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**Exposure to anthrax toxin alters human leukocyte expression of
Anthrax toxin receptor 1**

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(1) Summary

Anthrax is a toxin-mediated disease; the lethal effects of which are initiated by the binding of Protective antigen (PA) with one of three reported cell surface toxin receptors (ANTXR). Receptor binding has been shown to influence host susceptibility to the toxins. Despite this crucial role for ANTXR in the outcome of disease, and the reported immunomodulatory consequence of the anthrax toxins during infection, little is known about ANTXR expression on human leukocytes. We characterised the expression levels of ANTXR1 (TEM8) on human leukocytes using flow cytometry. In order to assess the effect of prior toxin exposure on ANTXR1 expression levels, leukocytes from individuals with no known exposure, those exposed to toxin through vaccination and convalescent individuals were analysed. Donors could be defined as either 'low' or 'high' expressers based on the percentage of ANTXR1 positive monocytes detected. Prior exposure to toxins appears to modulate ANTXR1 expression, exposure through active infection being associated with lower receptor expression. A significant correlation between low receptor expression and high anthrax toxin-specific IFN γ responses was observed in previously infected individuals. We propose that there is an attenuation of ANTXR1 expression post-infection which may be a protective mechanism that has evolved to prevent re-infection.

(2) Introduction

Anthrax is a toxin-mediated disease caused infection with by the opportunistic Gram-positive bacterial pathogen, *Bacillus anthracis* [1]. The anthrax toxin is a tripartite A–B toxin, comprising two alternative A-subunits, lethal factor (LF) and edema factor (EF), and a single receptor-binding B-subunit, consisting of heptamers of protective antigen (PA). PA combines with LF to form lethal toxin (LT) or with EF to form edema toxin (ET). PA consists of four folding domains [2]; domain 1 acts as a binding site for LF or EF, domain 2 forms the transmembrane pore and participates in receptor binding while domain 3 is involved in heptamerisation and domain 4 binds to the host cell receptor [2–4]. PA alone is not toxic and is the principal component of existing licensed vaccines for anthrax in the UK and US.

PA binds in a 1:1 ratio [5] with either one of three known cell surface receptors: tumour endothelial marker 8 (TEM8 or ANTXR1), capillary morphogenesis protein 2 (CMG2 or ANTXR2) [6,7] , more recently it was reported that beta1-integrin can also function as a receptor [8]. Both ANTXR1 and ANTXR2 are highly expressed in epithelial cells lining the sites of entry favoured by *B. anthracis* - the lungs, skin and intestine [7,9,10]. The physiological functions of these receptors are associated with binding to extracellular matrix components and are believed to include regulation of endothelial cell–matrix interactions, adhesion, migration, cell spreading on collagen and angiogenesis [11–13].

The interaction of the anthrax toxins with their receptors has significant impact on the disease process. A mutant cell line lacking ANTXR1/2 is resistant to the effects of purified toxin [14], while cells that over express either ANTXR show increased

susceptibility to lethal toxin and rapid apoptosis [14,15]. These effects are also seen during anthrax infection *in vivo*: mice supplemented with mutant macrophages lacking ANTXR1/2 expression are able to clear a dose of *B. anthracis* spores which is lethal in mice supplemented with wild-type macrophages [16].

Despite the clear role of ANTXR in the disease process [16] and the reported immunomodulatory consequence of the anthrax toxins during infection [17], little is known about the expression of these receptors on leukocytes. Recently, it has been demonstrated that PA preferentially binds NKT cells rather than NK cells or T cells [18]. Furthermore, *in vitro* exposure of macrophages to ET has been shown to upregulate mRNA expression of both receptor types [19], whereas mRNA levels for ANTXR were down-regulated in the lungs of mice injected intra-nasally with *B. anthracis* Sterne strain spores.

