1 cells did not bind Hsp70 to the cell surface whether or not they expressed Siglec-5 and Siglec-14.

#### Hsp70 interacts with the amino-terminal V-set domains of Siglec-5 and Siglec-14

Our prediction of a Sia-independent endogenous ligand for Siglec-5 and Siglec-14 was based on the bidirectional gene conversion events that have occurred between the V-set and first C2 domains throughout primate evolution, which explains why the two human Siglecs have 100% sequence identity in the V-set domain and differ by only one amino acid in the first C2 domain (Angata *et al*, 2006). Hsp70 binding to both Siglec-5 and Siglec-14 suggests that the interaction occurs through the first two near-identical extracellular domains of the receptors, but analysis of individual domain involvement in the interaction was undertaken for further validation. The essential arginine residue for Sia binding resides in the V-set domain. We found that the R119A mutation, which ablates Siglec-14 binding to Sia, also partially disrupts its binding to Hsp70 (Fig 4A). Amino acid sequence alignment of the V-set domains of Siglec-5 and Siglec-14 (Hsp70-binding) with Siglec-7 (Hsp70-non-binding) reveal several differences between these receptors. For example lysine 134 on Siglec-5 and Siglec-14 was not present in Siglec-7 (Fig 4B). A Siglec-14 K134A Fc mutant displayed relatively weaker affinity for Hsp70 in comparison with Siglec-14 Fc (Fig 4B), again implying a key role for the V-set domain in Hsp70



**Figure 3. Direct interaction between Hsp70 and Siglec-5 and Siglec-14.**

**A** Immunoblot probed with Siglec-5 Fc and Siglec-14 Fc to detect direct binding to Hsp70. B6N domain of GBS  $\beta$ -protein was used as a positive control, and clusterin was used as the negative control.

**B** ELISA demonstrates direct binding of Siglec-5 Fc and Siglec-14 Fc, but not Siglec-7 Fc, to Hsp70. Bovine serum albumin (BSA) was used as the negative control.

**C** Siglec-5 or Siglec-14 Fc immobilized on magnetic beads was incubated with heat-shocked HEK293A cell lysates. A Western blot of the captured proteins using an anti-Hsp70 antibody demonstrates Hsp70 endogenously produced by HEK293A cells interacts with Siglec-5 and Siglec-14.

**D** HEK293A cells were transfected with expression plasmids for Siglec-7, Siglec-5 or Siglec-14, and then subsequently incubated with (red) or without (gray) recombinant Hsp70 (bottom panels). Flow cytometry analysis reveals Hsp70 binding to cell surfaces of HEK293A cells expressing Siglec-5 and Siglec-14, but not Siglec-7. Histogram of Siglec expression after transfection is shown as blue lines (top panels).

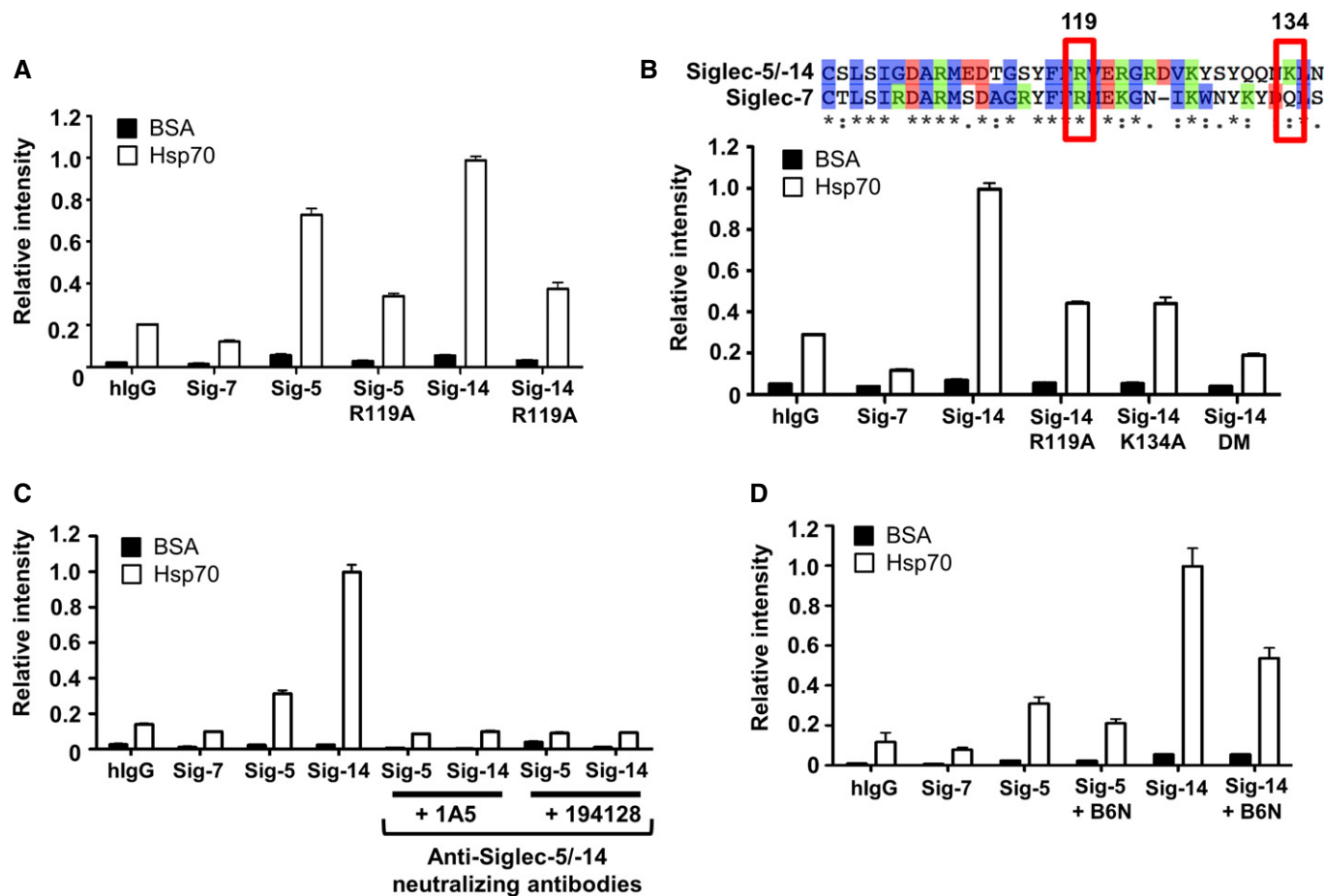
**E** Using flow cytometry, undifferentiated or PMA-differentiated THP1 cells with empty vector (EV), Siglec-5 (Sig5), or Siglec-14 (Sig14) overexpression were assessed for the presence of endogenously secreted Hsp70 bound on the cell surface. The mean fluorescence intensity (MFI) of signals from an anti-Hsp70 antibody on unpermeabilized THP1 cells is shown.

Data information: Data are representative of at least 2–3 replicates. Graphs display mean  $\pm$  SD. \* $P$  < 0.05, as determined by unpaired *t*-test.

binding. Extending this analysis, a double mutant R119A + K134A completely eliminated the binding interaction between Siglec-14 to Hsp70. The influence of these Siglec-14 V-set mutations on Hsp70 binding suggest the specific amino acids are either directly responsible for the interaction or are essential in maintaining a global V-set domain architecture involved in binding. Monoclonal antibodies against Siglec-5 and Siglec-14 have been previously identified that block their interaction with Sia-bearing ligands (Carlin *et al*, 2009a). Pre-incubation of these antibodies with

Sig5-Fc and Sig14-Fc also completely inhibited binding of these Siglecs to Hsp70 (Fig 4C).

