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# The interaction between CD300a and phosphatidylserine inhibits tumor cell killing by NK cells

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**The activity of NK cells is controlled by inhibitory and activating receptors. The inhibitory receptors interact mostly with MHC class I proteins, however, inhibitory receptors such as CD300a, which bind to non-MHC class I ligands, also exist. Recently, it was discovered that phosphatidylserine (PS) is a ligand for CD300a and that the interaction between PS expressed on apoptotic cells and CD300a inhibits the uptake of apoptotic cells by phagocytic cells. Whether PS can inhibit NK-cell activity through CD300a is unknown. Here, we have generated specific antibodies directed against CD300a and we used these mAbs to demonstrate that various NK-cell clones express different levels of CD300a. We further demonstrated that both CD300a and its highly homologous molecule CD300c bind to the tumor cells equally well and that they recognize PS and additional unknown ligand(s) expressed by tumor cells. Finally, we showed that blocking the PS–CD300a interaction resulted in increased NK-cell killing of tumor cells. Collectively, we demonstrate a new tumor immune evasion mechanism that is mediated through the interaction between PS and CD300a and we suggest that CD300c, similarly to CD300a, also interacts with PS.**

**Keywords:** CD300 · Ligand · Phosphtidylserine · Tumor cell

## Introduction

Natural killer (NK) cells represent the third (following B and T cells) largest lymphoid cell population in mammals [1]. The function of NK cells occurs naturally and unlike T or B cells, NK cells do not require sensitization for their activity, although recent reports demonstrate that NK cells possess a certain type of memory [2–5]. NK cells are characterized by the expression of activating and inhibitory receptors that mediate their function [6]. The inhibitory receptors recognize mainly MHC class I proteins [7, 8], however, inhibitory receptors that interact with proteins other than MHC class I, such as CD300a, also exist [9]. The CD300a molecule contains four immune receptor tyrosine based inhibitory motif (ITIM) sequences in its cytoplasmic domain. It possesses a single V-like Ig domain that is 80% similar at the amino acid level to another family member, CD300c. However, unlike CD300a, CD300c contains a short cytoplasmic domain that lacks ITIM sequences and also includes a glutamic acid residue in its transmembrane domain, suggesting an association with as yet an undefined signaling molecule [10–13]. Because of the high similarity between the extracellular portion of CD300a and CD300c, none of the commercially available antibodies that are directed against these proteins can discriminated between them [14, 15].

Until recently, the ligand(s) recognized by CD300a were unknown, however, Nakahashi-Oda et al. [16] and Simhadri et al. [17] recently reported that phosphatidylserine (PS) is a ligand for CD300a. PS is a phospholipid that is ubiquitously present in membranes; it is normally asymmetrically distributed in the plasma membrane of mammalian cells so that essentially all of the PS is restricted to the cytosolic surface [18]. During several important biological processes, this asymmetry collapses and PS

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is exposed on the cell surface. For example, PS becomes externalized on the cell surface during activation of platelets, during the blood coagulation cascade [19, 20], and during the early stages of apoptosis [18, 21, 22]. The externalization of PS appears to be the signal by which apoptotic cells are recognized and subsequently removed by phagocytes [23–25]. The recognition of PS by a phagocyte cell occurs through several different mechanisms: via direct recognition by members of the TIM family of receptors (TIM-1, TIM-3, and TIM-4) [26–29], BAI1 [30], and Stabilin-2 [31], and via indirect recognition by soluble PS-binding molecules including MFG-E8 [32], Gas6 [33], and protein S [34]. Several studies have shown that in the tumor microenvironment, there is a significant stress imposed on the tumor endothelium by acidity, reactive oxygen species (ROS), and by transient hypoxia, which results in the redistribution of PS to the cell surface [35, 36]. Indeed, expression of PS was detected in gastric carcinoma [37], ovarian carcinoma [38], and melanoma [39].

Here, we identified a new tumor immune evasion mechanism that is based on the inhibition of NK-cell activity through the CD300a–PS interaction.

## Results

### Specific recognition of CD300a by newly generated mAbs

Currently, there is no mAb that is able to discriminate between CD300a and CD300c (data not shown and [14, 15]). Therefore, to study the function of CD300a and CD300c, we generated specific anti-CD300a and CD300c antibodies. Mice were immunized with fusion proteins that include the extracellular portions of CD300a and CD300c proteins fused to human IgG1 and hybridomas were generated according to the standard techniques.

To test the mAb specificity, we stained YTS cells transfected to express CD300a, BW cells transfected to express CD300c, and the corresponding parental cell lines (that are negative for CD300a and CD300c, Fig. 1A) with three hybridomas (for an unknown reason, we could not obtained transfectants of YTS cells expressing CD300c or tranfectants of BW cells expressing CD300a and, therefore, we screened for the expression of CD300a and CD300c on two different cell lines). As can be seen in Figure 1A, Hybridoma 1 and Hybridoma 4 specifically recognize CD300a and not CD300c, while Hybridoma 2 specifically recognizes CD300c.

We next stained primary bulk NK-cell cultures derived from various donors with the different antibodies and as can be seen in Figure 1B, while all bulk NK cells were stained with the CD300a-specific mAbs, Hybridoma 1 and Hybridoma 4, no staining was detected with Hybridoma 2 (Fig. 1B and data not shown). We have therefore continued our research with the CD300a-specific Hybridomas 1 and 4.

### Anti-CD300a antibodies inhibit NK-cell killing in redirected cytotoxicity assays

To test whether the triggering of CD300a by Hybridomas 1 and 4 will inhibit NK-cell cytotoxicity, we performed redirected killing