We have previously reported the detailed characterisation of immune responses to anthrax toxins in cohorts of naturally infected, vaccinated and unexposed individuals [20,21]. These cohorts offer a unique opportunity to determine the modulatory impact of prior toxin exposure *in vivo* in humans in a controlled comparison. Thus, the aim of the research presented here was to carry out the first detailed characterisation of the surface expression of ANTXR1 on human leukocytes; and more specifically, to assess the effect of prior toxin exposure by profiling ANTRX1 expression levels in convalescent individuals, by comparison with non-toxin exposed individuals.

(3) Materials and Methods

Isolation of peripheral mononuclear blood cells from whole blood

As previously described [21], blood samples were obtained from each of 3 cohorts: patients treated for and recovered from cutaneous anthrax (n=10), volunteers routinely vaccinated every 12 months for a minimum of 4.5 y with the U.K. Anthrax Vaccine Precipitated vaccine (U.K. Department of Health) (n=10), and healthy controls with no known exposure to PA or anthrax toxins (UK n = 14, Turkey n = 10). Full informed consent was provided by each subject and ethical approval for the study was granted respectively by Ericyes University Ethical Committee, Chemical and Biological Defence Independent Ethics Committee for the U.K. Ministry of Defence and the Research Ethics Committee reference number 08/H0707/173.

Peripheral blood mononuclear cells (PBMCs) were prepared from sodium heparinized blood, using Accuspin tubes (Sigma-Aldrich, Dorset, U.K.), and centrifuged at $800 \times g$ for 30 min, after which the cells were removed from the interface and washed twice in AIM V serum-free media (GIBCO Invitrogen, Carlsbad, CA).

Antibody and protein conjugation

Polyclonal TEM8 (ANTXR1), goat IgG isotype control (both Santa Cruz Biotechnology, USA) recombinant PA (DSTL, UK) and a control of bovine serum albumin (Sigma, UK) were fluorescently labelled using an Alexa Fluor 488 protein-labelling kit (Invitrogen, UK) following the manufacturer's protocol.

Analysis of Anthrax Toxin Receptor 1 expression and Protective Antigen binding by flow cytometry

Isolated PBMC were washed twice in FACS buffer [PBS (Invitrogen, UK), 10% foetal bovine serum (Autogen Bioclear, UK)] by centrifuging at 500 x g for 10 minutes. They were then stained with the following antibodies CD56PE, CD3PECy5, CD19 PECy5, CD14 PE (all eBioscience, UK), Alex 488 conjugated TEM8 (ANTXR1), IgG isotype control, PA or control BSA. All antibodies were used at optimal titrated concentrations as recommended by the manufacturers. Post-staining, the cells were washed with FACS buffer, fixed with 2% paraformaldehyde and stored at 4°C until analysis. Approximately 100,000 events within the lymphocyte gate were acquired using a FACScalibur (BD Bioscience, USA) and analysed with FlowJo software (Treestar, USA). The lymphocyte and monocyte gates were identified based on their forward/side scatter properties and the cell populations further defined as T cell (CD3+ CD56-), NK cells (CD3- CD56+), NKT cells (CD3+ CD56+), B cells (CD19+) and monocytes (CD14+). The isotype control antibody or BFA protein binding control were used to establish levels of non specific binding and set the gates for positive PA binding or ANTXR1 expression (Figure 1A).

Statistical analysis

As flow cytometric data are inherently non-parametric, the Kruskal-Wallis test, with Dunns post hoc testing, was used to compare the levels of PA binding and ANTXR1 expression level between cell types and cohorts. During analysis, it could be seen that there were distinct groupings of individuals based on the percentage of ANTXR1 positive monocytes, using the boundaries of these groupings, the populations were categorized as low ($\leq 35\%$) or high ($> 35\%$); a comparison of the number of subjects falling into each of these categories was made using a two-tail Chi-squared test. The expression levels of ANTXR1 were logged before linear regression analysis with

previously published IFN γ responses to PA detected by ELISpot [20] from the same individual. Graphpad prism 4.0 software (Graphpad Inc, USA) was used for all analyses.

