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Sensing of cell stress by human γδ TCR-dependent recognition of Annexin A2

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Human γδ T cells comprise a first line of defense through TCR recognition of stressed cells. However, the molecular determinants and stress pathways involved in this recognition are largely unknown. Here we show that exposure of tumor cells to various situations of stress induced tumor cell recognition by a Vδ8Vδ3 TCR. Using a strategy that we previously developed to identify antigenic ligands of γδ TCRs (Willcox et al, Nat Immunol, 2012, 13:872), Annexin A2 was identified as the direct ligand of Vδ8Vδ3 TCR, and was found to be expressed on tumor cells under the stress situations tested in a reactive oxygen species-dependent manner. Moreover, purified Annexin A2 was able to stimulate the proliferation of a Vδ2negγδ T cell subset within PBMC incubated with IL-2 and other Annexin A2-specific Vδ2negγδ T cell clones could be derived from PBMC. We thus propose membrane exposure of Annexin A2 as an oxidative stress signal for some Vδ2negγδ T cells that could be involved in an adaptive lymphoid stress surveillance.

Introduction:

It is well established that one of the major roles of conventional γδ T lymphocytes is to protect the host against microorganisms. The molecular cornerstone of this function is the recognition by their antigen receptor of microbial moieties presented in the context of classical MHC molecules (1). In contrast, γδ T lymphocytes do not recognize peptides presented by classical MHC molecules and are biased against self-reactive recognition. Consistent with the Ig-like structure of γδ TCRs (2) and the diversity of their repertoire, the self-antigens so far described to be directly recognized by γδ TCRs are structurally highly diverse, including MHC-related or unrelated molecules (for recent reviews see (3) and (4)). Intriguingly, most of those self-antigens are constitutively expressed on cells and healthy tissues, implying mechanisms to control the γδ T cell response in appropriate situations and avoid autoimmunity. Some of these mechanisms have been described such as increased self-antigen expression upon cell activation (e.g. T10/T22 in mice, (5)), dependence of recognition on a multi-molecular stress signature in CMV-infected cells and tumor cells (EPCR (6)), presentation of, or conformational modification by, metabolites (CD1d (7, 8) and BTN3A1 (9–11)), and ectopic localization in tumor cells (F1-ATPase/Apo1 (12)).

Although the pathophysiological contexts associated with the expression of these self-antigens in the appropriate environment or conformation leading to γδ T cell response remains elusive for most of them, the contribution of γδ T cells to host protection is thought to rely on recognition of cell dysregulation. The so-called lymphoid stress surveillance response has been described as rapid, weakly specific and resulting from activation of large numbers of pre-activated or pre-programmed γδ T cells without necessary clonal expansion (13). Stress surveillance by γδ T cells is considered important for tissue repair (14), rapid local containment of microbes or tumors (15–17), and activation of downstream conventional immune responses (18).

Given their implication in the control of tumors and infections, understanding the molecular basis of stress surveillance by γδ T cells could have important impacts on their use in immunotherapy. Such understanding has been hindered by the limited characterization of bona fide stress-induced antigens recognized by γδ TCRs and of the stress pathways leading to the expression of these antigens. The objective of the present study was to provide novel insights into these issues. We focused on Vδ2negγδ T cell clones isolated from healthy donors previously shown to react against a broad panel of B cell lymphoma in an ILT2-dependent pathway (19). We elucidated here the antigenic specificity of one of these clones as being Annexin A2, a molecule expressed on the cell surface in response to oxidative stress and able to activate a subset of Vδ2negγδ T cells.

Results:

Expression of 73R9 ligand by U373MG glioblastoma cell line

We focused on the Vγ8Vδ3 T cell clone (73R9) that was reactive against transformed B cells (19). HLA-I engagement on 73R9 by ILT2 expressed on B cells was previously shown to be necessary for T cell activation. Since such stress-induced antigen was recognized by the conventional TCR (20), we investigated whether it was also targeted by the γδ T cell clone 73R9. Consistent with this, our results indicate that the γδ T cell clone 73R9 recognizes transformed B cells.