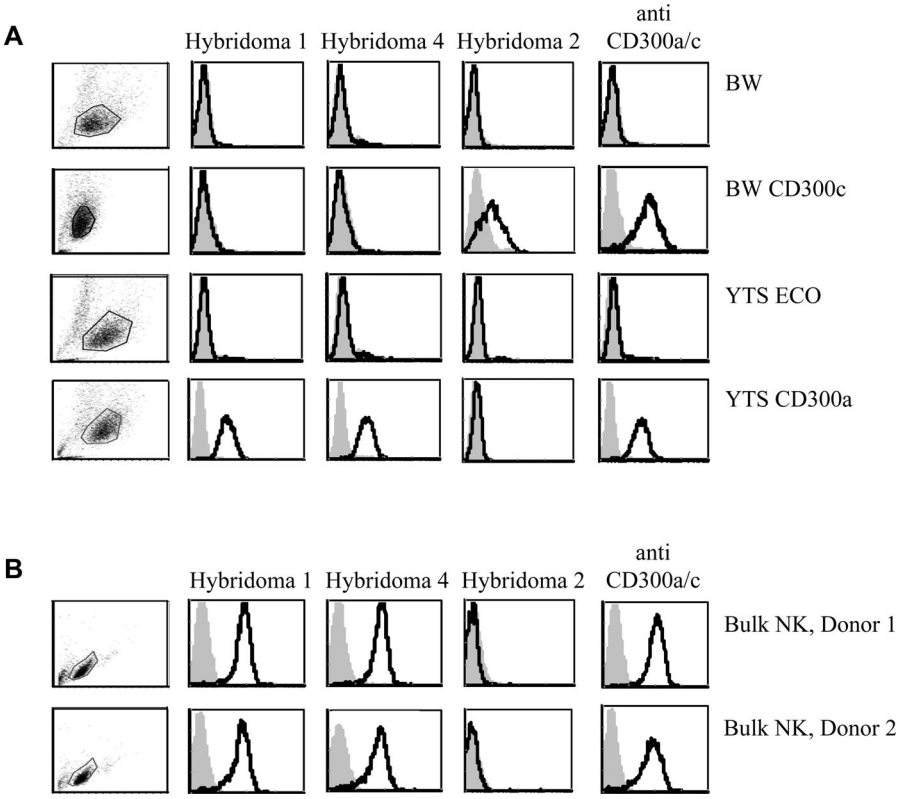



**Figure 1.** Specific recognition of CD300a by newly generated hybridomas. (A, B) FACS analysis of (A) BW cells, BW expressing CD300c, YTS ECO cells, and YTS ECO expressing CD300a cells, and of (B) bulk NK-cell cultures derived from two different donors. The various hybridomas are indicated above the histograms. The anti-CD300a/c antibody recognizes both CD300a and CD300c. Staining is indicated by black-line histogram and background (filled gray histogram) is the staining with allophycocyanin-conjugated F(ab')2 goat anti-mouse IgG only. The gating strategy is shown in the left part of the figure. Data shown are representative of one out of three independent experiments performed.

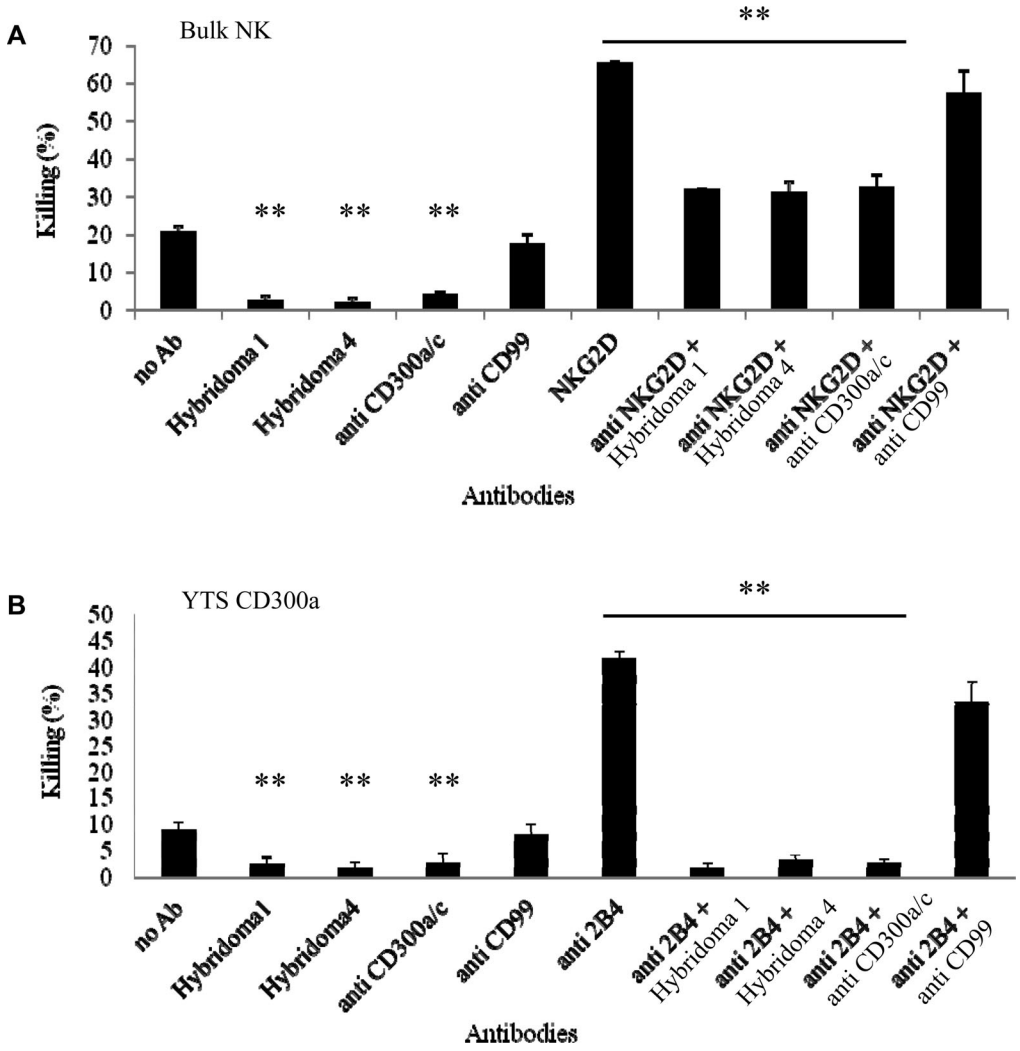



**Figure 2.** The new CD300a hybridomas inhibit redirected NK cytotoxicity. (A) Bulk NK cells and (B) YTS CD300a cells were incubated with P815 cells that were precoated with the various antibodies (indicated in the x axis). The E:T ratio was 3:1 for the bulk NK and 20:1 for the YTS CD300a cell line. Data are shown as means percentage and ±SD of three replicates and are representative of one out of three independent experiments performed. $^{**}p \leq 0.02$, *t*-test.