Like Hsp70, GBS  $\beta$ -protein also interacts with Siglec-5 and Siglec-14 near its V-set domain (Nordstrom *et al*, 2011). Therefore, we evaluated whether the  $\beta$ -protein could competitively inhibit binding of Siglec-5 and Siglec-14 to Hsp70. The relative binding capacity of Sig5-Fc to Hsp70 was determined in the presence or absence of B6N, the  $\beta$ -protein domain responsible for Siglec interaction. Reduced Sig5-Fc binding to Hsp70 was observed in the



**Figure 4.** Hsp70 interacts with V-set domain of Siglec-5 and Siglec-14.

- A** A comparison of the relative binding affinities between Siglec-5 WT versus R119A Fc, and Siglec-14 WT versus R119A Fc to Hsp70 by ELISA. The arginine-mutated Siglecs display lower affinity for Hsp70 in comparison with their wild-type sequence.
- B** A lysine-to-alanine mutation in residue 134 in Siglec-14 Fc (K134A) also abrogates its affinity for Hsp70, as determined by ELISA. Lysine 134 was identified as a possible residue that changes binding affinity by aligning the sequences of Siglec-5 and Siglec-14 with Siglec-7. The Siglec-14 R119A and K134A double mutant (DM) Fc reduces the interaction with Hsp70 to background levels.
- C** Antibodies against Siglec-5 and Siglec-14 neutralized the interaction between Siglec-14 Fc to Hsp70, as determined by ELISA.
- D** Co-incubation of B6N peptide with Siglec-5 and Siglec-14 Fc decreased binding of each Siglec to Hsp70 in an ELISA.

Data information: Data are representative of at least 2–3 replicates. Graphs display mean  $\pm$  SD.

presence of B6N, suggesting a direct competition between these two ligands to the Siglec (Fig 4D). Taken together, these complementary data indicate that Hsp70 interacts with Siglec-5 and Siglec-14 through their V-set domains.

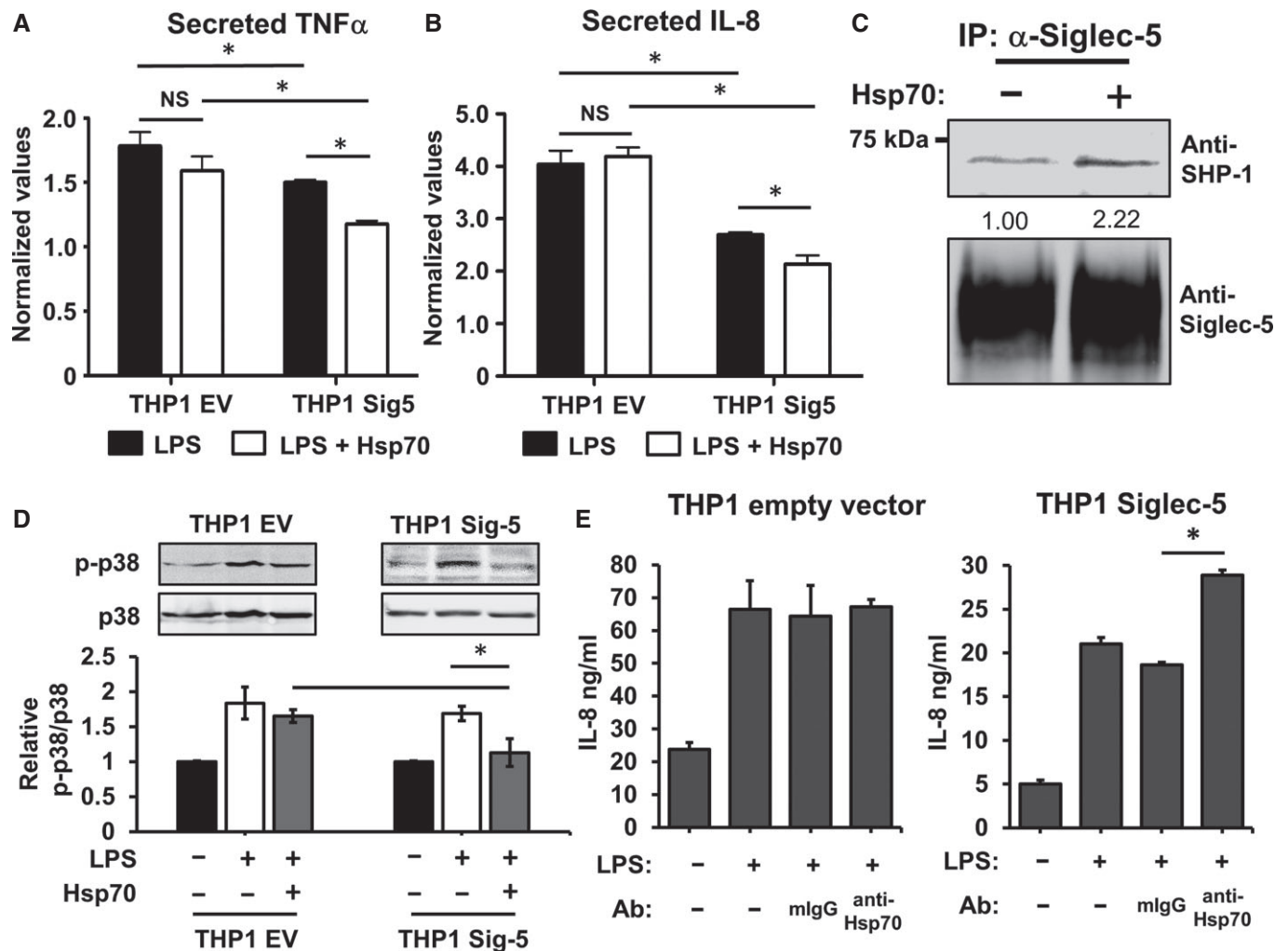
### Hsp70 suppresses inflammation through Siglec-5 in monocytic cells

Next, we investigated whether Hsp70 modulates inflammation and the associated intracellular signaling responses through Siglec-5 and Siglec-14. Myelomonocytic lineage cells secrete pro-inflammatory cytokines such as TNF $\alpha$  and IL-8 in response to PAMPs such as bacterial lipopolysaccharide (LPS). THP1+EV and THP1+Sig5 cells were differentiated with PMA and stimulated with LPS in the presence or absence of Hsp70. Cytokine secretion was determined by sandwich ELISA of the cell culture supernatant. Notably, we found that expression of Siglec-5 on the cell surface

without additional Siglec-5 ligands is sufficient to decrease the production of TNF $\alpha$  and IL-8. However, the addition of Hsp70 further reduced TNF $\alpha$  and IL-8 secretion by THP1+Sig5 cells, while having negligible influence on production of these cytokines by control THP1+EV cells (Fig 5A and B). The intracellular ITIM domains of Siglec-5 are phosphorylated when the receptor engages its ligand, and leads to recruitment of Src Homology Phosphatase 1 (SHP1). THP1+Sig5 cells were incubated in the presence or absence of Hsp70 (Carlin *et al*, 2009a; Chang *et al*, 2012). Western blot indeed showed more SHP1 co-immunoprecipitating with Siglec-5 from cells incubated in the presence of Hsp70 compared to the control group (Fig 5C). We also previously demonstrated that Siglec-5 suppresses inflammation by inhibiting the pro-inflammatory p38 MAP kinase signaling pathway (Ali *et al*, 2014). Adding Hsp70 with LPS to THP1+Sig5 cells decreased phosphorylation of p38 (p-p38) compared to the addition of LPS alone in THP1+Sig5 cells but not THP1+EV cells (Fig 5D).

We and others have previously noted that Siglec-5 and Siglec-14 can modulate the cell's response to inflammation even in the absence of exogenously added Siglec ligands (Angata *et al*, 2006; Ali *et al*, 2014). As Hsp70 is released from cells after stimulation, we asked whether this endogenously secreted Hsp70 feeds back to engage Siglec-5 and Siglec-14 on the cell surface (Tulapurkar *et al*, 2015). Differentiated THP1+EV or THP1+Sig5 cells were exposed to LPS or also with an anti-Hsp70 (or

isotype-matched IgG control) simultaneously. Consistent with our previous findings, we found that THP1+Sig5 cells secreted less IL-8 in comparison with the control THP1 cells when these cells were exposed to equivalent concentrations of LPS. However, we found that with the addition of the anti-Hsp70 antibody, the THP1+Sig5 cells secreted more IL-8 in comparison with the isotype control, but this neutralizing effect was not observed in THP1+EV cells (Fig 5E).



