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# NK cell expression of Tim-3: *First impressions matter*

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ABSTRACT

Given the heightened interest in manipulation of co-signaling cascades for cancer immunotherapy, we sought to determine how/whether tumors decorated with therapeutic monoclonal antibodies (mAbs) impact the expression of co-signaling molecules on human NK cells. Stimulation of NK cells with aggregated IgG1 resulted in the upregulation of HAVCR2 – the gene encoding T-cell immunoglobulin and mucin-containing domain (Tim)-3 – known to be involved in the induction of peripheral T cell tolerance. This upregulation of HAVCR2 was recapitulated at the protein level, following NK cell stimulation by either mAb opsonized tumors, recombinant human IgG1 Fc multimer, and/or non-Fc stimuli e.g. IL-12/IL-18. The patterns of Tim-3 expression were temporally distinct from the FcR mediated induction of the co-signaling molecule, 4-1BB (CD137), with Tim-3 increases observed twenty minutes following exposure to Fc multimers and remaining at high levels for at least six hours, while increases in CD137 expression were first observed at the four-hour time point. Importantly, these Tim-3+ NK cells were functionally diverse, as evidenced by the fact that their ability to produce IFN-γ in response to an NK cell responsive tumor was strictly dependent upon the stimuli employed for Tim-3 induction. These data suggest that Tim-3 upregulation is the common end-result of NK cell activation by a variety of unique and overlapping stimuli and is not an independent marker of NK cell exhaustion. Furthermore, our observations potentially explain the diverse functionality attributed to Tim-3+ NK cells and should be considered prior to use of anti-Tim-3 inhibitory mAbs for cancer immunotherapy.

## 1. Introduction

Monoclonal antibodies (mAbs) targeting defined antigens are effective tools for the treatment of a variety of malignant and autoimmune diseases (Adams and Weiner, 2005; Montalban et al., 2017). For example, anti-CD20 mAbs can effectively treat both B cell lymphomas and rheumatoid arthritis, while anti-EGFR mAbs have utility in the management of both colorectal carcinoma and squamous cell carcinoma of the head and neck (SCCHN) (Bonner et al., 2006; Oflazoglu and Audoly, 2010; Cutsem et al., 2011; Du et al., 2017). Despite such demonstrable benefits, anti-tumor responses induced by these mAbs are subject to immune escape, resulting in relapse and development of secondary resistance (Stolz and Schuler, 2009; Rezvani and Maloney, 2011; Zarour and Ferrone, 2011; Pérez-Callejo et al., 2015). One approach to overcome such immune evasion is to identify and modulate the function of immunoregulatory molecules on the surface of immune effector cells that are induced by aggregated Fc domains of targeted mAbs displayed on opsonized tumors.

*Abbreviations:* mAb, monoclonal antibody; SCCHN, squamous cell carcinoma of the head and neck; ADCC, antibody dependent cellular cytotoxicity; FcR, Fc receptor; Tim-3, T-cell immunoglobulin and mucin-containing domain; PBMC, peripheral blood mononuclear cell; IVIG, intravenous immunoglobulin; MFI, mean fluorescence intensity

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Natural killer cells are bone-marrow derived lymphocytes capable of spontaneously responding to antibody-opsonized targets, as well as to virally-infected and stressed cells (Caligiuri, 2008; Vivier et al., 2011). Engagement of FcγRIIIa (CD16) on NK cells with the Fc domains of mAbs aggregated on the target surface, induces release of cytolytic granules, such as perforin and granzyme, mediating lysis of the opsonized target cell via a mechanism termed antibody-dependent cell-mediated cytotoxicity (ADCC) (Wang et al., 2015a,b). NK cells also function through the release of immunomodulatory cytokines, such as IFN-γ and TNF-α (Juelke et al., 2010; De Maria et al., 2011). An evolving body of literature now suggests that in addition to their cytolytic functions, NK cells are perhaps better characterized as immunomodulatory, with their situational response dictated by their cognate receptor ligand interactions in combination with input from the microenvironment (MacDonald, 2005).

There is growing support for the idea that NK cells can mediate both the death of opsonized tumor and arbitrate secondary tolerogenic effects – tolerogenic effects that likely stem from the conserved need to maintain immune homeostasis (Zhang et al., 2006; Deniz et al., 2008; Olson et al., 2010). From a cancer therapeutic perspective, the induction of secondary tolerance potentially contributes to cancer relapse and development of resistance to therapy. Our laboratory has sought to identify molecules induced on NK cells by antibody-decorated targets, which can be targeted to overcome these tolerogenic properties. For instance, we previously demonstrated that cross-linking Fc receptors (FcRs) with human IgG1 Fc fragments induced expression of the co-signaling molecule, 4-1BB (CD137), on NK cells (Lin et al., 2008). Subsequent studies revealed that tumors opsonized with targeted mAbs bearing functional Fc fragments can enhance NK cell expression of CD137 and that ligation of CD137 on these Fc activated NK cells augments their antitumor responses (Kohrt et al., 2014, 2012). Given the inherent redundancy in the immune system, we reasoned that the immunoregulatory molecules upregulated on NK cells in response to Fc aggregates would not be restricted to CD137.

Tim-3 (T-cell immunoglobulin and mucin-containing domain) is a co-signaling molecule expressed by several distinct lymphocyte populations including, but not limited to, T cells, monocytes, dendritic cells and NK cells (Monney et al., 2002; Ju et al., 2010; Chiba et al., 2012; Huang et al., 2015). Ligands reported to bind Tim-3 are Galectin-9, HMGB1, and CEACAM-1 (Chiba et al., 2012; Huang et al., 2015; Zhu et al., 2005). On T cells, Tim-3 is recognized as a marker of functional exhaustion (Sánchez-Fueyo et al., 2003), however, the role Tim-3 plays in regards to NK cell function remains elusive. For example, while Tim-3 has been described as a negative regulator of NK cell function (Ndhlovu et al., 2012; Golden-Mason et al., 2015; da Silva et al., 2014; Wang et al., 2015a,b), it has also been associated with enhanced IFN-γ production following cognate ligand interactions (Gleason et al., 2012). Moreover, Tim-3 expression may influence the state of NK cell activation and its receptiveness to mediate effector responses (Golden-Mason et al., 2015; Cheng et al., 2015).