assays (Fig. 2). Primary bulk NK cells (Fig. 2A) or YTS CD300a cells (Fig. 2B) were incubated with P815 cells that were precoated with and without anti-NKG2D mAb (Fig. 2A) and anti-2B4 (Fig. 2B), in the presence or absence of the Hybridoma 1, Hybridoma 4, Hybridoma that recognizes both CD300a and CD300c (anti-CD300a/c, CMRF02.6 [15]), and anti-CD99 mAb (which was used as a negative control). Basal killing of P815 cells was observed with both the primary bulk NK cultures and the YTS CD300a cells (the identity of the killer receptor(s) involved in the killing of P815 cells by NK cells or by YTS is unknown), and this basal killing was inhibited by all anti-CD300a antibodies (Fig. 2); the two specific ones, Hybridomas 1 and 4, and the anti-CD300a/c mAb. Furthermore, the NKG2D-mediated redirected killing of the bulk NK-cell cultures was inhibited by all anti-CD300a and anti-CD300a/c antibodies (Fig. 2A). Similar results were obtained when the redirected killing of the bulk NK-cell cultures was induced by other antibodies such as anti-CD16 and anti-2B4 (data not shown).

The killing of the YTS cell line is executed mainly by the 2B4 receptor [40] and as can be seen in Figure 2B, the 2B4-mediated redirected killing of YTS CD300a cells was also inhibited by all antibodies apart from the control anti-CD99 mAb.

## Human NK-cell clones express various levels of CD300a

To study the clonal distribution of CD300a, we grew NK clones derived from four different donors at various time points over a period of 1 year (between 25 and 48 NK-cell clones were obtained from each donor, Fig. 3A). The different NK clones expressed CD300a at various levels and we thus divided them into

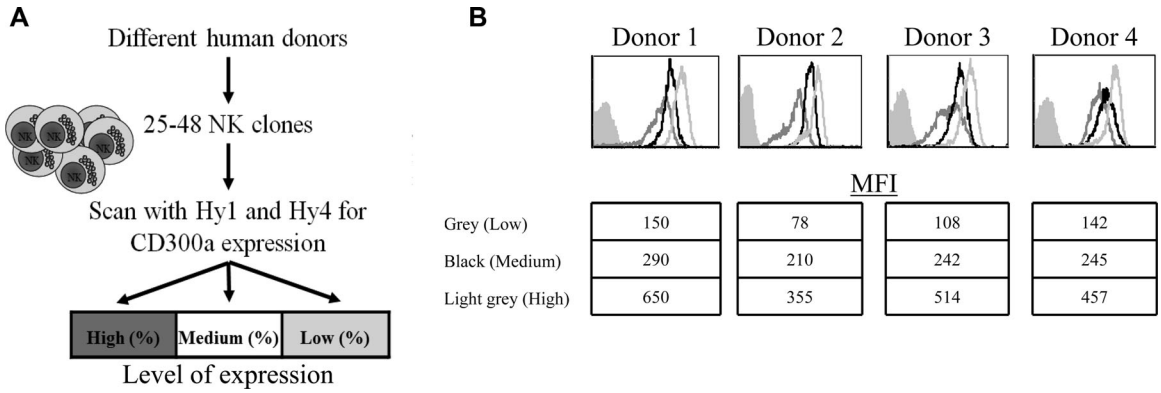



**Figure 3.** CD300a exhibits clonal pattern of expression on different NK-cell clones. (A) Schematic representation of the experimental procedure. (B) Representative staining of the low (dark gray line histogram), medium (black line histogram), and high (light gray line histogram) clones from each donor. Background (filled gray histogram) is the staining with allophycocyanin-conjugated F(ab')2 goat anti-mouse IgG only.

**Table 1.** Three levels of expression of CD300a on NK cells[a)]

| Level of expression | High (%) | Medium (%) | Low (%) |
|---|---|---|---|
| Donor 1 | 36.67 | 10 | 53.33 |
| Donor 1 | 10.42 | 52.08 | 37.5 |
| Donor 1 | 44.44 | 48.15 | 7.41 |
| Donor 1 | 85.71 | 10.71 | 3.57 |
| Donor 2 | 6.98 | 34.88 | 58.14 |
| Donor 2 | 22.22 | 66.67 | 11.11 |
| Donor 3 | 54.17 | 31.25 | 14.58 |
| Donor 3 | 19.05 | 57.14 | 23.81 |
| Donor 4 | 13.04 | 58.7 | 28.26 |
| Donor 4 | 18.18 | 72.73 | 9.09 |

[a)] Summary of the percentages of clones expressing CD300a derived from four different donors at various time points during a 1-year period. NK-cell clones were divided into three groups based on the levels of CD300a expression.

three groups based on the levels of CD300a expression (Fig. 3A). NK-cell clones expressing CD300a at levels below MFI of 200 were considered low expressing, clones expressing CD300a in MFI of 200–300 were considered medium, and clones expressing CD300a in MFI higher than 300 were considered high. The percentages of NK-cell clones in each group are indicated in Table 1 and examples of the expression of CD300a on the various clones are shown in Figure 3B.

As can be seen in Table 1, the expression of CD300a varies significantly among different donors. For example, 13.04 and 18.18% of the NK-cell clones of donor 4 (NK cells from this donor were isolated at two time points during the 1-year period of this study) expressed CD300a at high levels, while at same time point 54.17% of NK cells derived from donor 3 and 85% of NK cells derived from donor 1 expressed CD300a at high levels.

Furthermore, even within the same donor the expression levels of CD300a vary significantly, depending on the time in which they were isolated. For example, at some point, 85.71% of donor 1 NK cells expressed CD300a at high levels while in other time point only 10.42% of the NK-cell clones of donor 1 expressed CD300a at high levels (Table 1).

## Tumor cells express a trypsin-sensitive ligand for CD300

A single nucleotide polymorphism in CD300a that encodes for a nonsynonymous mutation in the IgV-like domain (the corresponding amino acids are either argenin (R) or glutamine (Q)) has been linked to susceptibility to psoriasis [17, 41]. To test whether CD300a variants having either R94 or Q94 in CD300a will be differentially recognized by Hybridomas 1 and 4, we constructed fusion proteins in which the extracellular portion of CD300a containing either R (CD300a R94 Ig) or Q (CD300a Q94 Ig) was fused to human IgG1. These fusion proteins were used in ELISA assays for recognition by the different anti-CD300a Hybridomas. As can be seen in Figure 4A, all mAbs tested, hybridomas 1 and 4, and the anti-CD300a/c antibody, recognized the CD300a R94 Ig and CD300a Q94 Ig proteins equally well. CD300c Ig was recognized by the anti-CD300a/c mAb only, whereas the control D1 Ig fusion protein was not recognized by any of the mAb tested (Fig. 4A).

