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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 Vγ8Vδ3 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 Vγ8Vδ3 TCR, and was found to be expressed on tumor cells under stress situations tested in a reactive oxygen species-dependent manner. Moreover, purified Annexin A2 was able to stimulate the proliferation of a Vδ2neg γδ T cell subset within PBMC incubated with IL-2 and other Annexin A2-specific Vδ2neg γδ T cell clones could be derived from PBMC. We thus propose membrane exposure of Annexin A2 as an oxidative stress signal for some Vδ2neg γδ 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 αβ 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 Vδ2neg γδ 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 Vδ2neg γδ 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

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 V83 TCRs and inhibited by blocking anti-VB3 chain 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 mAb specifically blocking 73R9 TCR reactivity 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 selected and cloned to produce a mAb called FMS-01 (Fig. 1D). Inhibition by FMS-01 was specific for the V83-73R9 TCR as it was not observed for other V8 TCRs (Fig 1E). Among glioblastoma, U373MG was the cell line constitutively expressing the most important level of antigen labelled by FMS-01 mAb (Fig. S1A), in accordance with JRT3-73R9 reactivity (Fig. 1A). We concluded that FMS-01 competed 73R9 TCR and most likely recognized the same antigen.

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 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

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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 (V8/V83 TCR) and JRT3-26 (V9/V93 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-V8, anti-V93 or anti-V01 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 (*p<0.05, ***p<0.0001). (D) CD69 expression by JRT3-73R9 incubated with or without U373MG cells in the presence or absence of a selection of hybridoma supernatants. (E) CD69 expression by JRT3 reporter cells expressing no TCR (JRT3 WT) or indicated V8/V83 TCRs, incubated with or without glioblastoma cells (U137MG, HT29, SKW6.4). Supernatant of FMS-01 or control hybridoma (25 % of culture volume) were added in indicated conditions. Data are representative of at least 3 independent experiments.

Fig. 2. CMV infection of glioblastoma cells enhances 73R9 TCR reactivity and FMS-01 expression. Activation of clone 73R9 (A) or JRT3-73R9 (B) by co-culture with CMV-infected or uninfected glioblastoma cells. (C) JRT3-73R9 activation by CMV-infected or uninfected U373MG with or without FMS-01 and/or control IgM. (D) Cell surface staining of CMV-infected (black line) and uninfected (grey histogram) U373MG cells (U373MG, HT29, SKW6.4). Supernatant of FMS-01 or control hybridoma (25 % of culture volume) were added in indicated conditions. Data are representative of at least 3 independent experiments. Statistical significance was tested using the Wilcoxon test, *p<0.05 and **p<0.005.
cell-mediated activation of JRT3-73R9 when increasing B cell: JRT3-73R9 ratio (Fig. S1C). 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 Vδ2-positive γδ T cells exhibit dual TCR-dependent reactivity towards tumour cell lines and CMV-infected cells (20). 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) (cell 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. 2D 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-blots (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...
was dependent on Annexin A2 expression but independent of S100A10 expression (Fig 4D and Fig S3B).

**Annexin A2 is recognized by the 73R9 TCR**

FMS-01 ligand was expected to be 73R9 TCR ligand, it was then important to ensure that 73R9 TCR recognized Annexin A2. EGTA membrane eluates from U373MG cells cultured at high confluence were able to activate JRT3-73R9, and not other TCR-transductants, in an Annexin A2-dependent manner, in contrast to eluates generated from cells cultured at low confluence (Fig. 5A and S4A). Annexin A2 was mandatory for recognition of glioblastoma cells by JRT3-73R9 since down regulation of its expression by RNA interference abrogated JRT3-73R9 activation while downregulation of S100A10 had no effect (Fig 5B).