**Figure 5. Hsp70 inhibits inflammation through Siglec-5 in THP1 cells.**

- A Secretion of pro-inflammatory cytokine TNF $\alpha$  by THP1 cells transfected with an empty vector (EV) or Siglec-5 (Sig5) expression plasmid was evaluated by ELISA after concurrent exposure with LPS and 10  $\mu$ g/ml Hsp70 or LPS alone. Secretion of TNF $\alpha$  was reduced only in THP1 Sig5 cells exposed to both LPS and Hsp70 but not LPS alone.
- B Similarly, IL-8 secretion from THP1 EV and Sig5 cells was evaluated by ELISA after concurrent exposure with LPS and 10  $\mu$ g/ml Hsp70. Secretion of IL-8 was also reduced in only THP1 Sig5 cells exposed to both LPS and Hsp70 but not LPS alone.
- C THP1 Sig5 cells were incubated with Hsp70, and the levels of SHP-1 recruitment to Siglec-5 were evaluated by immunoprecipitation of Siglec-5 and Western blot for SHP-1. Higher levels of SHP-1 were co-immunoprecipitated with Siglec-5 when the cells were incubated with Hsp70.
- D Western blots demonstrate phosphorylated p38 (p-p38) was reduced in THP1 Siglec-5 cells when exposed to both LPS and Hsp70 in comparison with LPS alone. This reduction was also greater in THP1 Siglec-5 cells in comparison with the control THP1 cells. Numbers below immunoblots indicate densitometric analysis of each band normalized to the respective cell line's unstimulated control group, divided by the respective loading control (total p38).
- E Secretion of pro-inflammatory cytokine IL-8 by PMA-differentiated THP1 EV or THP1 Sig5 cells was evaluated by ELISA after concurrent exposure with 10 ng/ml LPS with an anti-Hsp70 antibody or isotype-matched antibody. Secretion of IL-8 was increased in only THP1 Sig5 cells exposed to LPS and an anti-Hsp70 antibody in comparison with the isotype control antibody, but this increase was not replicated in THP1 EV cells.

Data information: Data are representative of at least 2–3 replicates. Graphs display mean  $\pm$  SD. \* $P$  < 0.05, as determined by unpaired  $t$ -test.

### Hsp70 augments inflammation through Siglec-14 in monocytic cells

THP1 cells that overexpress either Siglec-5 or Siglec-14 (Yamanaka *et al*, 2009) were differentiated and stimulated with Hsp70. Although the recombinant Hsp70 had undergone endotoxin removal before cell stimulation, cells were exposed to Hsp70 in the presence of endotoxin-neutralizing compound polymyxin B as an extra precaution. Increased release of TNF $\alpha$  and IL-8 were detected in THP1+Sig14 cells, but not THP1+Sig5 cells, upon incubation with Hsp70 (Fig 6A and B). Moreover, in comparison with empty vector control cells, THP1 Siglec-14 cells demonstrated a greater increase of phosphorylated p38 in a time-dependent manner (Fig 6C). Under basal conditions, the pro-inflammatory transcription factor NF- $\kappa$ B is restricted from entering the nucleus by its inhibitor, I $\kappa$ B $\alpha$ . After an inflammatory stimulus, however, I $\kappa$ B $\alpha$  is degraded, consequently allowing NF- $\kappa$ B translocation to the nucleus and activating transcription of pro-inflammatory genes. In a time-dependent manner after stimulation with Hsp70, we found that THP1+Sig14 cells had a greater reduction of I $\kappa$ B $\alpha$  compared to THP1 EV cells (Fig 6D).

Next, we asked whether endogenously secreted Hsp70 feeds back to augment the pro-inflammatory response through Siglec-14. Consistent with our previous findings, THP1+Sig14 cells stimulated with PMA alone secreted higher levels of pro-inflammatory cytokine IL-8 in comparison with control THP1+EV cells. With addition of an antibody against Hsp70, IL-8 secreted from THP1+Sig14 cells was reduced compared to the THP1+Sig14 cells co-incubated with an isotype control antibody (Fig 6E). The antibody against Hsp70 also slightly reduced the levels of IL-8 produced by THP1+EV cells, but not in a statistically significant manner.

### Impact of the human *SIGLEC14* deletion polymorphism on Hsp70 modulation of inflammation

Possibly due to the rapidly evolving nature of the CD33rSIGLEC genes, there are no known Siglec-5 and Siglec-14 homologs in any experimental rodent model (Angata *et al*, 2006). To determine whether the Hsp70-Siglec-14 interaction is biologically significant in primary cells under *ex vivo* conditions, human monocytes isolated from *SIGLEC14*<sup>+/+</sup> individuals were stimulated with Hsp70. We detected TNF $\alpha$  secreted from *SIGLEC14*<sup>+/+</sup> primary monocytes after stimulation with human Hsp70 free of endotoxin contaminants (Fig 7A). Furthermore, this pro-inflammatory stimulation appears specific for human Hsp70 but not DnaK, the *Escherichia coli* Hsp70 homolog. Pre-incubation of primary monocytes with an antibody that recognizes both Siglec-5 and Siglec-14 diminished the amount of TNF $\alpha$  produced from *SIGLEC14*<sup>+/+</sup> monocytes, but not pre-incubation with the isotype control (Fig 7B). Next, we asked whether monocyte activation by Hsp70 is affected by the *SIGLEC14* allele dosage. Since individuals could harbor other polymorphisms unrelated to Siglec-5 and Siglec-14 that affect the immune response, we compared TNF $\alpha$  production between *SIGLEC14*<sup>+/+</sup>, *SIGLEC14*<sup>+/-</sup>, and *SIGLEC14*<sup>-/-</sup> monocytes relative to the individual response to LPS rather than comparing the absolute value of TNF $\alpha$  production between the three genotypes. After stimulation with Hsp70, we found that *SIGLEC14*<sup>+/+</sup> monocytes secreted the highest amount of TNF $\alpha$ , followed by *SIGLEC14*<sup>+/-</sup>

monocytes, with the least amount of TNF $\alpha$  secreted by *SIGLEC14*<sup>-/-</sup> monocytes across all tested concentrations of Hsp70 (Fig 7C).

The *SIGLEC14* null allele expresses a protein product identical to immunosuppressive Siglec-5, in place of Siglec-14; as a consequence, *SIGLEC14*<sup>-/-</sup> monocytes display the Siglec-5-like receptor on the cell surface. We asked whether the addition of Hsp70 would invoke an anti-inflammatory response instead of a pro-inflammatory response in *SIGLEC14*<sup>-/-</sup> monocytes. *SIGLEC14*<sup>-/-</sup> primary human monocytes were stimulated with LPS along with a dose range of Hsp70, and TNF $\alpha$  secretion by these cells was indeed suppressed compared to untreated cells starting at 10  $\mu$ g/ml of Hsp70 (Fig 7D).