Significance

Human γδ T lymphocytes have both innate-like and adaptive-like functions and can circulate in blood or reside in tissues. They are activated by specific antigens recognized by their TCR and recognize infected and transformed cells, suggesting that cellular stress is involved in specific antigen expression. However, molecular characterization of stress-induced antigens remains elusive, hampering our understanding of γδ T cell role in cancer and infections. In the present study we identify Annexin A2 as such stress-induced antigen known as a phospholipid-binding protein involved in tumorigenesis, redox potential regulation and wound healing. Stress-mediated membrane exposure of Annexin A2 could thus constitute a novel danger signal for γδ T cells to recognize various cell dysregulations and protect the host against cancer and infections.

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to stimulate clone cytolytic function (19). However, the role of the TCR in 73R9 recognition of B cells remained unclear. To address this issue we transduced the 73R9 TCR into the TCR-deficient human JRT3 T cell, producing the JRT3-73R9 reporter cell line. Unexpectedly, when assayed against transformed B cell lines the activation of JRT3-73R9 was markedly low (Fig 1A), except against the 721-221 cell line. Among forty-two other tumor cell lines tested, only the U373MG glioblastoma cell line induced a strong JRT3-73R9 cell activation, which was TCR-specific since not observed with other Vγ8 TCRs and inhibited by blocking anti-Vδ3 mAb (Fig. 1A-C). Two other glioblastoma cell lines (U343MG and U251MG) also weakly induced JRT3-73R9 cell activation (Fig. 1A). Altogether, our results suggested that, by contrast to transformed B cells, the U373MG cell line expressed an antigen specifically recognized by the 73R9 TCR.

**Generation of a mAb specifically blocking 73R9 TCR reactivity**

To characterize 73R9 TCR antigenic ligand, we generated a specific blocking monoclonal antibody using the strategy we previously described (6). Mice were immunized with U373MG and B cell hybridoma supernatants screened for their ability to decrease JRT3-73R9 reactivity against U373MG. Such a hybridoma was obtained when using C91 or U373MG cells (Fig S1A). Accordingly, anti-Vδ3 mAb, but not by FMS-01 mAb, inhibited activation of 73R9 by B cells, and inverted results were obtained when using C91 or U373MG cells (Fig S1B). A slight additive effect of combining both mAbs on B cell recognition suggested a low-grade 73R9-TCR engagement by B cells (Fig S1B), which was consistent with upregulated B cell expression of IL2 (Fig S1B).

In agreement with the discrepancy between the results obtained with JRT3-73R9 versus clone 73R9 on B cell recognition, B lymphoma cells were found to express very low levels of FMS-01 antigen, except for 721-221 cells, compared to glioblastoma and C91 T lymphoma cells (Fig. S1A). Conversely, B cell lines expressed high level of ILT2 whereas C91 and glioblastoma cells did not (Fig S1A). Accordingly, anti-ILT2 mAb, but not by FMS-01 mAb, inhibited activation of 73R9 by B cells, and reversed results were obtained when using C91 or U373MG cells (Fig. S1B). A slight additive effect of combining both mAbs on B cell recognition suggested a low-grade 73R9-TCR engagement by B cells (Fig S1B), which was consistent with upregulated B cell expression of IL2 (Fig S1B).

**Fig. 1.** FMS-01 mAb inhibits 73R9 TCR-mediated recognition of U373MG (A) CD69 expression by JRT3-73R9 co-cultured 4h with different target cells. Results shown are fold increase of CD69 MFI in the presence of target cells versus in medium alone (dotted line). (B) CD69 expression by JRT3-73R9 (VγVδV3 TCR) and JRT3-26 (VγVδV3 TCR) co-cultured 4h of with glioblastoma cells or with anti-CD3 mAb. (C) CD69 expression by JRT3-73R9 incubated with U373MG and with or without anti-Vδ3, anti-Vγ3 or anti-Vδ1 mAbs. In (A) to (C) bars represent the mean+SEM of at least 3 independent experiments and Mann Whitney test was used to compare conditions (⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎⁎ ⁣
cell-mediated activation of JRT3-73R9 when increasing B cell: JRT3-73R9 ratio (Fig. 5.1C). Altogether, these results indicated that the same γδ T cell clone can use either TCR-dependent or independent molecular pathways of activation to respond to different target cells.