Remarkably, recombinant soluble Annexin A2 alone, but not recombinant S100A10, was able to activate very efficiently JRT3-73R9 (Fig. 5C left panel). Activation was not observed when using Annexin A6 (Fig 5C right panel) or another TCR-transductant (Fig S4B), and was inhibited in the presence of the FMS-01 mAb (Fig S4C). Moreover, soluble Annexin A2 was at least as efficient as anti-CD3 mAb to activate the 73R9 clone (Fig 5D). Finally, Annexin A2, 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 ERK1/2 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 Vδ2Vγ6 T cell subset**

Finally, we tested the effect of Annexin A2 on Vδ2Vγ6 T cells isolated from the blood of healthy donors. When co-cultured with autologous PBMC, a small population of proliferating Vδ2Vγ6 T cells appeared in the presence of Annexin A2 plus IL-2 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 Vδ2Vγ6 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 Vδ2Vγ6 T cell clones. From 72 Vδ2Vγ6 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-Vδ and anti-Vγ TCR chain antibodies and flow cytometry analysis, we showed that one clone (# 24.2) expressed a Vδ3 TCR (the Vγ chain could not be determined using the panel of available antibodies but was neither Vγ4, Vγ8 nor Vγ9), and the other one (# 33.20) expressed a Vγ4Vδ1 TCR.

These results indicate that Annexin A2 specificity is not restricted to the 73R9 TCR nor to Vδ8Vδ3 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, Vγ6 T cells are considered to be important players in lymphoid stress surveillance. However, the nature of the cellular disregulation 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 disregulations and specifically recognized by the Vγ6 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 Vγδ8Vδ3 TCR.

The Vγ8Vδ3 Vγ6 T cell clone 73R9 used in this study is representative of a panel of Vδ2Vγ6 Vγ6 T cells previously described to recognize a large panel of B lymphoma cell lines through an atypical ILT2/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 IRT2 on B lymphoma cells and the VγVδ3 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 VγVδ3 TCR-mediated recognition of glioblastoma cells. Together with the observation that purified Annexin A2 was able to activate the Vγ8Vδ3 TCR specifically, this result demonstrates that Annexin A2 is critical for VγVδ3 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 heterotetrameric 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 also decreased stress-induced Annexin A2 surface 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 Vγδ 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 increase 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

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γδ and low frequency of Annexin A2-specific cells.

(A) Representative dot-plot of flow cytometry staining, (B) percentages labelled Vγδ2 T cells among Annexin A2 labelled Vγδ2 T cells within stressed tissues. Annexin A2 could be considered as a Vγδ T cell activator, 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 Vγδ 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 V52^{m/c} Vγδ T cell proliferation by soluble Annexin A2 should foster further investigations to evaluate the interest of this antigen in immunotherapeutic settings aiming at stimulating Vγδ T cell control of cancer or infections.

**Materials and Methods:**

For further details see SI Materials and Methods.

**Generation of effectors:**

Human γδ T clone 73R9 (expressing a TCR Vγ8Vδ3) 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 Vγ8Tδ3 chain and particles expressing Vδ3 TCR chain. Amino acid sequences of the V53-JD0-J01 and Vδ8-J2 junctional regions of 73R9 TCR are: CAFTGGLCSTHDKLFP ATDWOSSKFSGLGTLV, respectively.

**Functional assays with stressed cells.**

(A-C) CFSE labelled Vγδ T cells by tumor cell lines at 1:1 (E:T) ratio was measured by expression of CD69 by flow cytometry. Activation of clone 73R9 was analysed using CD107a mobilization assay or cytokine production. TCR signalling was also analysed by flow cytometry in some experiments, tumor cells were infected with CMV clinical strain T8404E for 4 days. Correct infection of the cells was confirmed by cytopathic effect observation. For hyperoxic stress, tumor cells were grown in 21% or 0.1% oxygen atmosphere and were released, counted and stained in normoxia. For heat shock assay, cell lines were grown for 48 hours, then detached and incubated at 42°C or 37°C for the indicated times. For all assays, target cells were washed twice before incubation with effector cells in a 1:1 ratio, or before staining with specific mAbs. In some experiments, glioblastoma cell lines were pre-incubated for 1h with 10 mM N-Acetyl-L-Cystein pH-adjusted solution, before stress induction (heat shock) or cell detachment.

**Expression of soluble TCR and binding studies.**

Soluble TCR was produced in drosophila cells and binding with proteins analysed by surface plasmon resonance.

**Generation of FMS-01 mAb.**

Balb/c mice were immunized with U373MG and hybridomas generated as previously described (6). Hybridomas that secreted antibody able to inhibit JRT3-73R9 reactivity against U373MG were cloned by limiting dilution, ending with selection of FMS-01 mAb because of its robust neutralizing activity.

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