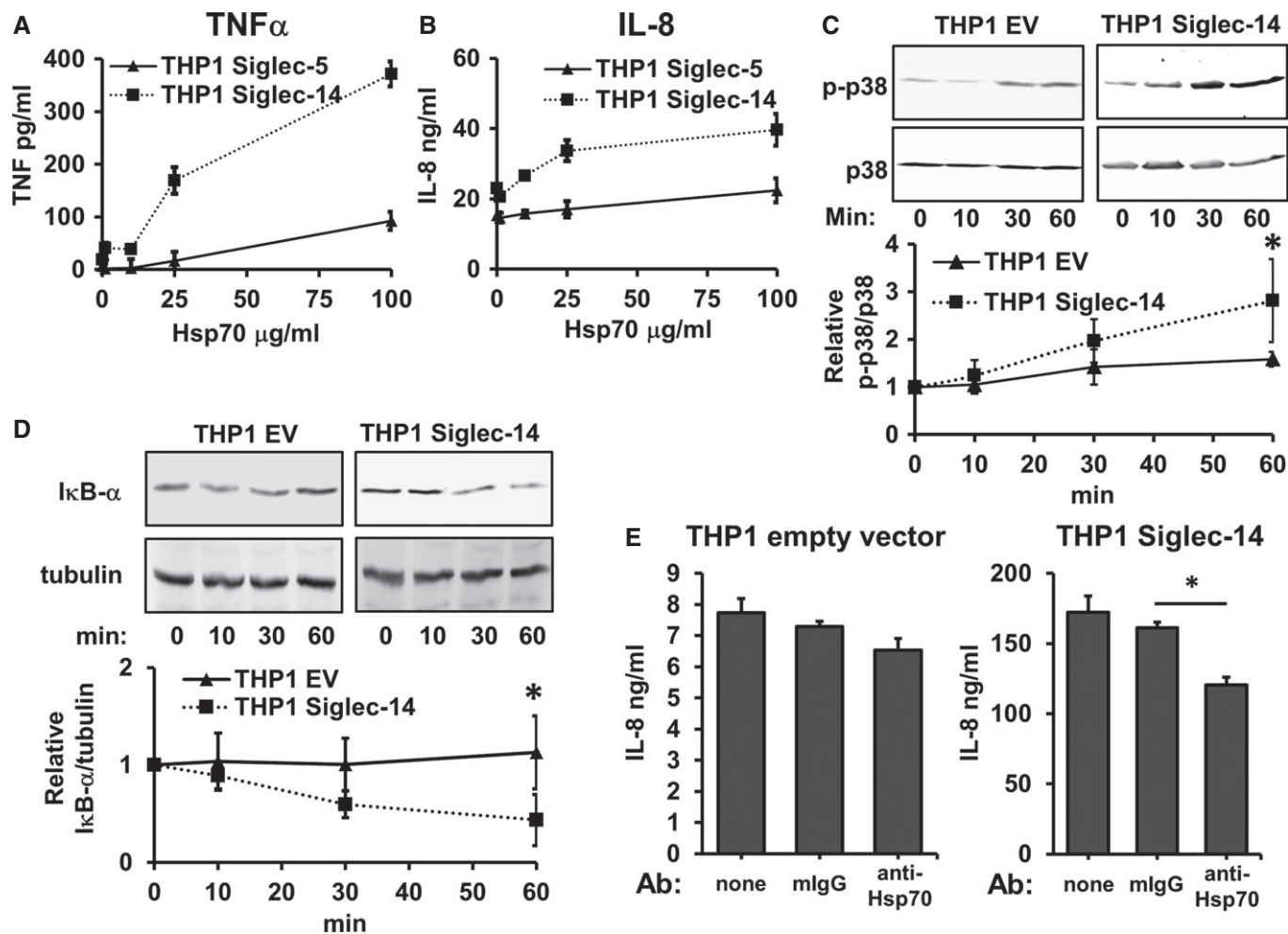
## Discussion

Certain highly conserved molecules with critical functions in intracellular compartments can also be released into extracellular spaces during stress and inflammation and then have the potential to serve as endogenous DAMPs. Examples include the well-studied high mobility group box 1 protein (HMGB1) (Wang *et al*, 1999; Chen *et al*, 2009) and the less well-explored Hsp70 (Asea *et al*, 2000). The eponymously named Siglec receptors were originally discovered as Sia-binding proteins and typically recognize features of the endogenous sialome as SAMPs (Varki, 2011). The logic of our search for a sialic acid-independent endogenous ligand of the paired receptors Siglec-5 and Siglec-14 was based on the bidirectional gene conversion between their ligand-binding domains throughout primate evolution, as well as the inability of several non-human primate Siglec-5 and Siglec-14 molecules to recognize Sias (Angata *et al*, 2006). The present study links these two areas of investigation, showing that Hsp70 can modulate inflammation by directly interacting with Siglec-5 and/or Siglec-14, thus acting either as a SAMP or a DAMP, depending on the expression patterns of these cognate receptors.

Previous work demonstrated that Hsp70 could both activate and suppress the immune response (Asea *et al*, 2000; Johnson & Fleshner, 2006; Rozhkova *et al*, 2010; Borges *et al*, 2012; Lee & Repasky, 2012). Our findings may provide help explain these seemingly contradictory results. In this regard it is notable that the initial study implicating Hsp70 as a DAMP used primary human monocytes, which are typically *SIGLEC14*<sup>+/+</sup> (Angata *et al*, 2006). However, many of the subsequent studies that implicated Hsp70 as an anti-inflammatory molecule used murine monocytes or macrophages instead, which do not express pro-inflammatory Siglec-14 (Gao & Tsan, 2003, 2004). Since some humans harbor a fusion polymorphism for *SIGLEC14* where the gene encodes a Siglec-5-like protein, genotyping the *SIGLEC14* allele is also critical for consistent results (Yamanaka *et al*, 2009).

The pro-inflammatory role of Hsp70 on innate immune cells remains controversial, as many groups have expressed concern that the pro-inflammatory observations are due to endotoxin contaminants in recombinant Hsp70 preparations (Asea *et al*, 2000; Gao & Tsan, 2003). As an extra measure of caution in this study beyond removing endotoxins from our recombinant Hsp70 preparations, we also performed our inflammatory response experiments in the presence of the endotoxin-neutralizing agent polymyxin B. Most





**Figure 6.** Hsp70 augments inflammation through Siglec-14 in THP1 cells.

- A Secretion of TNF $\alpha$  by THP1 cells transfected with a Siglec-5 or Siglec-14 expression vector was evaluated by ELISA after exposure to Hsp70 at the various concentrations in the presence of endotoxin-neutralizing agent polymyxin B. Only THP1 Siglec-14 cells exhibited significant secretion of TNF $\alpha$  in an Hsp70 dose-dependent manner.
- B IL-8 secretion was also evaluated after THP1 Siglec-5 and Siglec-14 cells were exposed to Hsp70. Similarly, only THP1 Siglec-14 cells exhibited significant secretion of IL-8 in an Hsp70 dose-dependent manner.
- C Western blot analysis demonstrates a greater increase in phosphorylated p38 (p-p38) from THP1 Siglec-14 cells after exposure to Hsp70 in comparison with control THP1 EV cells in a time-dependent manner. Graph below immunoblots indicates densitometric analysis of each band normalized to the respective cell line's unstimulated control group, divided by the respective loading control.
- D Exposure to Hsp70 also yielded greater degradation of the NF- $\kappa$ B inhibitor, I $\kappa$ B $\alpha$ , in THP1 Siglec-14 cells in comparison with control THP1 cells, augmentation of the pro-inflammatory response. Graph below immunoblots indicates densitometric analysis of each band normalized to the respective cell line's unstimulated control group, divided by the respective loading control.
- E Secretion of pro-inflammatory cytokine IL-8 by PMA-differentiated THP1 EV or THP1 Sig 14 cells was evaluated by ELISA after concurrent exposure with an anti-Hsp70 antibody or isotype-matched antibody. Secretion of IL-8 was decreased in only THP1 Sig 14 cells exposed to LPS and an anti-Hsp70 antibody in comparison with the isotype control antibody, but this change was not observed in THP1 EV cells.

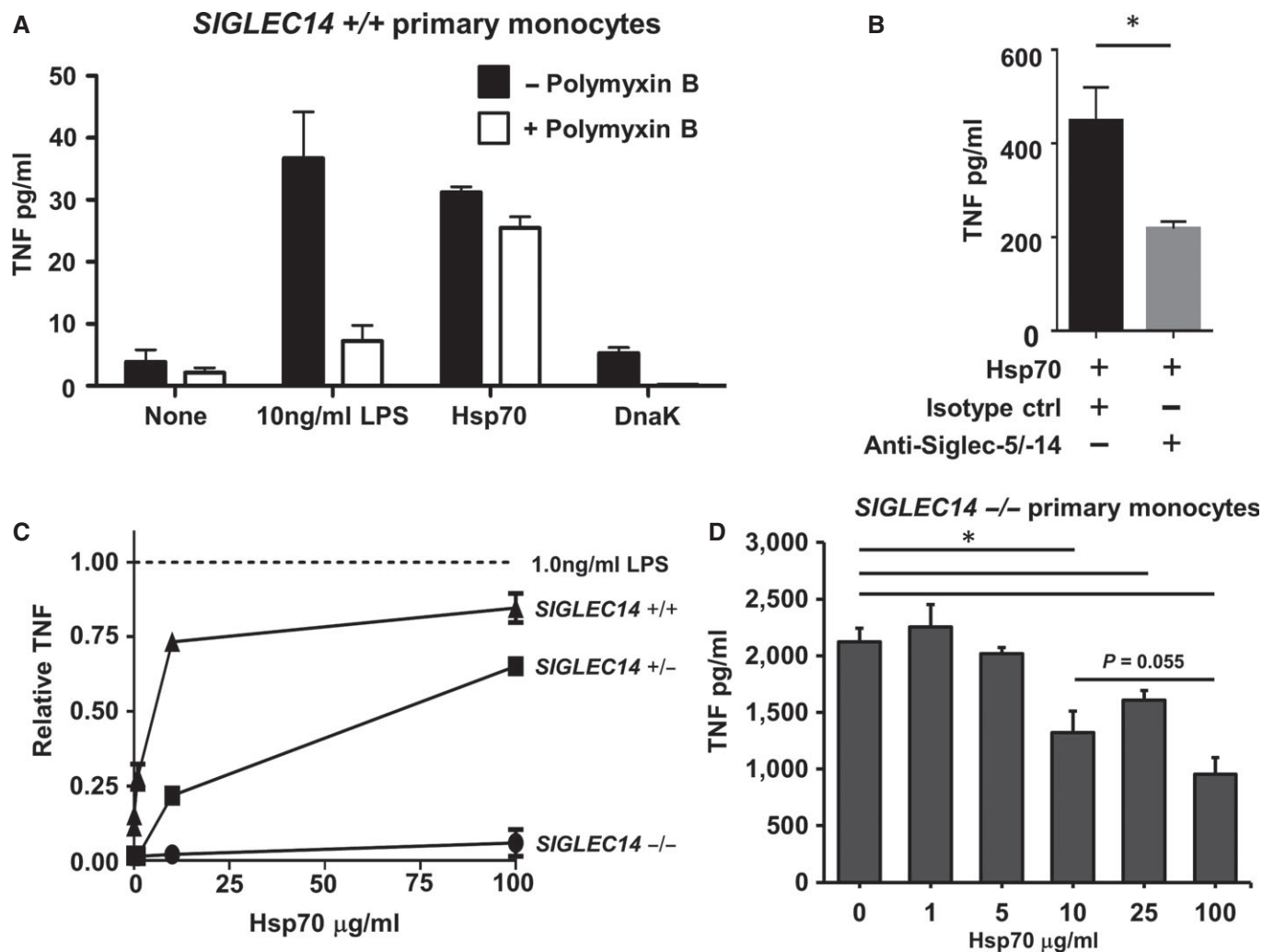
Data information: All figures are representative of at least 2–3 replicates. Graphs display mean  $\pm$  SD. \* $P$  < 0.05, as determined by unpaired  $t$ -test.