(4) Results

A detailed characterization of PBMC for ANTXR1 expression and binding of the anthrax toxin component PA was performed (Figure 1A). Although no significant differences in the percentage of T, NK and NKT cells binding PA were observed (Figure 1B), the median fluorescent intensity (MFI) was significantly higher on NKT cells compared to both NK cells ($p = 0.02$) or T cells ($p = 0.008$) (Figure 1E). The highest levels of both PA binding and MFI was observed on monocytes (Figure 1D), mirrored by a high percentage of monocytes expressing ANTXR1, although B cells showed the highest percentage of expression (Figure 1C).

During the analysis of ANTXR1 expression on monocytes, it was apparent that individuals could be divided into two main groupings termed 'low' and 'high' expressers (Figure 2A). As ANTXR expression is known to relate to the susceptibility of a cell to anthrax toxins (1, 4, 16), it could be hypothesised that the 'high' expresser population would be more susceptible to anthrax infection. To examine this further, we examined ANTXR1 expression levels of the monocytes of individuals who had previously been naturally infected with anthrax due to interaction with livestock in an anthrax endemic region of Turkey [21]. Contrary to the predicted results, all previously infected individuals were categorised as low expressers (Figure 2B, Table 1). In order to ensure this wasn't due to a generic local genetic variation in ANTXR1 expression levels, a cohort of local volunteers with no known prior *B. anthracis* was also examined. There was no difference in the percentage of high and low expressers in the unexposed volunteers from the UK and Turkey ($p=0.63$) (Figure 2B, Table 1). However the proportion of individuals defined as low or high expressers was significantly different in the Turkish individuals based on exposure ($p = 0.003$) (Table 1), with 60% of the unexposed controls defined as high expressers, whilst 100% of the convalescent

subjects were low expressers. To establish if the over representation of low expressers in the previously exposed subjects was as a result of their exposure to the components of the anthrax toxins, a cohort of AVP vaccinated individuals were tested. It was shown that there were significantly more 'high' expressers (figure 2B, Table 1) compared to the UK healthy control cohort ($p = 0.008$), and the convalescent individuals ($p < 0.0001$) (Figure 2B). Furthermore, in a subset of the samples both the percentage of PA binding and the median fluorescent intensity (MFI) of PA on the positive cells was measured post exposure (Figure 3). There was a significant reduction in the convalescent individuals ($n = 4$) in both the percentage of positive cells and the MFI of PA binding compared to the vaccinated subjects ($n = 10$) ($p = 0.03$ and 0.09 respectively), however only the percentage of PA binding was reduced in comparison to the unexposed controls ($n = 7$) ($p = 0.007$).

We have previously published IFN γ ELISpot responses to PA from these cohorts of naturally infected and vaccinated individuals [21]. When the response of each individual is correlated with their ANTXR1 expression no significant correlation was observed ($p = 0.87$, $r^2 = 0.003$) in AVP vaccinated individuals (Figure 4A), even when only those individuals who mount an immune response to PA were considered ($p = 0.13$, $r^2 = 0.75$). In contrast, there was a significant negative correlation in the naturally infected individuals ($p = 0.016$, $r^2 = 0.58$) (Figure 4B).

(5) Discussion

This study represents the first comprehensive characterization of ANTXR1 expression on human leukocytes. The observation that NKT cells preferentially bind PA compared with NK or T cells concurs with previously published research [18]. However, PA binding to monocytes was far higher, mirrored by higher percentage of cells expressing ANTXR1. Two distinct groups could be identified among our control blood donors based on the percentage of ANTXR1 positive monocytes and these were termed 'low' and 'high' expressers. These results are substantiated by the recent demonstration that there is a striking diversity in the sensitivity of human lymphoblastoid cell lines to anthrax toxin which results, at least in part, due to expression levels of ANTXR2 [22]. Using parent-child trios Martchenko et al [22] demonstrated that this variability in expression levels is genetically inherited. It was proposed in that study that lethal *B. anthracis* clades may have exerted evolutionary selection pressure on the incidence of toxin receptor polymorphisms in human populations. However, we consider it unlikely that anthrax, which is not easily spread between humans, has imposed strong selection pressure during human evolutionary history.