It was shown recently that PS is a ligand for CD300a [16]. To test whether tumor cells such as RKO, 293T, and 8866 cells, which serves as targets for NK cells, express a ligand for CD300a and CD300c, we stained them with the two CD300a fusion proteins (containing either R94 or Q94), with CD300c Ig, and with the control D1 Ig. The adherent cells RKO and 293T were harvested with and without trypsin. As can be seen in Figure 4B, both adherent cells expressed a ligand for CD300a (as they were recognized by CD300a Ig containing R94 or Q94) and CD300c (since they were recognized by CD300c Ig), whereas the nonadherent cells 8866 did not express ligand(s) for these receptors. Interestingly, following trypsin treatment the expression of the CD300a/c ligand(s) was significantly reduced (Fig. 4B).

It was demonstrated that the binding of CD300a to PS is calcium dependent [17]. Therefore, we incubated the various tumors indicated in Figure 4C in medium with and without calcium and observed an enhancement in the binding of all fusion proteins except from the control Ig fusion protein, D1 Ig. The binding of CD300a R94 Ig and CD300a Q94 Ig to tumor cells was very similar, almost identical (Fig. 4D), regardless of whether the cells

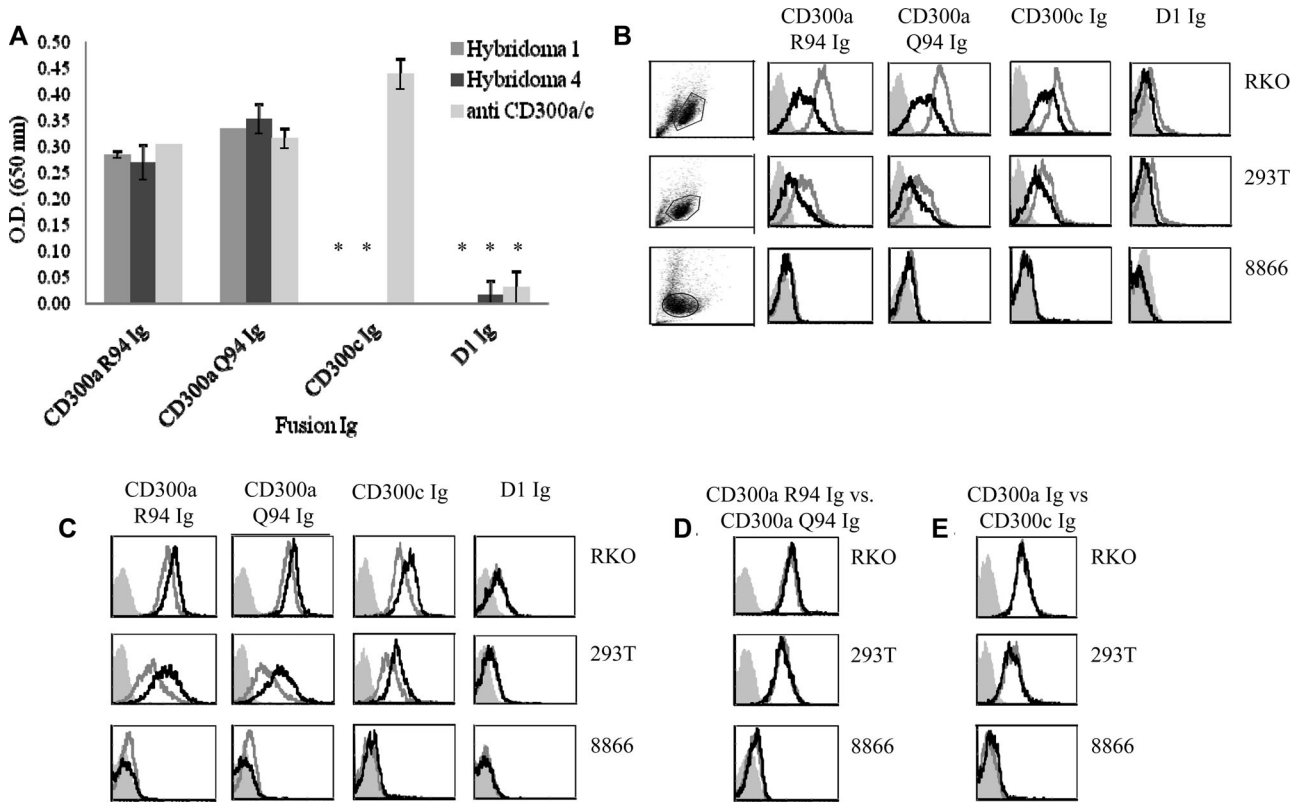



**Figure 4.** Tumor cells express a trypsin sensitive ligand for CD300. (A) Fusion proteins (indicated in the *x* axis) were incubated on ELISA plates and ELISA assays were performed with the indicated mAbs. Data are shown as means and ±SD of three replicates and are representative of one out of three independent experiments performed. $^{**}p \leq 0.004$, $^{***}p \leq 0.001$, *t*-test. (B) 8866, RKO, and 293T cells were harvested with or without trypsin (black and gray line histogram, respectively) and FACS staining was performed. Background (filled gray histogram) is the staining with allophycocyanin-conjugated F(ab')2 goat anti-human IgG only. (C) The various tumor cells indicated in the figure were harvested without trypsin and treated with medium with or without calcium (black and gray open histograms, respectively). Background (filled gray histogram) is the staining with allophycocyanin-conjugated F(ab')2 goat anti-human IgG only. (D) Binding of CD300a R94 Ig and CD300a Q94 Ig (black and gray open histogram, respectively) to various tumor cell lines. (E) Binding of CD300a Ig and CD300c Ig (black and gray open histogram, respectively) to various tumor cell lines. (B–D) Data shown are representative of one out of three independent experiments performed.

were treated with or without trypsin and with or without calcium. Interestingly, the binding of CD300c was also very similar to that of CD300a (Fig. 4E) and it remained similar in the presence or in the absence of the various treatments.