significantly, we have demonstrated both the anti-inflammatory and pro-inflammatory roles of Hsp70 endogenously secreted from THP1 cells in a sterile endotoxin-free environment.

Of course, these findings do not negate the previous work implicating other Hsp70 receptors (Asea *et al*, 2000, 2002; Delneste *et al*, 2002; Theriault *et al*, 2005; May *et al*, 2013). Certain scavenger receptors can engage Hsp70, including LOX-1, FEEL-1, SREC-1, NGK2A, and NGK2D (Theriault *et al*, 2006). Hsp70 ligation to LOX-1 on dendritic cells promotes antigen cross-presentation, but

the immunomodulatory role of the other scavenger receptors in relation to Hsp70 have not yet been defined (Delneste *et al*, 2002).

TLR4 has also been implicated as an innate immune receptor for Hsp70 (Asea *et al*, 2000, 2002). Since Siglecs may also engage TLRs and CD14, it is possible that Hsp70 binds Siglecs and other receptors together for a higher affinity interaction (Chen *et al*, 2014; Ishida *et al*, 2014). Hsp70 may also ligate directly with TLR4 or other pro-inflammatory receptor in the absence of Siglec-14, which may explain why we still observed some signs of the pro-inflammatory



**Figure 7. Hsp70 modulation of human monocytes is dependent on the SIGLEC14 genotype.**

- A TNF $\alpha$  secretion from SIGLEC14<sup>+/+</sup> monocytes was measured after exposure to LPS or Hsp70 in the presence or absence of endotoxin-neutralizing compound polymyxin B. Hsp70 was able to induce secretion of TNF $\alpha$  even in the presence of polymyxin B, suggesting a mechanism independent of endotoxin contaminants. DnaK, the *E. coli* homolog of human Hsp70, did not induce significant secretion of TNF $\alpha$  above background levels.
- B TNF $\alpha$  secretion from SIGLEC14<sup>+/+</sup> monocytes by Hsp70 was reduced when the cells were exposed to an anti-Siglec-5/Siglec-14 blocking antibody.
- C Human monocytes isolated from SIGLEC14<sup>+/+</sup>, SIGLEC14<sup>+/-</sup>, and SIGLEC14<sup>-/-</sup> individuals were exposed to varying concentrations of Hsp70, and TNF $\alpha$  secretion was evaluated. The amount of TNF $\alpha$  secreted was normalized to the respective cells' response to 1 ng/ml LPS to minimize the impact of other possible polymorphisms and factors that contribute to variation between individuals.
- D TNF $\alpha$  secretion from SIGLEC14<sup>-/-</sup> monocytes stimulated with 15 ng/ml LPS and various concentrations of Hsp70. TNF $\alpha$  secretion was reduced with increasing concentrations of Hsp70.

Data information: Data are representative of at least 2–3 replicates. Graphs display mean  $\pm$  SD. \* $P < 0.01$ , as determined by unpaired *t*-test.

response in cells that do not express Siglec-14. More studies will be needed to understand the mechanistic crosstalk between Siglecs and TLRs with respect to Hsp70, and even other unrelated ligands at the receptor level, as well as the intracellular signaling pathways.

Since Hsp70 also ligates to Siglec-5 and signals for immune suppression, it dually serves as a DAMP and SAMP. In this regard, it plays a similar role as HMGB1 through the Siglec-G/-10 and CD24 axis (Chen *et al*, 2009; Liu *et al*, 2009). In contrast to HMGB1 though, the dual function of Hsp70 is achieved through engaging paired receptors, which share identical ligand-binding domains but send opposite intracellular responses. An interaction between murine Siglec-G and Hsp70 was also previously insinuated, but not

concretely established (Chen *et al*, 2009). Furthermore, that study demonstrated that the sialoglycoprotein CD24 was essential for the interaction between Hsp70 and Siglec-G. In this study, however, we show a direct interaction between human Siglec-5 and Siglec-14 to Hsp70.

In our immunohistochemical assays, we found extracellular Hsp70 densely localized to the mucosal epithelium in the bronchial and prostate tract lumen. Furthermore, Hsp70 has been observed to oligomerize on tumor cell surfaces (Nimmervoll *et al*, 2015). Since our experimental methods required microgram per milliliter concentrations of Hsp70 in solution to achieve a detectable change in immune response, we speculate that the most physiologically

relevant interaction between Hsp70 with Siglec occurs on mucosal epithelia or through direct cell contact, where local concentrations can be very elevated, but not necessarily in blood or amniotic fluid, where concentrations have been reported in the picogram to nanogram per milliliter range (Zhu *et al*, 2003; Chaiworapongsa *et al*, 2008; Nakhjavani *et al*, 2010). Given the initial phosphorylation of ITAM receptors occurs after clustering due to a stimuli (Bezradica & Medzhitov, 2012), the dense presentation of Hsp70 on the mucosal layer may assist in a more rapid and efficient Siglec-14-dependent immune activation. Thus, the Siglec-5 and Siglec-14 interaction with Hsp70 may be most relevant to tissue resident macrophages rather than circulating monocytes, due to relative abundance of extracellular Hsp70.

Plasmacytoid and monocyte-derived dendritic cells are reported to express Siglec-5 and/or Siglec-14 (Lock *et al*, 2004; Angata *et al*, 2006), but due to cross-reactivity between the two receptors from all known commercially available antibodies, it is unclear whether only one or both Siglecs are present on these cells or tissue resident dendritic cells. Dendritic cells bind extracellular Hsp70 through the LOX-1 receptor, but anti-LOX-1 blocking antibodies only partially reduce Hsp70 binding to the dendritic cell surface (Delneste *et al*, 2002). It is possible that Siglec-14 may also engage Hsp70 on dendritic cells. These observations supports that the notion the physiologically significant sites of Hsp70-Siglec-5/-14 interaction reside in areas where Hsp70 can be densely localized or aggregated. However, at this time it is difficult to test these physiologically based questions *in vivo* since no known small animal models express paired Siglec receptors similar to Siglec-5 and Siglec-14.