CMV-induced 73R9 TCR activation through up-regulation of FMS-01 ligand expression

Our previous studies demonstrated that some Vγ2Vδ2 γδ T cells exhibit dual TCR-dependent reactivity against tumour cell lines and CMV-infected cells (21). In accordance with this, CMV infection of U373MG and U343MG, but not that of U251MG, significantly upregulated 73R9 activation (Fig. 2A). This effect of CMV was TCR-dependent since observed also when using JRT3-73R9 (Fig 2B), since inhibited by FMS-01 mAb (Fig 2C) and since associated to increased FMS-01 ligand expression on U373MG (Fig. 2D). These results supported the hypothesis that CMV-induced stress in host cells increased antigenic ligand expression and subsequent TCR-mediated activation of γδ T cells.

Different cell stress conditions trigger 73R9 TCR reactivity

We then investigated other conditions of cellular stress that could modulate target cell recognition by γδ T cells. First, pre-incubation of U343MG in hypoxia (0.1% O2) induced both JRT3-73R9 and 73R9 clone reactivity when compared to pre-incubation in normoxia (Fig. 3A and 3B). All activation assays were done at 21% O2, 37°C and with same number of target cells). JRT3 transduced with other γδ TCRs did not respond to hypoxia treated glioblastoma cells. Hypoxia-induced JRT3-73R9 and 73R9 clone activation was inhibited by FMS-01 mAb (Fig. 3A and 3B) and associated to increased FMS-01 ligand expression on U373MG cells (Fig. 3C), suggesting γδ TCR-mediated stress sensing. Similar results were obtained when U343MG were pre-exposed to high confluence (Fig. 3D and Fig. S2A-D) or to heat shock (Fig. 3E and Fig. S2C-D), and when using U373MG or U251MG. TCR 73R9 activation always consistently correlated with FMS-01 staining on the target cell surface for each of these stress conditions (Table S1). Oxidative burst could be a common trigger to induce FMS-01 ligand expression because treating glioblastoma cells with the free radical scavenger N-Acetyl-L-Cysteine (NAC) during stress exposure partially inhibited the increased FMS-01 ligand expression by all stress conditions tested on the three glioblastoma cell lines (Fig. 3F-H). In conclusion, different cell stress conditions enhance γδ TCR-mediated sensing of target cells through an increased expression of membrane ligand which is at least partially dependent on ROS production.

Identification of Annexin A2 as the ligand for FMS-01 mAb

The nature of the membrane moiety bound by FMS-01 was then identified through immunoprecipitation. FMS-01 specifically immunoprecipitated a protein of approximately 35 kDa from all glioblastoma cell lysates but not from a FMS-01-negative control cell line (Fig 4A). Proteins contained within the specific ~35 kDa band were digested with trypsin and analysed by Fourier transform-ion-cyclotron resonance mass spectrometry. This identified Annexin A2, a 35 kDa intracellular protein known to bind anionic phospholipids in a Ca2+-dependent manner, and to translocate to the cell surface as a heterotetrameric complex with the 11 kDa protein S100A10 expression in different cell types (Fig. 4B). Moreover, proteins immunoprecipitated with FMS-01 mAb were also detected with anti-Annexin A2 and anti-S100A10 Abs by western-blotting (Fig S3A). Western blots using recombinant forms of both proteins, or U373MG EGTA eluates containing Annexin A2/S100A10 complex from the cell surface as previously shown (21) Deora 2004, demonstrated that FMS-01 bound Annexin A2 only (Fig 4C). Finally, downregulation of Annexin A2 or S100A10 expression in glioblastoma cell lines using specific sh-RNA showed that surface staining by FMS-01
Remarkably, recombinant soluble Annexin A2 alone, but not A6, was able to induce multiple functions on the clone such as cytotoxicity (assessed by CD107a expression and granzyme B production) but also TNFα, IFNγ and GM-CSF secretion (Fig 5D).