## Tumor cells express PS

As previous reports have shown that PS is a ligand for CD300a [16, 17], we next tested whether PS is expressed by tumor cells and as can be seen in Figure 5, it is indeed expressed by both adherent (Fig. 5A) and nonadherent cells (Fig. 5B). The expression of PS is variable and some adherent cells express PS at low levels, while others express PS at high levels (Fig. 5A). A similar picture is observed with regard to the nonadherent cells (Fig. 5B). Furthermore, some mouse cells (such as RMAS, EL4, and PD1.6) also express PS, while others (such as YAC1) are PS negative (Fig. 5B). All cells that express PS at high levels were recognized by CD300a Ig and CD300c Ig (data not shown).

Since we have demonstrated above that the expression of the tumor ligand(s) for CD300a is trypsin-sensitive and calcium-dependent, we have next tested whether the expression of PS will be influenced by these treatments. Adherent cells were harvest with or without trypsin and as can be seen in Figure 5A, the levels of PS were significantly reduced following trypsin treatment. The calcium treatment did not affect the expression of PS either on the adherent cells (Fig. 5A) or on the nonadherent cells (data not shown), suggesting that calcium is probably important for the CD300a and CD300c binding to PS and not for the expression of PS itself.

## PI-negative tumor cells are recognized by CD300a and CD300c

It was reported that PS is expressed on dying cells and that it inhibits the engulfment of the apoptotic cells by macrophages [17]. To test whether the tumor cells that are recognized by CD300a are apoptotic, we double stained the tumor cells with anti-PS and with propidium iodide (PI). As can be seen in Figure 6A, both 293T and RKO cells lines express PS and are recognized by CD300a Ig, however, most of the cells were PI negative, indicating

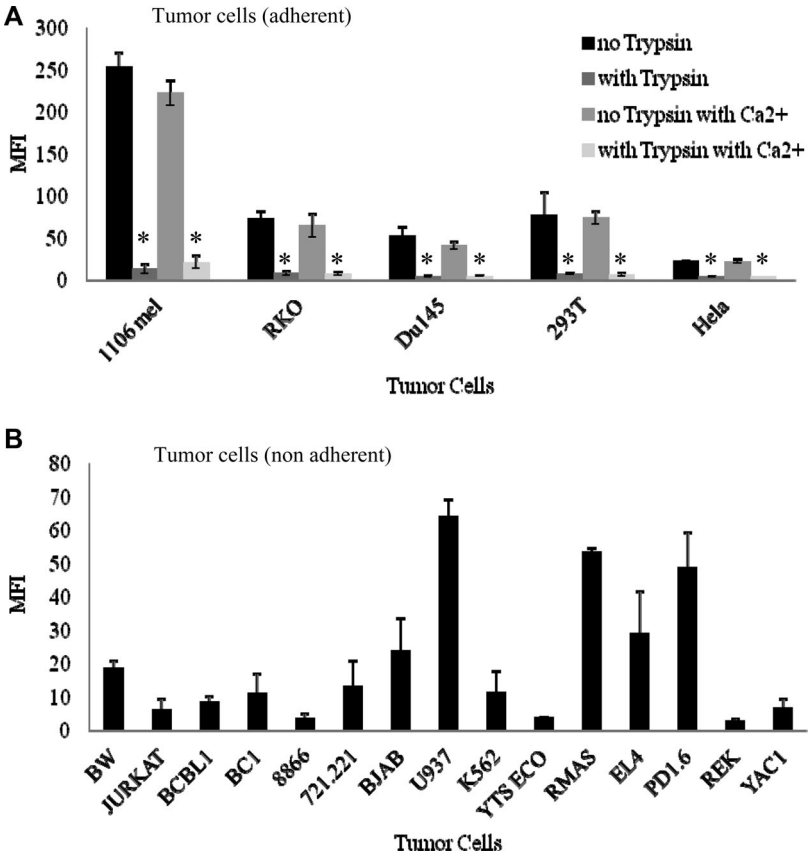



**Figure 5.** Tumor cell lines express phosphatidylserine (PS). Various tumor cell lines, (A) adherent and (B) non-adherent were stained with anti-PS antibody. (A) The adherent cells were harvested with or without trypsin and incubated with medium with or without calcium. The MFI average of three independent experiments is shown as means and ± SD. $^{*}p \leq 0.01$, *t*-test.

that these cells are not found in a late apoptotic stage. The 8866 cell line that was not recognized by CD300a Ig did not express PS (Fig. 6A). Thus, we concluded that either 293T and RKO are always a bit apoptotic or that alternatively PS is expressed by these tumor cells naturally, regardless of their apoptotic status.

Another reason accounting for the expression of PS on RKO and 293T cells might be that the anti-PS antibody we used is not specific to PS only. Therefore, we confirmed the PS expression on the tumor cells by using annexin V. As can be seen in Figure 6B, PS is indeed expressed by RKO and 293T cells as they were stained by annexin V, while 8866 cells were negative.