Using a transcriptomics approach to screen human NK cells for expression of immunoregulatory molecules induced after antibody stimulation, we observed that HAVCR2, the gene encoding Tim-3, was upregulated following stimulation with aggregated IgG. This increase in HAVCR2 gene expression correlated with elevated cell surface expression of Tim-3 on NK cells following ADCC and could be specifically induced by Fc: FcR interactions, select cytokines (IL-2, IL-12, and IL-15) and NK responsive tumors (K562). Of perhaps greater interest, the function of NK cells expressing Tim-3 was variable and was dependent upon the stimulus used for Tim-3 induction. Collectively, these data support the ideas that Tim-3 is not an independent marker of NK cell exhaustion and that Tim-3 expressing NK cells are functionally diverse.

## 2. Material and methods

### 2.1. Cell culture

Cells were cultured at 37 °C with 5% CO2 in RPMI-1640 (Mediatech Inc., USA) supplemented with 10% heat-inactivated fetal bovine serum (Atlanta Biologicals, USA), 10 mM HEPES buffer (Mediatech Inc., USA), 2 mM L-glutamine (Life Technologies, USA) and penicillin (100 U/ml)–streptomycin (100 μg/ml) (Life Technologies, USA). All experiments were performed using RPMI-1640 complete medium unless noted otherwise.

### 2.2. PBMCs and NK cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy human buffy coats (Biological Specialty Corporation, USA) by density gradient centrifugation (2000 RPM for 20 min at room temperature, no brakes) using Ficoll-Paque PLUS medium (GE Healthcare, Sweden). PBMCs were washed with PBS (Mediatech Inc., USA) at least two times at 1500–2000 RPM for five minutes at room temperature and NK cells were isolated using the MACS human NK cell negative selection kit (Miltenyi Biotec, USA) according to manufacturer's recommendations. NK cells were assessed phenotypically as CD56 + CD3- with a NK purity > 90%. All experiments were performed using fresh PBMCs or negatively selected NK cells unless noted otherwise.

### 2.3. Antibodies and flow cytometry

In some experiments, NK cells were cultured with human IgG1 (Sigma, USA) intravenous immunoglobulins (IVIG; Atlantic Biologicals, USA), recombinant IgG1 Fc homodimers (G001; Gliknik, USA), recombinant IgG1 Fc multimers (GL-2045; Gliknik, USA), Rituximab (anti- CD20; Genentech, USA), and/or mouse IgG1 (Biolegend, USA). The following staining antibodies were used in these studies as specified: mouse anti-human Tim-3-PE (Clone: F38-2E2), mouse anti-human CD137-APC or PE (Clone: 4B4-1), mouse anti-human CD3-PerCP (Clone: UCHT1) mouse anti-human IFN-γ-APC (Clone: 4S.B3), mouse anti-human CD107a-PE or FITC (Clone: H4A3) (all from Biolegend, USA), and mouse anti-human CD56-APC (Clone: B159; BD Biosciences, USA). Isotype controls (all from Biolegend, USA) were used to assess non-specific staining. For extracellular staining, samples were washed at least two times with FACS buffer (PBS supplemented with 2% FBS) at 1500 RPM for five minutes at 4 °C. Samples were stained for 30 min at 4 °C. For intracellular staining, samples were fixed with Fixation Buffer (Biolegend, USA) and permeabilized with Intracellular Staining Permeabilization Wash Buffer (Biolegend, USA) according to manufacturer's instructions. Samples were resuspended in FACS buffer prior to flow acquisition. Analytical flow cytometry analyses were performed using a LSRII flow cytometer (BD Biosciences, USA) with FACSDiva software (BD Biosciences, USA) and analyzed using FlowJo software (Tree Star Inc., USA).

### 2.4. PBMC stimulation assays

PBMCs were stimulated overnight with soluble Rituximab or soluble IVIG. The next day, PBMCs were collected and the expression of Tim-3 and CD137 on NK cells (CD56 + CD3 −) were assessed by flow cytometry.

### 2.5. NK stimulation assays

In some experiments, wells were coated one day prior to experimentation with human IVIG (50

μg/ml) at 37 °C. Wells were then washed with PBS at least three times before experimentation. NK cells were stimulated overnight with media only, soluble IVIG, soluble G001, soluble GL-2045, immobilized

IVIG as specified. In some experiments, NK cells were co-cultured with K562 target cells (10:1 E:T; ATCC, USA) for four hours. Expression of Tim-3 and CD137 on NK cells were assessed by flow cytometry.

### 2.6. Transwell assay

Cell culture plates with polycarbonate transwells (0.4 μm pore size; Corning, USA) were used in order to divide the well into a bottom and top compartment, which prevents cell movement between the compartments while allowing soluble mediators to traverse freely. One day prior to experimentation, bottom compartments were coated with human IVIG (50 μg/ml) at 37 °C. Wells were washed with PBS at least three times before experimentation. NK cells were added to bottom and top compartments in the presence or absence of immobilized IVIG in the bottom compartment for overnight stimulation. The next day, NK cells were collected and the expression of Tim-3 and CD137 were assessed by flow cytometry.

### 2.7. Pharmacodynamic assays

For dose-response assays, NK cells were stimulated overnight with either media only, soluble G001, soluble GL-2045, or soluble IVIG at specified concentrations ranging from 0.001 to 1000 μg/ml. For time-course assays, NK cells were stimulated with either media only or soluble GL-2045 at the indicated time points. After stimulation, NK cells were collected and the expression of Tim-3 and CD137 were assessed by flow cytometry.