Some stress response proteins have also been categorized as resolution-associated molecular patterns (RAMPs) (Shields *et al*, 2011), and the Hsp70-Siglec-5 interaction is perhaps physiologically significant in this context. RAMPs are defined as endogenous proteins released from intracellular compartments during inflammation, similar to DAMPs. In contrast, however, RAMPs are anti-inflammatory and help counterbalance the DAMPs and PAMPs activating the immune response. Since most of the rapidly evolving CD33rSiglecs are immunosuppressive receptors that have been hypothesized to distinguish self from non-self, it is possible that Siglec-5 also evolved into a RAMP receptor to balance the inflammatory response. In this system of the paired Siglec-5 and Siglec-14 receptors, balancing inflammation may be achieved by coordinating expression and display of these receptors, even in the constant presence of an identical ligand. Since Sia-independent binding by Siglecs has been demonstrated before, such as Siglec-5 and Siglec-14 with the GBS  $\beta$ -protein, and Siglec-6 with leptin (Patel *et al*, 1999; Carlin *et al*, 2009a), it is conceivable that other Sia-independent endogenous ligands exist that influence innate immune and inflammatory responses through Siglecs.

A further complexity arises from the fusion polymorphism of *SIGLEC14* in human populations (Yamanaka *et al*, 2009). The ancestral/wild-type *SIGLEC14* allele, which allows expression of the Siglec-14 receptor, is already a known risk factor for developing acute exacerbations of chronic obstructive pulmonary disease (Angata *et al*, 2013). Although a potential mechanism for this linkage involving Sia-displaying pathogens was put forth in the original study, we also now propose that augmenting inflammation by secreted Hsp70 through Siglec-14 is another contributing factor

for exacerbation of COPD. Furthermore, since *SIGLEC14* null individuals display an immunosuppressive Siglec-5-like receptor on their monocytes instead, the Hsp70-Siglec-5 interaction in this case possibly assists in downregulating the pro-inflammatory response in COPD. The combined observations of Hsp70 densely localizing to the bronchiole epithelium and the upregulation of Hsp70 in BAL of individuals with lung inflammation also support this hypothesis (Pastor *et al*, 2013). Similarly, since Hsp70 is found in amniotic fluid, our previous finding of the human specific expression of Siglec-5 and Siglec-14 on the amnion becomes relevant. We speculate as to whether the Hsp70-Siglec-5/-14 interaction also plays a role in regulating human labor (Jean-Pierre *et al*, 2006).

In conclusion, our study demonstrates that extracellular Hsp70 directly engages Siglec-5 and Siglec-14 to modulate innate immunity in a sialic acid-independent manner. Due to the nature of the paired receptors, this interaction may contribute to why earlier studies on Hsp70 yielded contradicting results. In light of the *SIGLEC14* deletion polymorphism, we speculate that this endogenous mechanism of balancing inflammation also contributes to the associations between the *SIGLEC14* polymorphism and inflammatory disease phenotypes. Not only do these findings present new paradigms for paired Siglec receptors evolving to engage sialic acid-independent ligands, but they also shed new light on the controversial role of extracellular Hsp70 as an immunomodulatory signaling molecule. Future studies may explore whether other CD33-related Siglecs can engage Hsp70, and whether other highly homologous members of the Hsp70 family also interact with Siglec immunoreceptors. Since extracellular heat-shock proteins outside the Hsp70 family have also been observed to regulate inflammation, such as Hsp60 (Quintana & Cohen, 2011; Van Eden *et al*, 2013), a thorough and systematic study to determine whether these other heat-shock proteins bind to Siglec immunoreceptors should prove fruitful.

## Materials and Methods

### Hsp70 and Siglec-Fc expression and purification

Hsp70 was acquired commercially (StressMarq Biosciences SPR-108) or prepared as previously described (Qi *et al*, 2013). Briefly, Hsp70 was cloned into a pSMT3 vector (a generous gift from Dr. Chris Lima, Sloan-Kettering Institute) and expressed as a Smt3 fusion protein in BL21 (DE3) Gold. After purifying the fusion protein on a HisTrap column, the Smt3 tag was removed by Ulp1 protease. The Smt3 tag was removed using a HisTrap column and Hsp70 was purified with a HiTrap Q and Superdex 200 16/60 column.

Siglec-Fc fusion proteins were also prepared as previously described (Angata *et al*, 2006; Yamanaka *et al*, 2009). Briefly, Siglec-Fc vectors were transfected into HEK293A cells in serum-free media with Nutridoma-SP (Roche). Culture supernatant was collected, and Siglec-Fc's were purified on a Sepharose Protein A column. After washing with Tris-buffered saline (20 mM Tris-HCl, 150 mM NaCl, pH 8.0; TBS), the Siglec-Fc's were desialylated by neuraminidase derived from *Arthrobacter ureafaciens* (Sigma-Aldrich) for 1 h at room temperature. After extensive washing with TBS, the Siglec-Fc's were eluted with 0.1 M glycine-HCl pH 3.0 and concentrated by Amicon centrifugation.

## Immunohistochemistry

Frozen or paraffin-embedded human tissue samples were sectioned onto glass slides. Slides were blocked with 1% BSA in TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), then incubated with 5 µg/ml of Siglec-Fc overnight at 4°C. After washing with TBS-T, the slides were incubated with anti-human IgG conjugated to alkaline phosphatase. The slides were washed, and then developed using an alkaline phosphatase developing solution. For immunofluorescence, slides were treated similarly.

## Bait-capture and protein identification

Siglec-Fc chimera proteins were immobilized onto Dynabeads Protein G (Life Technologies) as per the manufacturer's instructions. After immobilization, the bead complex was incubated with human tissue homogenates or bronchoalveolar lavage fluid for 2 h at room temperature to capture Siglec-Fc interacting proteins. The bead complex was subsequently washed in TBS-T.

For visualization, the proteins were eluted by boiling for 10 min at 100°C in denaturing sample buffer. Proteins were separated on a SDS-PAGE gel, transferred onto PVDF, and then probed with an anti-Hsp70 antibody for immunoblot.

For protein identification, the proteins were retained on the beads and submitted to the University of California, San Diego Biomolecular/Proteomics Mass Spectrometry Facility.

## Western blot

THP1 cells were incubated with 25 µg/ml Hsp70 at the indicated time points, collected, lysed, and boiled in Laemmli buffer. Samples were separated on Tris-glycine SDS-PAGE gels, and then transferred to PVDF membrane. Membranes were blocked in 1:1 Odyssey blocking buffer (Li-Cor):TBS, as per the manufacturer's instructions. After blocking, the membranes were incubated overnight at 4°C with Siglec-Fc or the appropriate corresponding antibody (p-p38, Cell Signaling 9216; p38, BioLegend Poly6224; Iκbα, BioLegend Poly6249). After washing, the membranes were incubated with the corresponding secondary antibody conjugated to either IRDye 680 or IRDye 800 for 1 h at room temperature. After washing, the membranes were scanned using the Odyssey scanner (Li-Cor).

## Flow cytometry

HEK293A (adherent) cells were washed and then lifted off the plate using 5 mM EDTA in PBS. After lifting, the cells were washed again in PBS. For cell surface Hsp70 binding, 10 µg/ml of Hsp70 was incubated with the cells for 2 h at 4 degrees in 1% BSA in PBS. After washing, the cells were incubated with a mouse anti-Hsp70 antibody (clone W27; BioLegend) for 30 min at 4°C. After another wash, the cells were incubated with an anti-mouse allophycocyanin (APC)-conjugated antibody for 30 min at 4°C. After washing, the cells were read by the BD FACSCalibur (BD Biosciences). THP1 (suspension) cells were treated similarly without the initial use of EDTA to lift cells off the plate.

To determine if endogenously secreted Hsp70 binds to the THP1 cell surface, differentiated THP1+EV, THP1+Sig5, and THP1+Sig14 cells (Angata *et al*, 2006; Ali *et al*, 2014) were stimulated with

50 ng/ml of PMA for 24 h. Undifferentiated THP1 cells did not undergo the PMA stimulation process. Cells were subsequently lifted off culture plates with cold 5 mM EDTA in PBS, washed, and incubated with an anti-Hsp70 antibody conjugated to FITC (clone W27, BioLegend) as per the manufacturer's recommendations. After washing, THP1 cells were analyzed by flow cytometry with the BD FACSCalibur. Mean fluorescence intensity (MFI) was calculated by FlowJo software analysis.