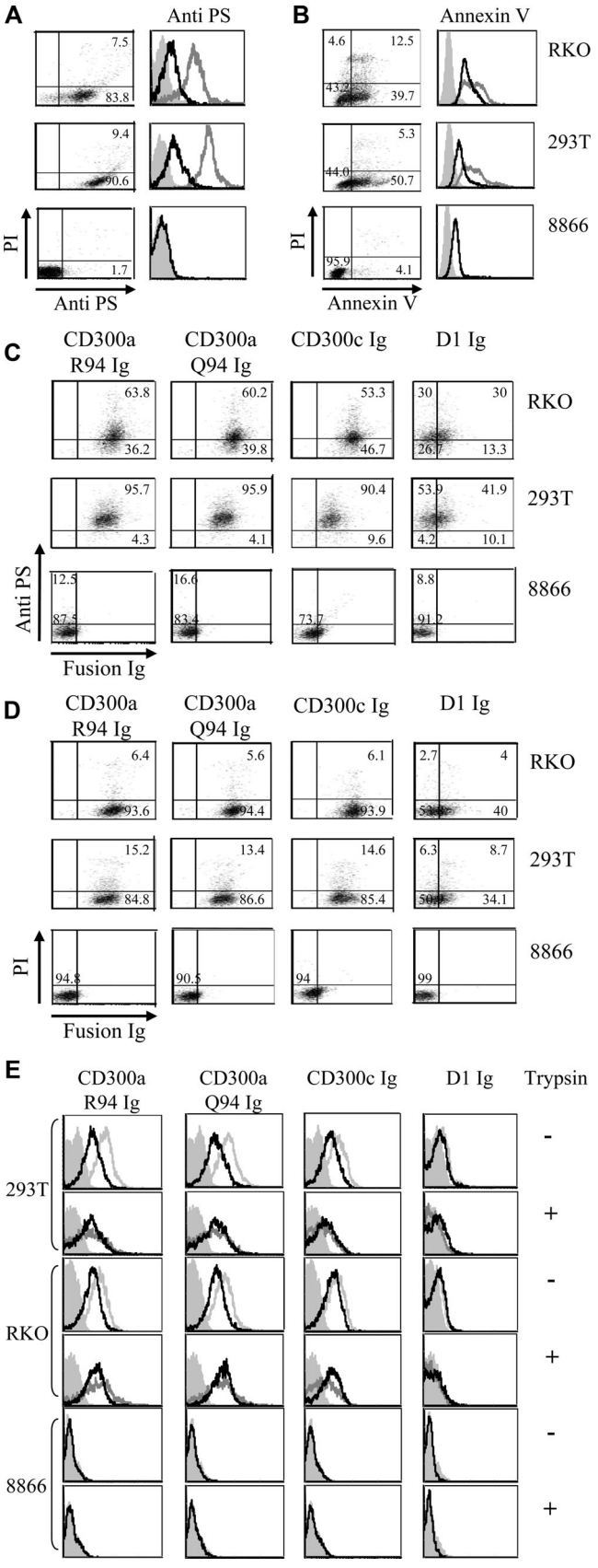
We showed above (Fig. 4 and 5) that the binding of the anti-PS mAb to the tumor cells was reduced by trypsin treatment. Therefore, we treated the tumor cells with trypsin, stained them with annexin V, and observed that the annexin V binding was reduced upon trypsin treatment (Fig. 6B, right). The 8866 cells were sometimes recognized by annexin V (Fig. 6B, right). However, because the 8866 cells were never recognized by the anti-PS antibody and the annexin V staining was not reduced following trypsin treatment (Fig. 6B, right), we considered this binding to 8866 cells nonspecific.

To further study the CD300a Ig and CD300c Ig binding to tumor cells, we next performed double staining of the tumor cells with PS together with CD300a Ig and CD300c Ig. As can be seen in Figure 6C, most of the PS-positive cells were recognized by CD300a and CD300c Ig. However, staining of CD300a Ig and CD300c Ig was detected also in a subset of PS-negative cells (especially in RKO, Fig. 6C), suggesting that CD300a and CD300c recognize additional ligand(s) other than PS that are expressed on RKO and 293T cells. Finally, we showed that the RKO and 293T cells that were recognized by CD300a and CD300c Ig are not found in the late apoptotic stage as they were almost entirely PI-negative (Fig. 6D).

To demonstrate directly that CD300a Ig and CD300c Ig interact with PS, we blocked the PS recognition by using MGF-E8 that was previously shown to block the CD300a–PS interaction on macrophages [16, 32]. Importantly, following incubation of RKO and 293T cells with MGF-E8, the binding of CD300a Ig and CD300c Ig was reduced (Fig. 6E), indicating that both proteins interact with PS. When RKO and 293T cells were treated with trypsin, the binding of CD300a Ig and CD300c Ig was reduced but still detected and this binding could not be blocked with MGF-E8 (Fig. 6E). No binding of CD300a Ig or CD300c Ig was detected to 8866 cells in the presence or absence of MGF-E8, with or without trypsin (Fig. 6E). Thus, we concluded that both CD300a and CD300c recognize two different ligands on RKO and 293T cells: (i) a trypsin-sensitive ligand PS and (ii) a trypsin-insensitive unknown ligand.

## Blocking of PS enhanced the killing of tumor cells by NK cells

We next performed NK-cell cytotoxicity assays. Because both anti-CD300a mAbs that we have generated (Hybridoma 1 and




Hybridoma 4) are not blocking antibodies (data not shown), we blocked the interaction of CD300a with PS by using MGF-E8. As can be seen in Figure 7A, when PS was blocked, the killing of the cells expressing PS (RKO and 293T) was significantly enhanced, while the killing of the PS-negative cell line, 8866, remained unchanged, suggesting that the interactions between CD300a and PS indeed inhibit NK-cell cytotoxicity.

The enhancement of NK-cell killing following MGF-E8 blocking can result from the interaction of PS with receptors other than CD300a that are present on human NK cells. Therefore, to demonstrate directly that the interaction between CD300a and PS inhibits tumor cell killing, we used the YTS cell line. As mentioned above, the killing of target cells by YTS cells is mediated via the 2B4–CD48 interactions. Because both RKO and 293T cells are CD48 negative (Fig. 7B), we expressed the CD48 protein in 293T cells and verified that the expression of PS was similar to the parental 293T cells (Fig. 7B). Next, we performed killing assays using YTS cells and the various 293T cells in the presence and absence of MGF-E8. As can be seen in Figure 7C, in the absence of CD48, the parental 293T cells were not killed by YTS cells. In contrast, a moderate killing was executed by the parental YTS ECO cells against 293T CD48 cells and this moderate killing was significantly reduced when YTS CD300a were used. Importantly, the reduction in YTS CD300a killing was partially dependent on PS as incubation of 293T CD48 cells with MGF-E8 partially restored the killing by YTS CD300a cells (Fig. 7C).

## Discussion

The CD300 family consists of seven protein members that coordinate leukocyte responses [42]. Members of the human CD300 family have broad expression patterns and the function of the various members is largely unknown [43].