Given that anthrax toxin receptor expression has been correlated with cell susceptibility to the effects of toxin both *in vitro* and in animal models [14–16,22], we postulated ANTXR1 'high' expressers would be more susceptible to either anthrax infection or to the development of severe disease. To test this premise, the expression levels of ANTXR1 were measured on cells from a cohort of individuals that received hospital treatment for cutaneous anthrax infection [21]. As the clinical picture of cutaneous anthrax ranges from mild to severe [23] we reasoned that a correlation between expression level and the severity of disease would be observed. However,

we found that all the exposed and recovered individuals were low expressers. To rule out that this was due to some form of generic low expression across this population, we also examined local healthy volunteers with no known history of exposure to anthrax, but the grouping of unexposed individuals was comparable in the UK and Turkish cohorts.

All the Turkish samples were collected by the same clinician, processed by a single researcher and analysed under the same flow cytometry conditions, arguing that the striking attenuation of ANTXR1 expression on the monocytes of exposed individuals is not an artefact of sample preparation or processing. The preponderance of low expressers in the exposed cohort could be theorised to result from modulation of receptor expression levels by exposure to the anthrax toxin components. Alternatively, low expressers may be at increased risk of infection. To examine these postulations, we tested a cohort of individuals receiving multiple boosts with AVP vaccine, thus exposed to PA, LF and EF without infection. If exposure to these anthrax proteins is capable of modulating ANTXR1 expression, then these individuals would also have reduced expression of ANTXR1 on their monocytes, whilst if low expression results in increased risk of infection, the expression profile of the vaccinated individuals should resemble that of the healthy controls. Surprisingly, the AVP vaccinated cohort were all classified as high expressers. This divergence in response may be due to the concentration and/or ratio in which the infected and vaccinated individuals are exposed to the individual anthrax toxin components. The vaccinees will have been primarily exposed to PA, with low concentrations of LF and EF therefore much lower toxin concentration than infected individuals. Alternatively, down regulation of ANTXR1 may require PA binding within the context of the inflammatory milieu resulting

from infection. It has been demonstrated that PA and EF exposure induces an up-regulation of ANTXR1 in vitro [19], and these condition perhaps more accurately model the immunological setting of vaccination as opposed to the highly inflammatory conditions that will be present during a natural infection. The infected individuals also received antibiotic treatment at the time of exposure to the anthrax toxins [21] which the vaccinated group did not. We therefore cannot exclude the possibility that the therapeutics play a role in the reduction of ANTXR1 levels seen in the convalescent cohort.

It is well established that anthrax LT induces pyroptosis in macrophages [24], this rapid killing occurs LT activation of the Nlrp1 inflammasome resulting in caspase-1 activation[25,26], it is therefore possible that this mechanism may have eliminated many of the ANTXR1 expressing cells in the cohort of individuals who have been previously exposed. It has been suggested that this LT-mediated activation of Nlrp1b and subsequent lysis of macrophages may be a protective host-mediated innate immune response as opposed to a virulence mechanism exploited by *B. anthracis* [26].

Although we demonstrated that unexposed individuals can be classified as high or low expressers based on the percentage of positive monocytes, universally high levels of PA binding to these cells was observed. This is likely to be due to the co-expression of ANTXR2 and/or beta1-integrin on the monocytes. Unfortunately due to the logistical complexity in obtaining these invaluable convalescent samples it is not feasible to go back and reassess the expression of these additional receptors. The rarity of these samples is reflected by the small sample number in which we were able to assess the

levels of PA binding. Despite this, we clearly see that the reduction in ANTXR1 expression is associated with a reduction both in the percentage of PA positive cells and the MFI of PA binding. While ANTXR2 is reported to bind PA with a far greater affinity than ANTXR1 [27], the pH at which binding of PA to the receptor occurs also differs; with ANTXR1 binding at the more physiologically relevant pH 6.8 in comparison to ANTXR2 which binds at pH5.6 [28]. Furthermore, it has been reported that a mutant form of ANTXR1, L56A, performs similarly if not slightly better than ANTXR2 mutants in both in vitro and in vivo toxin protection assays [29]. Taken together, this is indicative that the reduction in the percentage of monocytes expressing ANTXR1 could have a biological role in the host response to anthrax toxins.