## ELISA for Siglec-Hsp70 interaction

Purified proteins were added to wells overnight in 50 mM sodium carbonate/bicarbonate pH 9.6 at 4°C at a concentration of 10 µg/ml, 100 µl per well. The wells were washed three times with TBS-T (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20, pH 8.0), between each subsequent step. After blocking with 1% BSA in TBS-T for 1 h at room temperature, 5 µg/ml Siglec-Fc was added to each well for 2 h at room temperature. Next, the wells were incubated with biotinylated anti-human IgG for 1 h, then incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 30 min. HRP development was assayed by the ELISA HRP Substrate 680 kit (Li-Cor) and scanned by the Odyssey reader as per the manufacturer's instructions.

## THP1 and human monocyte stimulation with Hsp70

Endotoxin removal was performed using Pierce High Capacity Endotoxin Removal Kit (as per the manufacturer's instructions), and final endotoxin concentration was measured at < 1 EU/ml in each assay, as evaluated by Pierce LAL Chromogenic Endotoxin Quantitation Kit.

Unless otherwise indicated, THP1 cells were differentiated by incubation with 5 ng/ml of phorbol 12-myristate 13-acetate (PMA) in RPMI 1640 for 48 h. Despite the low EU concentration, cells stimulation with Hsp70 occurred in the presence of 200 µg/ml Polymyxin B except when used in conjunction with LPS. Unless otherwise indicated, the concentration of Hsp70 used in each assay was 10 µg/ml. The culture supernatant was collected after 24 h to determine cytokine secretion by ELISA (BioLegend) as per the manufacturer's instructions.

In experiments utilizing an anti-Hsp70 antibody to neutralize pro-inflammatory effects through Siglec-14, THP1+EV or THP1+Sig14 cells were stimulated and differentiated with 50 ng/ml of PMA for 24 h in the presence or absence of the anti-Hsp70 antibody (clone N27F34) or isotype control antibody at 5 µg/ml. The culture supernatant was collected to determine cytokine secretion by ELISA. For experiments utilizing the anti-Hsp70 antibody to neutralize its anti-inflammatory effects through Siglec-5, THP1+EV or THP1+Sig5 cells were stimulated and differentiated with 50 ng/ml of PMA for 24 h. After the differentiation period, the culture supernatant was removed and replaced with RPMI 1640 + 10% FCS without PMA or other additives for another 24 h. Next, the cells were stimulated with 10 ng/ml LPS in the presence or absence of the anti-Hsp70 antibody (clone N27F34) or isotype control antibody at 5 µg/ml for 24 h, and the culture supernatant was collected for ELISA to determine cytokine secretion.

To enrich for primary human monocytes, whole blood was collected from donors and peripheral blood mononuclear cells

(PBMCs) were separated by Ficoll-Paque Premium (GE Healthcare) centrifugation, as per the manufacturer's recommendations. After isolation of PBMCs, the monocytes were enriched by negative selection with the Human Monocyte Isolation Kit (Miltenyi Biotec) and then incubated with corresponding concentrations of Hsp70 over 24 h. Supernatant was collected at the end of the incubation, and cytokine secretion was determined by ELISA (BioLegend) as per the manufacturer's instructions.

### Ethics and approvals

Human blood samples were acquired from volunteers who gave informed consent. The protocols for sample acquisition were approved by and obtained from the Institutional Biosafety Committee at the University of California, San Diego.

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### Author contributions

JJF, TA, VN and AV conceived the original idea. JJF, KS, LD, TA and QL developed the reagents and performed the experiments. JJF, QL, NMV, VN and AV analyzed and interpreted the data. JJF, VN and AV wrote the manuscript with suggestions provided by all other authors.

### Conflict of interest

The authors declare that they have no conflict of interest.

## References

- Ali SR, Fong JJ, Carlin AF, Busch TD, Linden R, Angata T, Areschoug T, Parast M, Varki N, Murray J, Nizet V, Varki A (2014) Siglec-5 and Siglec-14 are polymorphic paired receptors that modulate neutrophil and amnion signaling responses to group B *Streptococcus*. *J Exp Med* 211: 1231–1242
- Angata T, Hayakawa T, Yamanaka M, Varki A, Nakamura M (2006) Discovery of Siglec-14, a novel sialic acid receptor undergoing concerted evolution with Siglec-5 in primates. *FASEB J* 20: 1964–1973
- Angata T, Ishii T, Motegi T, Oka R, Taylor RE, Soto PC, Chang YC, Secundino I, Gao CX, Ohtsubo K, Kitazume S, Nizet V, Varki A, Gemma A, Kida K, Taniguchi N (2013) Loss of Siglec-14 reduces the risk of chronic obstructive pulmonary disease exacerbation. *Cell Mol Life Sci* 70: 3199–3210
- Asea A, Kraeft SK, Kurt-Jones EA, Stevenson MA, Chen LB, Finberg RW, Koo GC, Calderwood SK (2000) HSP70 stimulates cytokine production through a CD14-dependant pathway, demonstrating its dual role as a chaperone and cytokine. *Nat Med* 6: 435–442
- Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK (2002) Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. *J Biol Chem* 277: 15028–15034
- Beutler BA (2009) TLRs and innate immunity. *Blood* 113: 1399–1407
- Bezbradica JS, Medzhitov R (2012) Role of ITAM signaling module in signal integration. *Curr Opin Immunol* 24: 58–66
- Borges TJ, Wieten L, van Herwijnen MJ, Broere F, van der Zee R, Bonorino C, van Eden W (2012) The anti-inflammatory mechanisms of Hsp70. *Front Immunol* 3: 95
- Carlin AF, Lewis AL, Varki A, Nizet V (2007) Group B streptococcal capsular sialic acids interact with siglecs (immunoglobulin-like lectins) on human leukocytes. *J Bacteriol* 89: 1231–1237
- Carlin AF, Chang YC, Areschoug T, Lindahl G, Hurtado-Ziola N, King CC, Varki A, Nizet V (2009a) Group B *Streptococcus* suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. *J Exp Med* 206: 1691–1699
- Carlin AF, Uchiyama S, Chang YC, Lewis AL, Nizet V, Varki A (2009b) Molecular mimicry of host sialylated glycans allows a bacterial pathogen to engage neutrophil Siglec-9 and dampen the innate immune response. *Blood* 113: 3333–3336
- Chaiworapongsa T, Erez O, Kusanovic JP, Vaisbuch E, Mazaki-Tovi S, Gotsch F, Than NG, Mittal P, Kim YM, Camacho N, Edwin S, Gomez R, Hassan SS, Romero R (2008) Amniotic fluid heat shock protein 70 concentration in histologic chorioamnionitis, term and preterm parturition. *J Matern Fetal Neonatal Med* 21: 449–461
- Chang YC, Uchiyama S, Varki A, Nizet V (2012) Leukocyte inflammatory responses provoked by pneumococcal sialidase. *MBio* 3: e00220–11
- Chang YC, Nizet V (2014) The interplay between Siglecs and sialylated pathogens. *Glycobiology* 24: 818–825
- Chen GY, Tang J, Zheng P, Liu Y (2009) CD24 and Siglec-10 selectively repress tissue damage-induced immune responses. *Science* 323: 1722–1725
- Chen GY, Brown NK, Wu W, Khedri Z, Yu H, Chen X, van de Vlekkert D, D'Azzo A, Zheng P, Liu Y (2014) Broad and direct interaction between TLR and Siglec families of pattern recognition receptors and its regulation by Neu1. *Elife* 3: e04066
- Collins BE, Blixt O, DeSieno AR, Bovin N, Marth JD, Paulson JC (2004) Masking of CD22 by cis ligands does not prevent redistribution of CD22 to sites of cell contact. *Proc Natl Acad Sci USA* 101: 6104–6109
- Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. *Nat Rev Immunol* 7: 255–266
- De Maio A (2011) Extracellular heat shock proteins, cellular export vesicles, and the Stress Observation System: a form of communication during injury, infection, and cell damage. It is never known how far a controversial finding will go! Dedicated to Ferruccio Ritossa. *Cell Stress Chaperones* 16: 235–249
- Delneste Y, Magistrelli G, Gauchat J, Haeuw J, Aubry J, Nakamura K, Kawakami-Honda N, Goetsch L, Sawamura T, Bonnefoy J, Jeannin P (2002) Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity* 17: 353–362
- Gao B, Tsan MF (2003) Endotoxin contamination in recombinant human heat shock protein 70 (Hsp70) preparation is responsible for the induction of tumor necrosis factor alpha release by murine macrophages. *J Biol Chem* 278: 174–179
- Gao B, Tsan MF (2004) Induction of cytokines by heat shock proteins and endotoxin in murine macrophages. *Biochem Biophys Res Commun* 317: 1149–1154
- Ishida A, Akita K, Mori Y, Tanida S, Toda M, Inoue M, Nakada H (2014) Negative regulation of toll-like receptor-4 signaling through the binding of glycosylphosphatidylinositol-anchored glycoprotein, CD14, with the Sialic acid-binding lectin, CD33. *J Biol Chem* 289: 25341–25350
- Janeway CAJ, Medzhitov R (2002) Innate immune recognition. *Annu Rev Immunol* 20: 197–216
- Jean-Pierre C, Perni SC, Bongiovanni AM, Kalish RB, Karasahan E, Ravich M, Ratushny V, Skupski DW, Witkin SS (2006) Extracellular 70-kd heat shock protein in mid-trimester amniotic fluid and its effect on cytokine production by *ex vivo*-cultured amniotic fluid cells. *Am J Obstet Gynecol* 194: 694–698