### 2.8. Cytokine stimulation assays

NK cells were cultured overnight with media only, soluble G001, soluble GL-2045, or soluble IVIG. In some wells, NK cells were also cultured with recombinant human IL-2 (Chiron, USA), recombinant human IL-15 (eBioscience, USA), recombinant human IL-12 p70 (Biolegend, USA), recombinant human IL-18/IL-1F4 (R&D Systems, USA), recombinant human IL-4 (R&D Systems, USA), recombinant human IL-6 (Biolegend, USA), or recombinant human IL-10 (Biolegend, USA). The following day, NK cells were collected and the expression of Tim-3 and CD137 were evaluated by flow cytometry.

### 2.9. CD107a degranulation and IFN-γ production assays

NK cells were stimulated with either media only or soluble GL-2045 in the presence or absence of IL-12 plus IL-18, as indicated. After overnight culture, K562 cells were added to the culture at an effector to target ratio (E:T) of 3:1 or 10:1 in the presence of brefeldin and anti-CD107a FITC (Biolegend). After a 4-hour incubation, NK cells were collected and stained for Tim-3-PE expression. For intracellular staining, NK cells were fixed and permeabilized and stained for IFN-γ-APC prior to FACS analysis.

### 2.10. Statistical analysis

Data are presented as mean ± SEM from at least three experiments. Paired *t*-test, one way or two way ANOVA were performed using Graphpad software. All hypothesis were tested with a two-sided analysis. Ordinary one-way ANOVA was carried out to compare the effects of different treatments versus controls. Two way ANOVA was used to compare expression of Tim-3 and CD137 on NK cells when treated with combination of GL-2045 and various cytokines. Tukey and Dunnett adjustment procedures were used to control type I errors in the presence of multiple comparisons. To test for a synergy between two treatments on the expression of Tim-3 and CD137, we calculated the log fold-change in mean fluorescence intensity (MFI) by taking the logarithm (base 2) of the ratio of the MFIs with and without GL-2045 in the presence of a specific cytokine compared with the same quantity without any cytokine present. The logarithmic transform was used to obtain an approximately normally distributed random variable. These log fold changes were calculated within each donor and compared using the paired *t*-test to test for a possible interaction or synergy. The difference estimated in log fold change was transformed back to a relative change in the MFI ratios. Differences were considered statistically significant when $p < 0.05$.

## 3. Results

### 3.1. Antibody stimulation induces Tim-3 expression on human NK cells

We sought to test the hypothesis that aggregated Fc fragments induce molecules on the NK cell surface that could be targeted to enhance NK cell function. As a first step, we performed whole-genome transcriptome analysis of human NK cells stimulated with coated human IgG1 for either 4 or 24 h. The transcriptional profile of NK cells clustered separately depending on type of stimulation (Supplemental Fig. 1A and B). Interestingly, although no significant changes were observed at four hours after immobilized IgG1 stimulation, 63 transcripts were significantly changed at 24 h after stimulation (Supplemental Table 1). Among them, 24 transcripts were involved in the cell death pathway and nine transcripts were related to the cytotoxicity pathway (e.g. HAVCR2, CCL5, CD244, CD300A, CD48, ITGAM, KLRF1, ADGRG1) (Supplementary Fig. 2A and B). HAVCR2 demonstrated a 1.98-fold upregulation in NK cells exposed to immobilized IgG1. Comparative real-time quantitative PCR confirmed a 2.15-fold-change ($p < 0.05$) in HAVCR2 on NK cells in the presence of immobilized IVIG compared to NK cells cultured in media only. Interestingly, there was no significant change in HAVCR2 gene expression on NK cells stimulated with soluble IVIG (Supplemental Fig. 2C). These data suggest that antibody aggregates induce HAVCR2 expression on NK cells.

HAVCR2 encodes for the Tim-3 protein — an activation induced receptor expressed on a variety of immune cells including, but not limited to, terminally differentiated T cells, antigen presenting cells and NK cells (Monney et al., 2002; Ju et al., 2010; Chiba et al., 2012; Huang et al., 2015; Kuchroo et al., 2008). Because Tim-3 expression on T cells is associated with peripheral tolerance (Sánchez-Fueyo et al., 2003), we postulated that blocking Tim-3 on NK cells might improve effector function. As a first step in this process, we sought to determine if the upregulation of HAVCR2 was associated with Tim-3 *protein* expression on NK cells following ADCC. Consistent with our hypothesis and prior data, Tim-3 and CD137 were upregulated on the surface of NK cells in PBMCs cultured with the anti-CD20 mAb, rituximab, but not soluble IVIG (Fig. 1A) (Lin et al., 2008; Kohrt et al., 2012). This up-regulation of Tim-3 was strictly dependent on receptor crosslinking as Tim-3 and CD137 upregulation occurred only after exposure to immobilized, but not soluble, IVIG (Fig. 1B). Collectively, these studies confirm that Tim-3 and CD137 are induced by exposure to antibodies capable of FcR crosslinking.

### 3.2. IVIG mediated expression of Tim-3 on human NK cells is contact-dependent

We next sought to investigate whether antibody mediated upregulation of Tim-3 was dependent on direct Fc: FcR interactions. We performed a transwell assay in which antibodies were coated in the lower well – allowing direct NK cell stimulation – while NK cells in the upper well were only exposed to conditioned media. Although a basal level of Tim-3 was present on unstimulated NK cells, significant upregulation of Tim-3 (BOTTOM: 25.5% ± 3.02%, TOP: 14.1% ± 3.42%) and CD137 (BOTTOM: 41.7% ± 2.26%, TOP: 1.70% ± 0.562%) were observed only on NK cells directly in contact with immobilized antibody (Fig. 1C and D). These data demonstrate that antibody-mediated upregulation of Tim-3 and CD137 on NK cells is contact dependent.