We started this research by generating two specific anti-CD300a antibodies and one specific mAb against CD300c. The anti-CD300c mAb specifically recognized the CD300c-Ig fusion

◀ **Figure 6.** CD300a and CD300c interact with phosphatidylserine (PS) expressed by tumor cell lines. (A) The indicated tumor cells were double stained with PI and PS (left). Values in quadrants indicate percentages. FACS analysis of the indicated different tumor cell lines harvested with or without trypsin (black and gray open histogram, respectively) and stained with anti-PS mAb (right histograms). Background (filled gray histogram) is the staining with allophycocyanin-conjugated F(ab')2 goat anti-mouse IgG only. (B) The indicated tumor cells were double stained with PI and with annexin V (left). Values in quadrants indicate percentages. FACS analysis of the indicated tumor cell lines harvested with or without trypsin (black and gray open histogram, respectively) and stained with annexin V (right). (C, D) The various tumor cells indicated in the figure were double stained with anti-PS (C) or PI (D) together with the indicated fusion proteins. The numbers in the figure indicate percentages. (E) The various tumor cells (each tumor cell line is represented by two histogram rows) were treated without trypsin (−, upper histogram row in each tumor cell line) or with trypsin (+, lower histogram row in each tumor cell line), incubated with or without 0.5 μg MGF-E8 (black and gray open histogram, respectively), and then incubated with the indicated fusion protein. Data shown are representative of one out of three independent experiments performed.

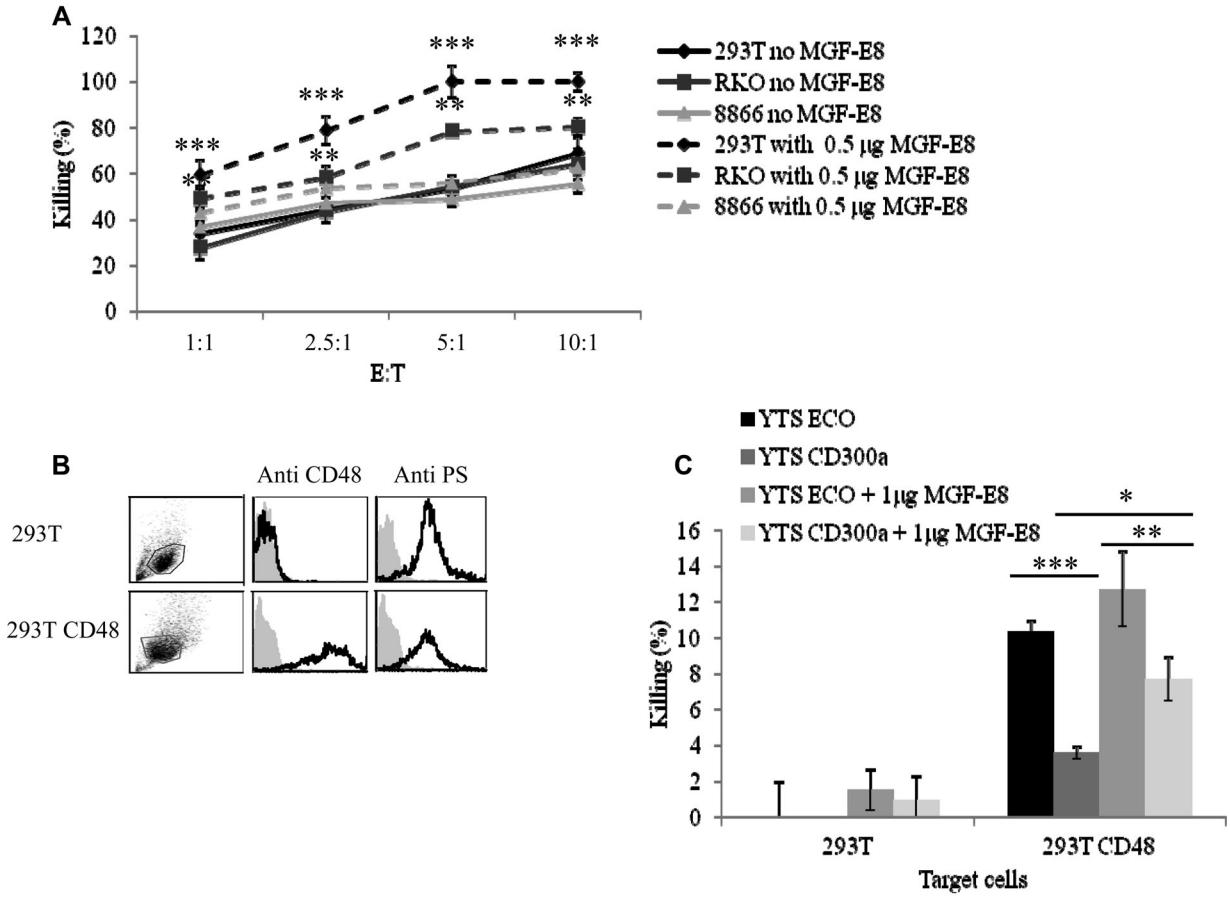



**Figure 7.** Blocking of phosphatidylserine (PS) enhance the killing of tumor cells by NK cells. (A) The indicated cells were incubated with or without 0.5 μg per well of MGF-E8 and then incubated with bulk NK-cell cultures at different E:T ratios. $^{**}p \leq 0.01$, $^{***}p \leq 0.0006$, *t*-test. (B) 293T and 293T CD48 were stained with anti-CD48 and anti-PS antibody (indicated above the histograms). The black line histogram represents the staining with the appropriate mAb, while the gray filled histogram is the background staining with allophycocyanin-conjugated F(ab')2 goat anti-mouse IgG only. The gating strategy is shown in the left part of the figure. (C) The parental YTS ECO cells and YTS CD300a cells were incubated with 293T or 293T CD48 in the presence or absence of 1 μg MGF-E8. The E:T ratio was 20:1. $^{*}p \leq 0.05$, $^{**}p \leq 0.04$, $^{***}p \leq 0.008$, *t*-test. Data are shown as means percentage and ±SD of three replicate and are representative of one out of three independent experiments performed.

and transfectants expressing CD300c, however, it did not recognize CD300c on NK cells. One possible explanation for discrepancy is that on the surface of the NK cells the epitope that is recognized by our anti-CD300c mAb is masked by another protein, maybe the one that is associated with it charged amino acid that is present in the transmembrane domain of the receptor.

We showed that NK cells can be divided into three groups based on the levels of CD300a expression, and that the percentages of such clones vary significantly among various individuals and even when the NK cells are taken from the same donor at various time points (the NK-cell clones were grown during the study period using the exact same conditions and staining were performed at exactly the same time following NK-cell isolation). It is possible that the levels of CD300a expression on the different NK clones might result from different cloning efficiencies. It will be interesting to characterize in the future the expression pattern of CD300a on NK cells derived from various tissues, such as lymph nodes, lungs, decidua, and liver, and to test which factors affect the levels of expression of CD300a.