Individuals in the convalescent cohort were infected at least one year previously, and in one case the over 7 years previously [21]. This suggests either that infection causes a permanent alteration in the expression of ANTXR1 or that there is ongoing exposure without subsequent re-infection which maintains the depressed expression levels, or that low-expressers among that population were more likely to become infected in the first place.

There was no correlation between the levels of ANTXR1 expression observed within this study and the IFN γ response to PA in same AVP vaccinated cohort [21], which suggests that a cellular recall response to PA is not related to ANTXR1 expression per se. However, only a minority of vaccinees responded to PA [21], only responsive individuals were included in the analysis a much stronger r^2 value was observed, this was not statistically significant but this may be more reflective of the small number of responders. In contrast, in naturally infected individuals a lower percentage of

ANTRX1 expression on monocytes significantly correlated with an increased PA T cell specific IFN γ response in that individual. Murine models have suggested that IFN γ responses by these CD4 T cells are protective against anthrax spores [30].

This is the first documentation of modulation of the expression of ANTXR1 in humans due to exposure to anthrax toxins. There is a significant correlation between low ANTXR1 expression and high cellular IFN γ recall responses to PA in individuals exposed to toxin in the context of natural infection, suggesting that the attenuation of ANTXR1 expression is one of the protective mechanisms to prevent re-infection in convalescent cutaneous anthrax patients.

(6) Acknowledgments

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Legends

Table 1. The percentage of subjects within each cohort defined as 'low' or 'high' expressors of ANTXR1 based on the percentage of positive monocytes detected by flow cytometry.

Figure 1; Representative flow cytometry data in a non-exposed control subject are shown depicting the levels of PA binding (A top panel) and ANTXR1 expression (A bottom panel) on NK cells (CD3⁻ CD56⁺), NKT cells (CD3⁺, CD56⁺), T cells (CD3⁺, CD56⁻), B cells (CD19⁺) and monocytes (CD14⁺). Cells were defined as positive if the levels of Alexa488 conjugated PA or ANTXR1 (TEM8) antibody (black line) were above the non specific background level of conjugated bovine serum albumin or an IgG isotype control respectively (grey line). The percentage of leukocytes binding PA (B) and positive for ANTXR1 (C) in the peripheral blood of health non-anthrax exposed volunteers was examined. The median fluorescent intensity (MFI, an indication of levels of binding per cell) of PA binding on NK, NKT and T cell was determined (D). PA binding to NKT is significantly higher than binding to T cells or NK cells ($p = 0.008$ and $p = 0.02$ respectively). However, a higher percentage of B cells and monocytes bound PA (D) and monocytes showed a significantly higher ANTXR1 MFI ($p = 0.002$) (E).

Figure 2. The percentage of ANTXR1 positive monocytes in UK healthy volunteers (A) and in naturally infected convalescent, vaccinated and unexposed (UK and Turkish) individuals (B).

Figure 3. The percentage of monocytes binding PA (A) and the MFI of levels PA in naturally infected convalescent ($n=4$), vaccinated ($n=10$) and unexposed (UK)

individuals (n=7) was determined by flow cytometry. Significantly lower levels of PA binding in the convalescent samples compared to both the unexposed controls and the AVP vaccinated individuals ($p = 0.03$ and 0.007 respectively), while PA MFI was significantly lower compare to the AVP vaccinated subjects ($p = 0.008$) but not unexposed controls ($p = 0.09$).