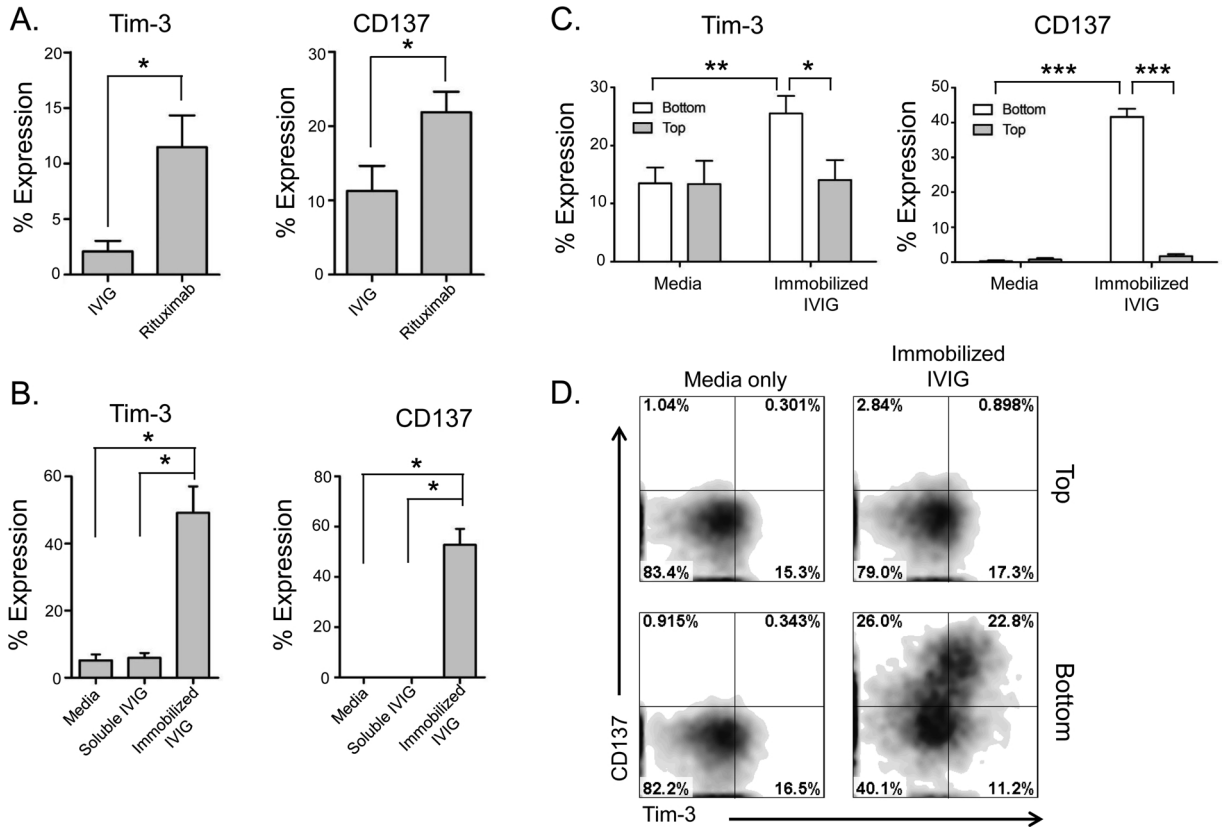



**Fig. 1.** Immobilized IVIG drives Tim-3 expression on human NK cells in a contact- dependent manner.
**(A)** PBMCs were cultured overnight in media containing Rituximab (anti-CD20; 10 μg/ml) or IVIG (100 μg/ml) and expression of Tim-3 and CD137 on NK cells were determined by FACS. **(B)** NK cells were cultured overnight with media only, soluble IVIG (10 μg/ml), or immobilized IVIG and expression of Tim-3 and CD137 were determined by FACS. **(C)** NK cells were cultured in transwells (0.4 μm pore size) overnight with complete media ± immobilized IVIG. Expression of Tim-3 and CD137 were determined by FACS. **(D)** Representative two-parameter density plots depict expression of Tim-3 and CD137 on NK cells cultured with media only or immobilized IVIG from the top or bottom compartments separated by transwells. The results are shown as the mean ± SEM from (A) five samples, (B) three samples, and (C) seven samples. $*p < 0.05$, $**p < 0.01$ or $***p < 0.001$.

### 3.3. Tim-3 upregulation occurs in a Fc:FcγR-dependent manner

The interactions of the Fc portion of IgG1 with CD16 on human NK cells mediates effector functions, such as ADCC, and triggers the release of immunomodulatory cytokines (Taylor et al., 2008). In order to understand the degree to which upregulation of Tim-3 was dependent on Fc:FcR interactions, we stimulated isolated human NK cells with soluble recombinant IgG1 Fc *homodimers* (G001), soluble recombinant IgG1 Fc *multimers* (GL-2045), or soluble IVIG (Sun et al., 2017; Zhou et al., 2017; Zhang et al., 2019). Exposure of NK cells to soluble GL-2045 at 10 μg/ml induced marked upregulation of Tim-3 and CD137, while identical concentrations of G001 or IVIG failed to mediate similar responses (Fig. 2A and B). While higher concentrations of G001 and IVIG could induce upregulation of Tim-3 and CD137, GL-2045 was significantly more potent (Fig. 2C and D). Upregulation of Tim-3 was associated with downregulation of CD16 and upregulation of CRTAM without reductions in the expression of CD56 (Supplemental Fig. 3A–C) (Qureshi et al., 2017). Collectively, these data demonstrate that multimeric Fc-dependent crosslinking of CD16 on NK cells induces Tim-3 upregulation.

### 3.4. Fc multimers induce distinct temporal patterns of Tim-3 and CD137

While Fc engagement of CD16 on NK cells induced both Tim-3 and CD137, their patterns of expression were overlapping and unique (Fig. 1D). In order to understand whether these distinct patterns of expression were temporal in nature, we analyzed the expression of Tim-3 and CD137 at various time points after stimulation. While Tim-3 (20 min; $p = 0.027$) upregulation occurred almost immediately following GL-2045 exposure, maximal upregulation of CD137 (four hours) was delayed (Fig. 3A). Furthermore, the patterns of expression of Tim-3 and CD137 changed over time, with a large population of cells expressing high levels of Tim-3 and lacking CD137 soon after stimulation, and an increased number of CD137 + Tim-3 + and CD137 + Tim-3- cells present at later time points (Fig. 3B).