- Johnson JD, Fleshner M (2006) Releasing signals, secretory pathways, and immune function of endogenous extracellular heat shock protein 72. *J Leukoc Biol* 79: 425–434
- Lee CT, Repasky EA (2012) Opposing roles for heat and heat shock proteins in macrophage functions during inflammation: a function of cell activation state? *Front Immunol* 3: 140
- Liu Y, Chen GY, Zheng P (2009) CD24-Siglec G/10 discriminates danger- from pathogen-associated molecular patterns. *Trends Immunol* 30: 557–561
- Lock K, Zhang J, Lu J, Lee SH, Crocker PR (2004) Expression of CD33-related siglecs on human mononuclear phagocytes, monocyte-derived dendritic cells and plasmacytoid dendritic cells. *Immunobiology* 209: 199–207
- Macauley MS, Crocker PR, Paulson JC (2014) Siglec-mediated regulation of immune cell function in disease. *Nat Rev Immunol* 14: 653–666
- Mambula SS, Calderwood SK (2006) Heat shock protein 70 is secreted from tumor cells by a nonclassical pathway involving lysosomal endosomes. *J Immunol* 177: 7849–7857
- Matzinger P (2002) The danger model: a renewed sense of self. *Science* 296: 301–305
- May LA, Kramarenko II, Brandon CS, Voelkel-Johnson C, Roy S, Truong K, Francis SP, Monzack EL, Lee FS, Cunningham LL (2013) Inner ear supporting cells protect hair cells by secreting HSP70. *J Clin Invest* 123: 3577–3587
- Nakhjavani M, Morteza A, Khajeali L, Esteghamati A, Khalilzadeh O, Asgarani F, Outeiro TF (2010) Increased serum HSP70 levels are associated with the duration of diabetes. *Cell Stress Chaperones* 15: 959–964
- Nimmervoll B, Chitchevlova LA, Juhasz K, Cremades N, Aprile FA, Sonnleitner A, Hinterdorfer P, Vigh L, Preiner J, Balogi Z (2015) Cell surface localised Hsp70 is a cancer specific regulator of clathrin-independent endocytosis. *FEBS Lett* 589: 2747–2753
- Nordstrom T, Mover E, Olin AI, Ali SR, Nizet V, Varki A, Areschoug T (2011) Human Siglec-5 inhibitory receptor and immunoglobulin A (IgA) have separate binding sites in streptococcal (beta) protein. *J Biol Chem* 286: 33981–33991
- Padler-Karavani V, Hurtado-Ziola N, Chang YC, Sonnenburg JL, Ronaghy A, Yu H, Verhagen A, Nizet V, Chen X, Varki N, Varki A, Angata T (2014) Rapid evolution of binding specificities and expression patterns of inhibitory CD33-related Siglecs in primates. *FASEB J* 28: 1280–1293
- Pastor MD, Nogal A, Molina-Pinelo S, Melendez R, Salinas A, Gonzalez De la Pena M, Martin-Juan J, Corral J, Garcia-Carbonero R, Carnero A, Paz-Ares L (2013) Identification of proteomic signatures associated with lung cancer and COPD. *J Proteomics* 89: 227–237
- Patel N, Brinkman-Van der Linden ECM, Altmann SW, Gish K, Balasubramanian S, Timans JC, Peterson D, Bell MP, Bazan JF, Varki A, Kastelein RA (1999) OB-BP1/Siglec-6 – A leptin- and sialic acid-binding protein of the immunoglobulin superfamily. *J Biol Chem* 274: 22729–22738
- Pillai S, Netravali IA, Cariappa A, Mattoo H (2012) Siglecs and immune regulation. *Annu Rev Immunol* 30: 357–392
- Pockley AG, Shepherd J, Corton JM (1998) Detection of heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal individuals. *Immunol Invest* 27: 367–377
- Qi R, Sarbeng EB, Liu Q, Le KQ, Xu X, Xu H, Yang J, Wong JL, Vorvis C, Hendrickson WA, Zhou L, Liu Q (2013) Allosteric opening of the polypeptide-binding site when an Hsp70 binds ATP. *Nat Struct Mol Biol* 20: 900–907
- Quintana FJ, Cohen IR (2011) The HSP60 immune system network. *Trends Immunol* 32: 89–95
- Rozhkova E, Yurinskaya M, Zatssepina O, Garbuz D, Karpov V, Surkov S, Murashev A, Ostrov V, Margulis B, Evgen'ev M, Vinokurov M (2010) Exogenous mammalian extracellular HSP70 reduces endotoxin manifestations at the cellular and organism levels. *Ann N Y Acad Sci*, 1197: 94–107
- Rudiger S, Buchberger A, Bukau B (1997) Interaction of Hsp70 chaperones with substrates. *Nat Struct Biol* 4: 342–349
- Schwarz F, Fong JJ, Varki A (2015) Human-specific evolutionary changes in the biology of siglecs. *Adv Exp Med Biol* 842: 1–16
- Shields AM, Panayi GS, Corrigan VM (2011) Resolution-associated molecular patterns (RAMP): RAMParts defending immunological homeostasis? *Clin Exp Immunol* 165: 292–300
- Theriault JR, Mambula SS, Sawamura T, Stevenson MA, Calderwood SK (2005) Extracellular HSP70 binding to surface receptors present on antigen presenting cells and endothelial/epithelial cells. *FEBS Lett* 579: 1951–1960
- Theriault JR, Adachi H, Calderwood SK (2006) Role of scavenger receptors in the binding and internalization of heat shock protein 70. *J Immunol* 177: 8604–8611
- Tsan MF, Gao B (2009) Heat shock proteins and immune system. *J Leukoc Biol* 85: 905–910
- Tulapurkar ME, Ramarathnam A, Hasday JD, Singh IS (2015) Bacterial lipopolysaccharide augments febrile-range hyperthermia-induced heat shock protein 70 expression and extracellular release in human THP1 cells. *PLoS ONE* 10: e0118010
- Van Eden W, Bonorino C, Van Der Zee R (2013) The immunology of cellular stress proteins. *Front Immunol* 4: 153
- Varki A, Angata T (2006) Siglecs—the major subfamily of I-type lectins. *Glycobiology* 16: 1R–27R
- Varki A, Schauer R (2009) Sialic acids. In *Essentials of Glycobiology*, Varki A, Cummings RD, Esko JD, Freeze HH, Stanley P, Bertozzi CR, Hart GW, Etzler ME (eds), pp 199–218. Cold Spring Harbor, NY: Cold Spring Harbor
- Varki A (2011) Since there are PAMPs and DAMPs, there must be SAMPs? Glycan “self-associated molecular patterns” dampen innate immunity, but pathogens can mimic them. *Glycobiology* 21: 1121–1124
- Varki A, Gagneux P (2012) Multifarious roles of sialic acids in immunity. *Ann N Y Acad Sci* 1253: 16–36
- Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 285: 248–251
- Yamanaka M, Kato Y, Angata T, Narimatsu H (2009) Deletion polymorphism of SIGLEC14 and its functional implications. *Glycobiology* 19: 841–846
- Zhu J, Quyyumi AA, Wu H, Csako G, Rott D, Zalles-Ganley A, Ogunmakinwa J, Halcox J, Epstein SE (2003) Increased serum levels of heat shock protein 70 are associated with low risk of coronary artery disease. *Arterioscler Thromb Vasc Biol* 23: 1055–1059