Figure 4. Whilst there was no significant relationship between the percentage of monocytes expressing ANTXR1 and IFN- γ ELISpot responses to PA in AVP vaccinated subjects (A), naturally infected individuals showed an inverse correlation between ANTRXR1 expression and IFN γ responses to PA (B), i.e. those individuals with the lowest percentage of ANTXR1 positive monocytes showed the highest levels of IFN- γ production in response to PA stimulation.

Table 1

cohort	Level of ANTXR1 Expression	
	Low	High
	≤35%	>35%
Naturally infected	100	0
AVP vaccinated	0	100
Non-exposed (Turkey)	40	60
Non-exposed (UK)	50	50

Figure 1

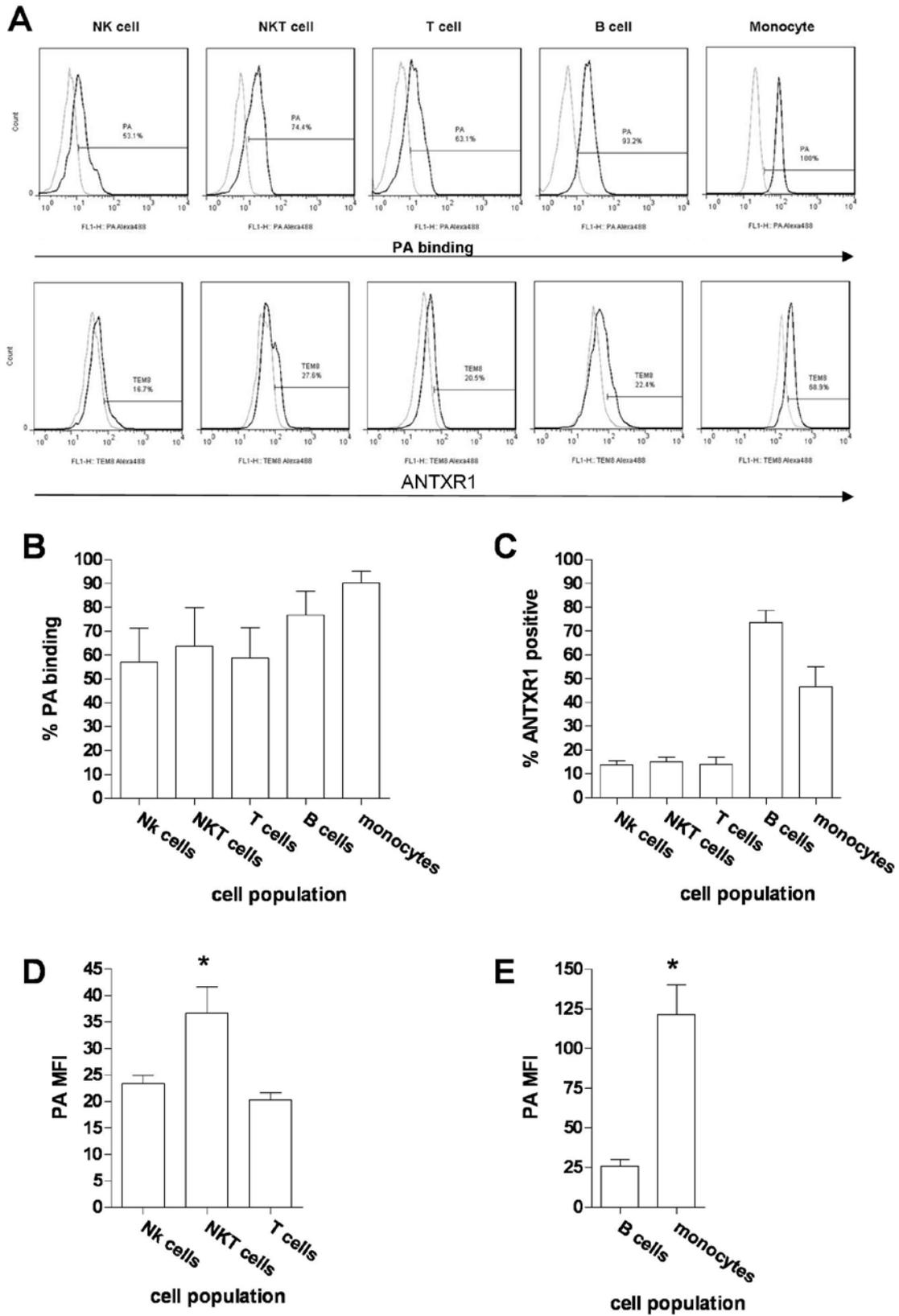


Figure 2

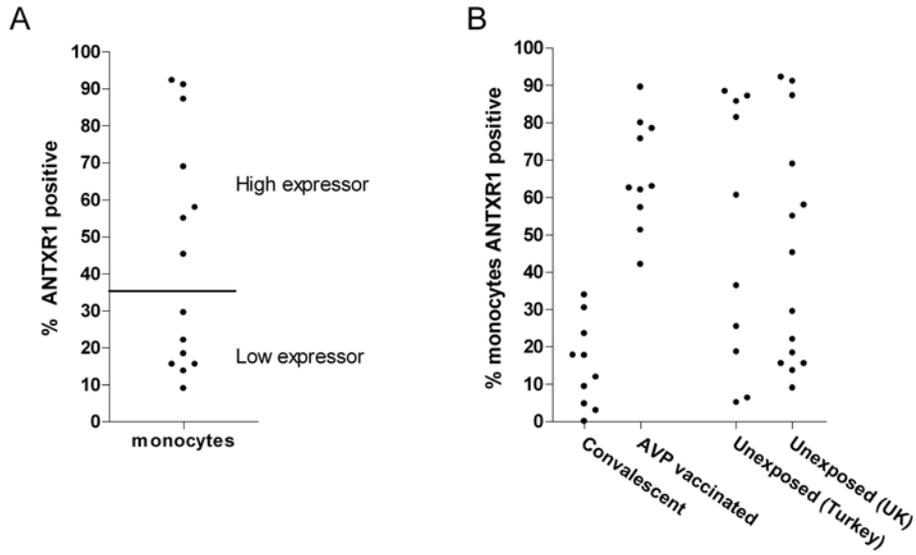


Figure 3

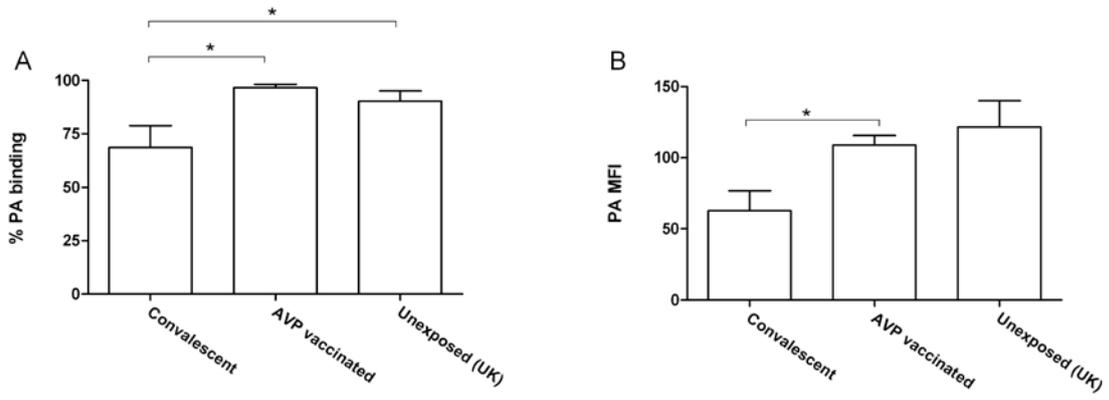


Figure 4