### 3.5. NK cells upregulate Tim-3 by a variety of stimuli

Previous studies have shown that IL-2, IL-15, IL-12 and IL-18 induce NK cell expression of Tim-3 (Ndhlovu et al., 2012; Gleason et al., 2012). In order to understand how these cytokines influenced FcR mediated expression of Tim-3, we screened a panel of cytokines for their ability to induce Tim-3, alone and in combination with GL-2045. These data revealed that GL-2045 mediated induction of Tim-3 is significantly enhanced by exposure to IL-15 and IL-12 (Fig. 4A). Furthermore, combination of GL-2045 with select cytokines (IL-2, IL-15, IL12, IL-18, IL-6, IL-10) stimulation further increased Tim-3 expression when compared with individual cytokines alone. However, none of the cytokines interacted synergistically with GL-2045 ($p > 0.2$) with the exception of IL-10 ($p = 0.057$). In this case, the fold-change in Tim-3 induced by GL-2045 was 1.60 times that seen without the cytokine, with 95% confidence limits 0.96 and 2.65. In all the other cases, the estimated effect of adding the cytokine was less than a 30% change in the fold-change induced by GL-2045 (Fig. 4A). Moreover, while GL-2045 in combination with either IL-2, IL-15, or IL-18 demonstrated enhanced CD137 expression compared with cytokine stimulation alone, none of the

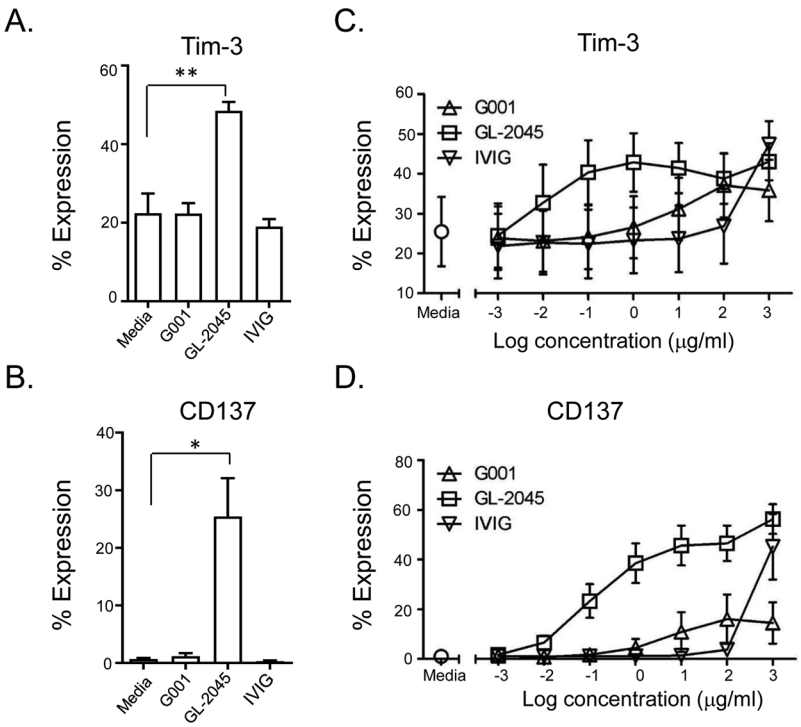



**Fig. 2.** Fc:FcγR engagement induces Tim-3 upregulation on NK cells.
**(A, B)** Isolated NK cells were cultured overnight in media only, soluble G001 (Fc homodimers; 10 μg/ml), soluble GL-2045 (Fc multimers; 10 μg/ml), or soluble IVIG (10 μg/ml) and expression of Tim-3 (A) and CD137 (B) were quantified by FACS. **(C, D)** NK cells were cultured overnight in the presence of media only, soluble G001, soluble GL-2045, or soluble IVIG with concentrations ranging from 0.001 μg/ml to 1000 μg/ml and expression of Tim-3 and CD137 were determined by FACS. The results are shown as the mean ± SEM from (A) three samples, (B) five samples, (C, D) four samples. *$p < 0.05$, **$p < 0.01$.

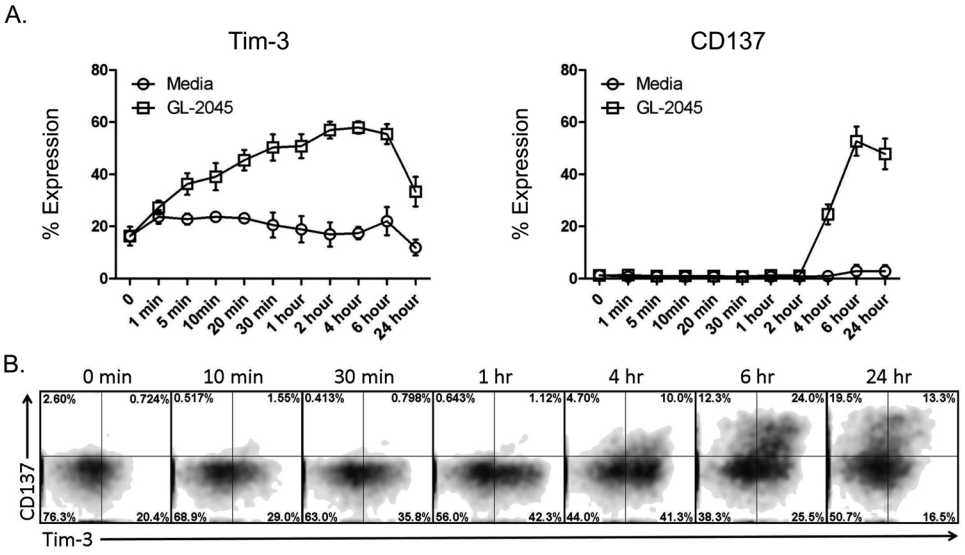



**Fig. 3.** Fc multimer induces distinct temporal patterns of Tim-3 and CD137 expression.
**(A)** NK cells were cultured with media only or soluble GL-2045 (10 μg/ml) for specific time intervals and expression of Tim-3 and CD137 were determined by FACS. **(B)** Representative two- parameter density plots depicting the temporal upregulation of Tim-3 and CD137 expression after GL-2045 stimulation. The results are shown as the mean ± SEM from four samples. *$p < 0.05$.

cytokines in the panel had a significant effect on CD137 induction by GL-2045 ($p > 0.2$) (Fig. 4B).