Molecular interaction between Annexin A2 and the 73R9 TCR was then confirmed by surface plasmon resonance (SPR). We observed greater responses when recombinant Annexin A2 was injected over immobilized 73R9 TCR compared to control TCRs or streptavidin alone, indicating specific binding (Fig 5E). Equilibrium binding analyses yielded an apparent dissociation constant (Kd) of ~3μM (Fig 5D). No specific binding of Annexin A6 was observed to 73R9 TCR when compared to control TCR. Annexin A2 was not only binding but also signalling through the TCR since ERK 1/2 and SLP76 phosphorylation was induced in the 73R9 clone (Fig 5F) - but not control clone (Fig S4E) - as well as in the JRT3-73R9 - but not in a control transductant - (Fig S4F). Altogether, these results provide evidence that the 73R9 TCR directly recognizes Annexin A2 independently of S100A10. Annexin A2 translocation to the cell surface represents a unified stress signal recognized by this TCR.

**Annexin A2 induces the proliferation of a V\(\delta^{2}\)neg\(\gamma\) T cell subset**

Finally, we tested the effect of Annexin A2 on V\(\delta^{2}\)neg\(\gamma\) T cells isolated from the blood of healthy donors. When cocultured with autologous PBMC, a small population of proliferating V\(\delta^{2}\)neg\(\gamma\) T cells appeared when compared to IL-2 alone (Fig 6A). Results obtained from 7 different donors are shown in Figure 6B, indicating statistically significant increase of proliferating V\(\delta^{2}\)neg\(\gamma\) T cells with Annexin A2. Moreover, the effect of Annexin A2 was specific as Annexin A6 had no effect (Fig 6C), in agreement with the results obtained with JRT3 73R9 activation and SPR. This prompted us to try to derive new Annexin A2-specific V\(\delta^{2}\)neg\(\gamma\) T cell clones. From 72 V\(\delta^{2}\)neg\(\gamma\) T cell clones expanded polyclonally from three different healthy donors, two clones were able to react to Annexin A2. Interestingly, one of them also reacted to Annexin A6, suggesting the recognition by this clone of a region shared by both Annexins. Using anti-V\(\delta\) and anti-V\(\gamma\) TCR chain antibodies and flow cytometry analysis, we showed that one clone (# 24.2) expressed a V\(\delta\)3 TCR (the V\(\gamma\) chain could not be determined using the panel of available antibodies but was neither V\(\delta\)4, V\(\gamma\)8 nor V\(\gamma\)9), and the other one (# 33.20) expressed a V\(\delta\)4V\(\beta\)1 TCR. These results indicate that Annexin A2 specificity is not restricted to the 73R9 TCR but to V\(\delta\)8V\(\beta\)3 TCRs.

**Discussion:**

Because they are able to react to infected, activated or transformed cells, and are involved in host response to diverse situations of stress, V\(\delta\) T cells are considered to be important players in lymphoid stress surveillance. However, the nature of the cellular dysregulation events that they respond to and the specific molecular stress stimuli that trigger their activation remain poorly understood. In particular, identification of the molecular signals associated with these dysregulations and specifically recognized by the V\(\delta\) TCR is still limited. As a contribution to this knowledge, we characterized Annexin A2 translocation to the cell surface as a common molecular stress signal recognized by a V\(\delta\)8V\(\beta\)3 TCR.