Only recently, it was found that PS interacts with CD300a and that this leads to inhibition of phagocytosis of apoptotic cells [17]. Our data support the above observations that the binding of CD300a-Ig to tumor cells is reduced when PS is blocked and that the blocking of PS enhances NK-cell-mediated cytotoxicity. Blocking of PS only partially blocked the binding of CD300a to tumor cells and only partially restored NK-cell cytotoxicity, indicating that tumor cells express an additional ligand for CD300a. Furthermore, we showed that the other ligand for CD300a expressed on tumor cells have different biochemical properties than that of PS as it was trypsin insensitive.

This additional ligand might be phosphatidylethanolamine (PE) that was also shown to be a ligand for CD300a [17]. We have tried to investigate this option and to study the function of PE with regard to NK-cell activity. Because antibodies against PE are unavailable, we used durmycin to block the PE interactions with CD300a; however, unfortunately, the drug was toxic to the cells.

The realization that CD300a present on NK cells interacts with PS might help in the discovery of new functions and interactions

between NK cells and other cells. For example, bone marrow cells express high levels of PS as a part of the bone assembling and disassembling processes [44]. PS also plays a major role in coagulation and platelets express high level of PS following activation [18, 45].

We demonstrated here that tumor cell lines are protected from NK-cell-mediated cytotoxicity via the CD300a–PS interactions and that PI-negative tumor cells express PS. Our results are supported by previous findings showing that tumor cells exhibit elevated expression of PS in the outer leaflet of the cell membrane [37, 46, 47].

The CD300a protein has two isoforms (R94 and Q94) and we showed here that the tumor ligand(s) for CD300a and CD300c is recognized equally well by both CD300a isoforms and by CD300c. In contrast, Simhadri et al. [17] demonstrated that CD300a R94 isoform binds PS at a higher affinity than that of the Q94 isoform. We think that the reasons for these differences are derived from the different systems used to evaluate the binding of the various isoforms. Simhadri et al. used primarily surface plasmon resonance (SPR) analysis and ELISA in which PS- and PE-containing liposomes were used and we have used cell culture systems. Indeed, when the CD300a isoforms were used by Simhadri et al. to stain dead PBLs, the differences were minor [17].

We further showed that the tumor ligand(s) for CD300a and also CD300c are sensitive to trypsin and since PS is a lipid, we wondered why it is influenced by the trypsin proteases.

Membrane lipid sidedness originates from vectorial biosynthesis of lipids in combination with transporter proteins that move lipids from one side of the membrane to the other [48, 49]. Thus, if PS is found on the cell membrane attached to these chaperons, it will be trypsin sensitive. Indeed, several studies reported on a connection between membrane proteins and PS. For example, a link between glycophorin-C binding (ligation) and PS expression on erythrocytes has been suggested by its appearance on *Plasmodium falciparum* infected erythrocytes [50]. In addition, it was shown that human gastric carcinoma cells that overexpress BCRP (an ABC transporter) also overexpress PS and that treating these cells with tryprostatin A decreased the expression of PS [37].

We demonstrated here that CD300c has binding properties similar to CD300a: the CD300c binding to tumor cells was trypsin-sensitive and calcium-dependent, the binding was correlated with expression of PS and in all cases the intensity of CD300c binding was almost identical to that of CD300a. Finally, we demonstrated that treating the tumor cells with MGF-E8 diminished the binding of CD300c Ig. Thus, we suggest that, by similarly to CD300a, CD300c also interacts with PS.

## Materials and methods

### Ethics statement

The NK cells that were used in this study were obtained from the blood of healthy voluntaries. The intuitional Helsinki committee of Hadassah approved the study (Helsinki number 0030-12-HMO). All subjects provided a written informed consent.

### Reagents, mAbs, and cells

The anti-CD300a: Hybridoma 1 and Hybridoma 4; anti-CD300a/c (CMRF2.06); and anti-CD300c: Hybridoma 2 were generated using standard protocols through the injection of CD300a-Ig and CD300c-Ig fusion proteins into mice. The following reagents were used: anti Phosphatidylserine (Millipore, clone 1H6) annexin V-FITC (IQ Products) and Milk fat globule EGF factor 8 (MGF-E8, R&D systems). The cell lines used in the present study were the mouse BW cells, BW transfected with CD300c, and YTS cells, transfected with either the ecotropic murine retrovirus receptor alone (YTS ECO) or with the ecotropic receptor and CD300a (YTS CD300a) [15, 51]. Additional tumor cell lines used in the present study were RKO, 293T, and 8866.

### Ig-fusion proteins

The following Ig fusion proteins CD300a R94-Ig, CD300a Q94-Ig CD300c-Ig, and D1-Ig were generated as described previously [15]. The CD300a R94 isoform was cloned from the CIR cell line cDNA and CD300a Q94 isoform was cloned from bulk NK-cell cDNA.

### Cytotoxicity assays

For the redirected killing assay, bulk NK or YTS cells were added to $^{35}$S-labeled P815 cells that were precoated with 0.2 μg per well of the various mAbs. The level of cytotoxicity was determined as previously described [52]. The cytotoxic activity of primary NK cells against the various targets were assessed in 5 h $^{35}$S release assays, as previously described. To block the PS–CD300a interaction, tumor cells were incubated with or without 0.5–1 μg per well of MFG-E8 (R&D Systems) for 30 min at RT and then incubated with the bulk NK or with YTS cells.

### Flow cytometry of tumor cells

Flow cytometry assays were performed as previously described [15]. Cells were harvested with or without trypsin and treated with or without calcium. Cells were incubated with calcium-containing solution (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, and 2 mM $MgCl_2$) at RT for 15 min and then washed. Blocking with MGF-E8 was performed as followed: cells were incubated with 0.5 μg per well MGF-E8 for 30 min at RT. Next, cells were incubated with 0.3 μg of fusion proteins for 30 min at RT, washed, and stained with a secondary allophycocyanin-conjugated $F(ab')_2$ goat anti-human antibodies (Jackson Immunoresearch Laboratories). Analysis of annexin V (IQ products) binding was performed according to manufacture instructions.

### Statistical analysis

*t*-test was used for the statistical analysis.

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*Abbreviations:* **PE**: phosphatidylethanolamine · **PS**: phosphatidylserine

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