We also hypothesized that Tim-3 expression may be regulated by other activating stimuli, e.g. MHC null tumor cells. NK cells co-cultured with K562 tumor cells, an MHC-null cell line that induces natural cytotoxicity, upregulated both Tim-3 and CD137 (Fig. 4C and D). These data suggest that Tim-3 upregulation is the common end-result of NK cell activation by a variety of unique and overlapping stimuli, raising the possibility that Tim-3 expression alone does not define a specific functional class of cells.

### 3.6. The function of Tim-3 expressing NK cells is dependent upon the initial activation stimuli

In order to directly test the function of Tim-3 on human NK cells, we used a modification of published approaches, testing how crosslinking and/or blockade of Tim-3 influences NK cell function. Tim-3 crosslinking failed to inhibit target lysis of Fc receptor-bearing P815 targets cells in redirected ADCC assays (Data not shown) (Ndhlovu et al., 2012). Furthermore, Tim-3 blockade did not enhance natural cytotoxicity responses against K562 target cells (Data not shown).

Based on our observations that a variety of stimuli induce Tim-3, the information that Tim-3 is expressed over a prolonged time-frame, and the fact that Tim-3 expression does not temporally correlate with that of other functional markers e.g. CD137, we hypothesized that the functional relevance of Tim-3 expression on NK cells might depend on the initial activation stimuli. In order to test this hypothesis, we incubated NK cells with three different types of stimuli, i.e. GL-2045, IL-12/IL-18, K562, alone or in combination. The function of NK cells was measured by flow cytometric analysis of IFN-γ production and CD107a expression. As shown in Fig. 4, all of these stimuli upregulated Tim-3 expression on NK cells. However, the ability of Tim-3 expressing NK cells to produce IFN-γ varied largely depending on the type of stimulation (Fig. 5). Specifically, NK cells stimulated with GL-2045 alone failed to

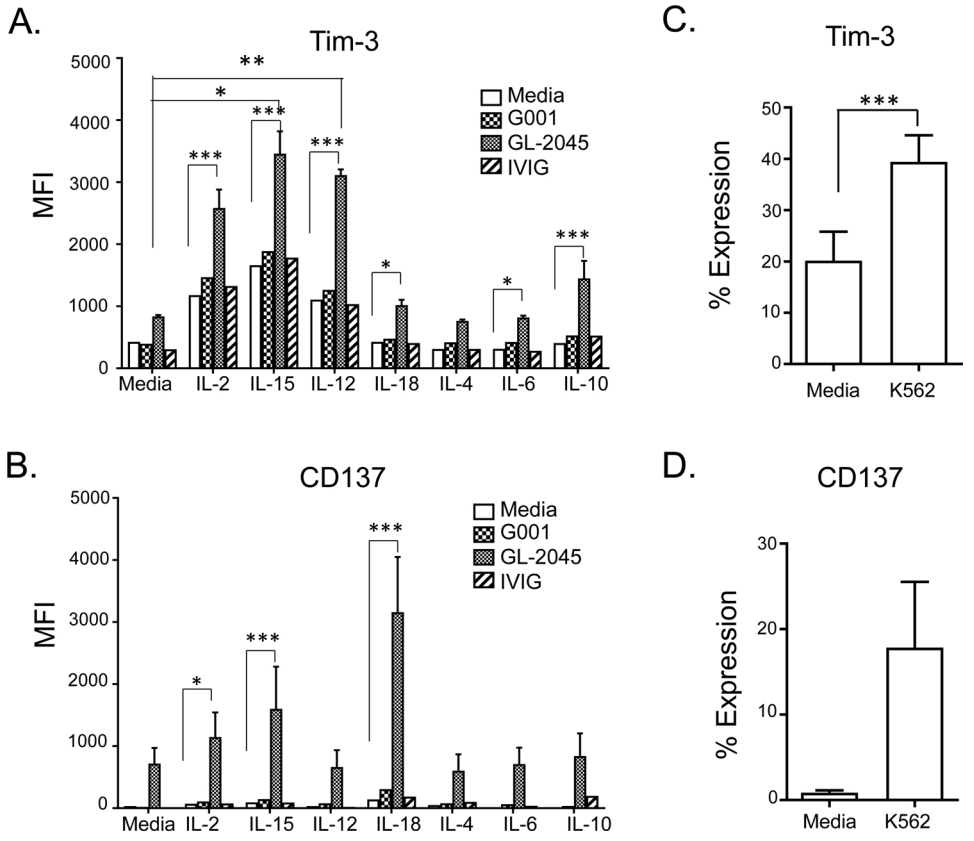



**Fig. 4.** NK cells upregulate Tim-3 by a variety of stimuli.
**(A, B)** Isolated NK cells were cultured overnight with IL-2 (500 IU/ml), IL-15 (100 ng/ml), IL-12 (50 ng/ml), IL-18 (50 ng/ml), IL-4 (50 ng/ml), IL-6 (50 ng/ml), or IL-10 (50 ng/ml) in the presence or absence of soluble G001 (10 μg/ml), soluble GL-2045 (10 μg/ml), or soluble IVIG (10 μg/ml) and the expression of Tim-3 and CD137 were determined by FACS. **(C, D)** NK cells were cultured with K562 tumor cells at a 10:1 E:T ratio for four hours and the expression of Tim-3 and CD137 were measured via FACS. The results are shown as the mean ± SEM from (A, B) three samples, (C) five samples (D) three samples. *$p < 0.05$, ***$p < 0.001$.