The V\(\delta\)8V\(\beta\)3 V\(\delta\) T cell clone 73R9 used in this study is representative of a panel of V\(\delta^{2}\)neg\(\gamma\) T cells previously described to recognize a large panel of B lymphoma cell lines through an atypical IL-12/HLA axis (19). We show here that stressed glioblastoma cells can also activate clone 73R9. Interestingly, different molecular mechanisms mediated recognition of distinct...
target cells. γδ T cell HLA molecules recognize ILT2 on B lymphoma cells and the Vy8V3 TCR is not (or weakly) involved in this process. In contrast, the TCR recognizes Annexin A2 on glioblastoma cells and ILT2 is not involved. The same γδ T cells can thus recognize different types of cellular dysregulation through distinct molecular pathways, making them able to integrate several and potentially separate contextual signals, in order for them to enlarge their functional diversity and responses to different situations.

Here, we identify Annexin A2 as the antigen targeted by FMS-01 mAb that specifically inhibited Vy8V3 TCR-mediated recognition of glioblastoma cells. Together with the observation that purified Annexin A2 was able to activate the Vy8V3 TCR specifically, this result demonstrates that Annexin A2 is critical for Vy8V3 TCR-dependent recognition of target cells. Annexin A2 belongs to the evolutionary ancient family Ca2+-regulated phospholipid-binding annexin proteins (22). Annexin A2 is present in the cytoplasm, associated with intracellular membranes of different organelles and with the internal or extracellular face of plasma membrane. It participates in a variety of membrane-related functions (endocytosis, exocytosis, membrane repair) in response to diverse cellular fluctuations including Ca2+ influx, pH variation, membrane phospholipid composition and its own post-translational modification. It can exist as a monomer or as heterotrimeric complexes with the S100A10 protein, which enhances its membrane phospholipid binding affinity. In our hands, the highest expression of Annexin A2 observed at the cell surface was achieved by placing cells under hypoxia, probably because it combines both membrane translocation and an increase in Annexin A2 gene expression which has been shown to be dependent on HIF-1 (23). Cellular reoxygenation after hypoxia is followed by ROS burst, and inhibiting ROS production using antioxidant NAC decreased stress-induced Annexin A2 expression. Oxidative stress could thus be a common pathway leading to Annexin A2 membrane translocation and γδ T cell activation since NAC also decreased heat shock and high-confluence-induced Annexin A2 expression.

Several features of Annexin A2 fulfill what we can expect from a canonical ligand of a Vγ2Vδ3 γδ TCR. First, Annexin A2 is overexpressed in many cancer cells including glioblastoma where it correlates positively with histologic grade and central nervous system dissemination (24). Secondly, despite the absence of a transmembrane domain, intracellular Annexin A2 can swiftly translocate to cell surface upon stress signals (25) in agreement with the increase of FMS-01 binding on glioblastoma cells treated for only 30 minutes at 42°C. In endothelial cells, Annexin A2 translocation is obtained, in vitro but also in vivo, within minutes in response to heat stress, thrombin exposure or hypoxia and relies on Annexin A2 phosphorylation (21–23). Thirdly, consistent with the γδ T cell responses to tissue injury (26) Annexin A2 plays a role in membrane repair and wound healing (27), which is supposedly due to an intracellular rise in Ca2+ upon membrane damage (28). Fourthly, in agreement with 73R9 TCR recognition of CMV-infected cells, CMV-infection has been shown to induce Annexin A2 phosphorylation which is necessary for translocation to cell surface and to further enhance CMV infection (29).

Annexin A2 appears to represent a bona fide stress antigen expressed on the cell surface only upon cellular dysregulation, and able to alert γδ T cells such as 73R9, 24.2 or 33.20 γδ T cells. Annexin A2 specific γδ T cells could thus contribute to lymphoid stress surveillance, a property that has rather been so
γδ and low frequency of Annexin A2-specific (A) Representative dot-plot of flow cytometry staining, (B) percentages are presented as percentages of CFSE-low cells among Vγδ presence of recombinant IL-2 with or without Annexin A2 for 5 days. Results comparison of Annexin A2 and Annexin A6 effects. (D) TNF inflammatory patterns in endothelial cells and macrophages. Annexin αβ rather represent an adaptive response requiring clonal expansion describe in this study suggest that response to Annexin A2 may far attributed to innate-like invariant \( T \) cells (13). The diversity and low frequency of Annexin A2-specific \( T \) cells that we describe in this study suggest that response to Annexin A2 may rather represent an adaptive response requiring clonal expansion in specific situations. This 'adaptable lymphoid stress surveillance' would probably be less immediately efficient than the massive response of invariant subsets but could be more rapid than a conventional \( T \) cell response because conceivably taking place within stressed tissues.