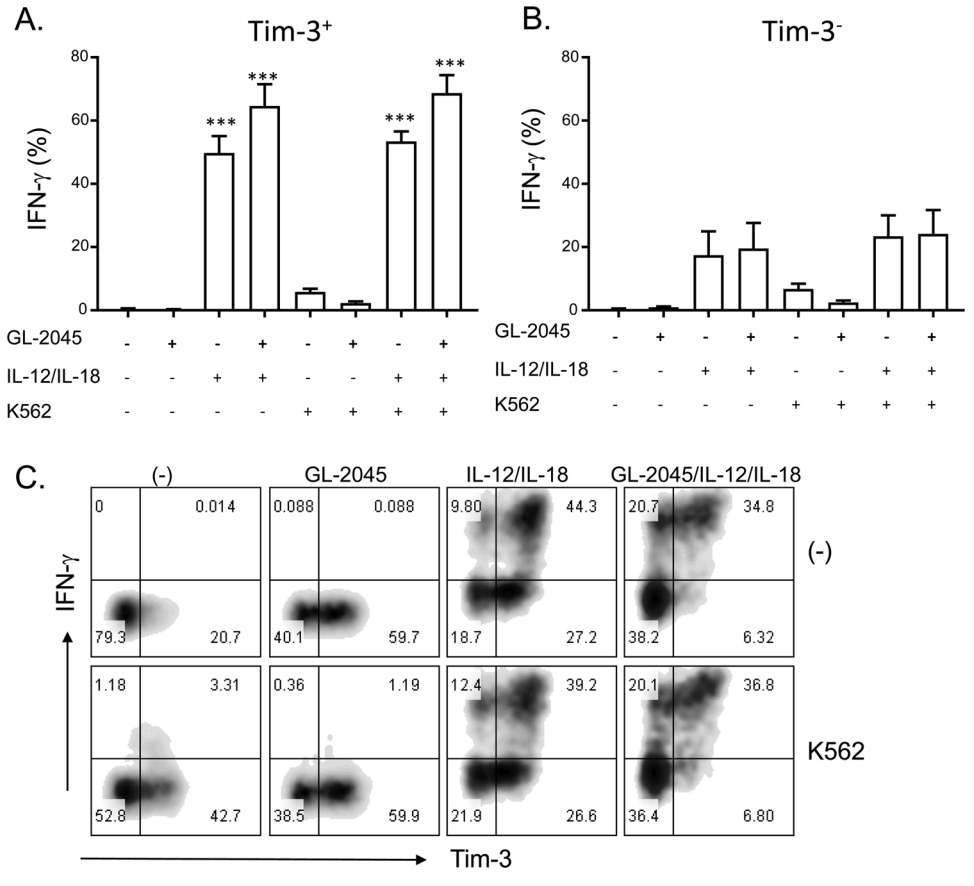
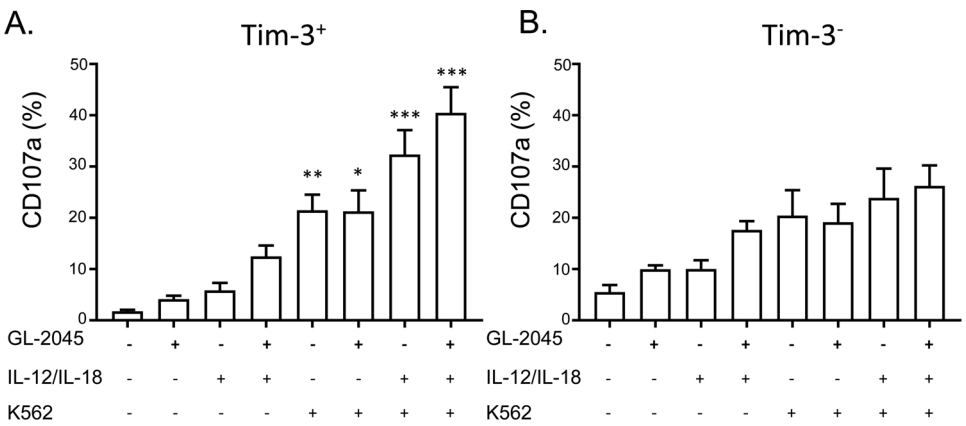



**Fig. 5.** Levels of IFN-γ expression on Tim-3$^+$ NK cells varies among the different stimuli.
NK cells were cultured with media only, GL-2045 (10 μg/ml), IL-12 (1 ng/ml)/IL-18 (10 ng/ml), or GL-2045/IL-12/IL-18 for overnight. K562 cells were added into selected conditions for an additional four hours and the percentage of Tim-3$^+$ (A) and Tim-3$^-$ (B) NK cells expressing IFN-γ was determined by FACS. **(C)** Representative two-parameter density plots depict expression of Tim-3 with IFN-γ under various conditions. The results are shown as the mean ± SEM from five samples. ***$p < 0.001$, compared with individual condition in the absence of IL-12/IL-18.



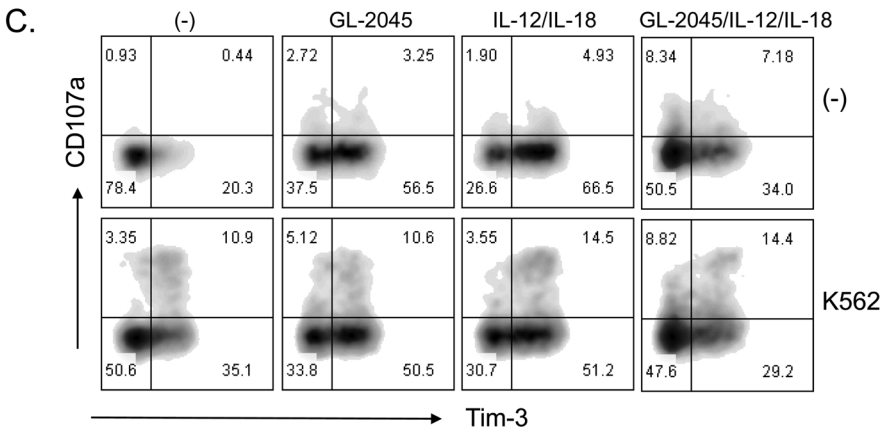




**Fig. 6.** Levels of CD107a expression on Tim-3$^+$ NK cells varies among the different stimuli. NK cells were cultured with media only, GL-2045 (10 μg/ml), IL-12 (1 ng/ml)/IL-18 (10 ng/ml), or GL-2045/IL-12/IL-18 for overnight. K562 cells were added into selected conditions for an additional four hours and the percentage of Tim-3$^+$ (A) and Tim-3$^-$ (B) NK cells expressing CD107a was determined by FACS. **(C)** Representative two-parameter density plots depict expression of Tim-3 with CD107a under various conditions. The results are shown as the mean ± SEM from five samples. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with individual condition in the absence of K562.

produce detectable levels of IFN-γ. IL-12/IL-18 significantly promoted IFN-γ production by Tim-3$^+$ NK cells, independent of GL-2045 or K562. While Tim-3$^-$ NK cells stimulated with IL-12/IL-18 demonstrated increased IFN-γ production, these changes were not significant.

Interestingly, CD107a, a marker of degranulation, and IFN-γ demonstrated distinct patterns of expression following NK cell exposure to unique stimuli. Specifically, K562 alone mediated strong induction of CD107a on Tim-3$^+$ NK cells, while IL-12/IL-18 alone showed no significant effect. Compared to the individual conditions in the absence of K562, the addition of K562 cells significantly enhanced CD107a expression on Tim-3$^+$ NK cells, but not on Tim-3$^-$ NK cells (Fig. 6). Collectively, these data suggest that it is the *manner* in which Tim-3 is induced on NK cells and not the expression of this molecule that influences NK cell function.

## 4. Discussion

The goal of our study was to define co-signaling molecules that are induced on the surface of NK cells by FcR crosslinking that could be targeted for manipulation to enhance NK cell function. We now demonstrate that Tim-3 is induced on NK cells by FcR engagement, as well as by other diverse activating stimuli. Moreover, we show that FcR mediated induction of Tim-3 on NK cells is temporally distinct from that of CD137.

Our initial decision to study NK cell expression of Tim-3 was based on our observation that immobilized IgG1 induced the upregulation of HAVCR2 mRNA, which encodes the Tim-3 protein. However, the fact that Tim-3 expression was upregulated twenty minutes after exposure to GL-2045, strongly suggests that early induction resulted from the mobilization of existing intracellular Tim-3 stores, rather than by *de novo* synthesis.

Following exposure to GL-2045, Tim-3 and CD137 displayed different temporal patterns of expression and were induced by distinct cytokines that depend on differing signaling pathways. Specifically, the IL-2/IL-12/IL-15 cytokines, capable of inducing Tim-3, depend on JAK-STAT signaling (Liu et al., 1998), while IL-18, a cytokine belonging to the IL-1 superfamily mediates its effects primarily through NFκB (Boraschi et al., 2018). These data suggest that while FcR stimulation induces both Tim-3 and CD137 on the NK cell surface, the expression of these molecules in vivo will vary depending on the cytokine milieu. Furthermore, these findings are consistent with the function of other co-signaling molecules whose functions depend on the biologic setting in which they are expressed (Dong et al., 1999, 2003).

Published studies using antibodies to define the function of Tim-3 on NK cells have shown that Tim-3 is both a negative regulator of NK cell mediated cytotoxicity (Ndhlovu et al., 2012) and a positive mediator of IFN-γ production (Gleason et al., 2012). In our study, anti-Tim-3 antibodies, in conditions conducive to either blocking or crosslinking, failed to define differences in NK cell function. We postulate that the differences between our findings and prior reports are largely attributable to our supposition that Tim-3 is expressed on functionally diverse NK cell populations. However, it is also possible, and indeed likely, that differences in the sources of NK cells, NK cell selection protocols, culture criteria and the limited availability of functionally well-defined anti-Tim-3 antibodies contributed to our observations.

In order to understand the functional relevance of Tim-3 expression on NK cells, we evaluated the ability of NK cells to produce IFN-γ and CD107a in response to various stimuli which can induce Tim-3 expression. Interestingly, Tim-3$^+$ NK cells demonstrated distinct patterns of IFN-γ and CD107a expression that were dependent on the type of initial stimulation. Consistent with previous reports (Mavropoulos et al., 2005), our data show that IL-12/IL-18 stimulates IFN-γ production by Tim-3$^+$ NK cells. In contrast, the expression of CD107a by Tim-3$^+$ NK cells was most pronounced following exposure to K562 target

cells. Despite the ability of GL-2045 to induce both Tim-3 and CD137, these cells produced no IFN-γ, and only low level of CD107a. These findings suggest that the function of Tim-3$^+$ NK cells depends on the stimuli employed to induce activation.

In summary, Tim-3 upregulation is the common end-result of NK cell activation by a variety of unique and overlapping stimuli. As such, Tim-3 + NK cells are functionally diverse – diversity that might explain some of the varying functional data attributed to Tim-3 ligation on the NK cell surface. These findings mandate caution when using Tim-3 as an independent marker for NK cell exhaustion and suggest that Tim-3 blockade will likely have diverse effects on NK cell activity in vivo.

## Authors contribution

E.C.S., X.Z., and S.E.S contributed to the experimental design, data interpretation, and manuscript preparation. E.C.S. performed a majority of the research. Y.J. and J.A. performed CD107a and IFN-γ assays. A.K., E.B., H.Z., H.S., and S.C. procured samples and helped collect data. R.H. and Y.S. were responsible for the microarray and gene expression analysis. S.B. provided statistical analysis. X.Z. and S.E.S. revised the manuscript. All authors had input into the final version of the manuscript.

## Conflict-of-interest disclosure

Dr. Strome is a Cofounder, consultant and stockholder in Gliknik Inc., a biotechnology company. He receives royalties for intellectual property, related to B7-H1 (PD-L1), licensed by the Mayo Clinic College of Medicine to third parties. He receives research support from Pfizer and Gliknik through sponsored research agreements through the University of Maryland, Baltimore. He serves on the scientific advisory board of Virion Inc. and has also served on Advisory Boards to Astra Zeneca and Genentech.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.imbio.2019.03.001.

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