Annexins and \( \alpha\beta \) molecules have been previously classified among alarmins (30) because of their ability to induce inflammatory patterns in endothelial cells and macrophages. Annexin A2 could be considered as a \( \alpha\beta \) \( T \) cell antigen, acting either through cell-cell contact or as soluble form since Annexin A2 can be released in the extracellular microenvironment (31). It is tempting to imagine that soluble Annexin A2 could alert distant specific \( \gamma\delta \) \( T \) cells and stimulate their proliferation. However, the affinity of Annexins for membrane phospholipids suggests that even when produced in soluble form Annexin A2 probably rapidly binds to proximal cell membranes and act in a membrane-bound fashion. Our results showing an induction of \( V_5^{\gamma\delta} \) \( \gamma\delta \) \( T \) cell proliferation by soluble Annexin A2 should foster further investigations to evaluate the interest of this antigen in immunotherapeutic settings aiming at stimulating \( \gamma\delta \) \( T \) cell control of cancer or infections.

Materials and Methods:

For further details see SI Materials and Methods.

Generation of effector cells.

Human \( \gamma\delta \) T clone 73R9 (expressing a TCR V\( \gamma \)V\( \delta \)) was obtained as previously described in (19). Reporter cells expressing TCR 73R9 (JRT3-73R9) were generated as previously described (6) by co-transduction with viral particles expressing V\( \gamma \)V\( \delta \) TCR chain and particles expressing V\( \delta \)V\( \gamma \) TCR chain. Amino acid sequences of the V\( \delta \)-D03-J01 and V\( \gamma \)-I-J2 junctional regions of 73R9 TCR are: CAFTGLGDTSHAKLIF and CATWDSSLFGSKTLVT, respectively.

Functional assays with stressed cells.

(T) NFlr production by two \( T \) cell clones isolated from healthy donors and incubated for 24h with soluble Annexins A2 or A6. Results from C to E are mean+SEM of at least 2 independent experiments.

Far attributed to innate-like invariant \( \gamma\delta \) \( T \) cells (13). The diversity and low frequency of Annexin A2-specific \( \gamma\delta \) \( T \) cells that we describe in this study suggest that response to Annexin A2 may rather represent an adaptive response requiring clonal expansion in specific situations. This 'adaptable lymphoid stress surveillance' would probably be less immediately efficient than the massive response of invariant subsets but could be more rapid than a conventional \( \alpha\beta \) \( T \) cell response because conceivably taking place within stressed tissues.

Annexins and \( \alpha\beta \) molecules have been previously classified among alarmins (30) because of their ability to induce inflammatory patterns in endothelial cells and macrophages. Annexin A2 could be considered as a \( \alpha\beta \) \( T \) cell antigen, acting either through cell-cell contact or as soluble form since Annexin A2 can be released in the extracellular microenvironment (31). It is tempting to imagine that soluble Annexin A2 could alert distant specific \( \gamma\delta \) \( T \) cells and stimulate their proliferation. However, the affinity of Annexins for membrane phospholipids suggests that even when produced in soluble form Annexin A2 probably rapidly binds to proximal cell membranes and act in a membrane-bound fashion. Our results showing an induction of \( V_5^{\gamma\delta} \) \( \gamma\delta \) \( T \) cell proliferation by soluble Annexin A2 should foster further investigations to evaluate the interest of this antigen in immunotherapeutic settings aiming at stimulating \( \gamma\delta \) \( T \) cell control of cancer or infections.

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