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Adenovirus vector-specific T cells demonstrate a unique memory phenotype with high proliferative potential and co-expression of CCR5 and integrin $\alpha_4\beta_7$

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Running title: Ad5-specific T cells express CCR5 and integrin $\alpha_4\beta$

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Brief Communication

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Abstract

Background: The Step Study was a randomized trial to reduce HIV infection through vaccination with an adenovirus type 5-based gag/pol/nef construct; analysis following early cessation of the trial revealed an excess of HIV seroconversion in Ad5 seropositive men. This led to the suggestion that the Ad based vector may boost the number of $CD4^+$ $CCR5^+$ T-cells, target cells for HIV infection.

Objectives: We sought to determine the immunophenotype and proliferative capacity of Ad5-specific T cells in the peripheral blood of adult donors to determine whether stimulation with replication defective Ad5 vectors could result in the significant expansion of a CD4+ CCR5+ T cell subset.

Methods: Ad5 specific T cells were identified in the peripheral blood of healthy donors by IFN-γ secretion assay and proliferative response was measured by CFSE labelling. Cells were analysed by flow cytometry to determine T cell differentiation marker, CCR5 and $\alpha_4\beta_7$ expression on memory and proliferated cells.

Results: Ad5-specific $CD4^+$ T cells within healthy adult donors exhibit a unique minimally differentiated memory phenotype with co-expression of CD45RA, CD45RO and CCR7. Stimulation with Ad vector leads to rapid expansion *in vitro* and a switch to an effector memory phenotype. Both short-term reactivated and proliferating Ad5-specific CD4+ T-cells express the HIV co-receptor CCR5 and the HIV gp120-binding integrin $\alpha_4\beta_7$.

Conclusion: Ad5-specific T cells demonstrate a phenotype and proliferative potential that would support HIV infection; these results are pertinent to the findings of the Step Study and future use of Ad5 as a vaccine vector.

Keywords: Adenovirus, HIV, vaccination, CD4 T lymphocytes, immunophenotype

Introduction

Replication deficient adenovirus (Ad) vectors have proven to be excellent vaccine vectors in pre-clinical animal models [1] but the question of how pre-existing immunity to Ad will impact on their efficacy in humans remains: Most individuals have been exposed to at least one of the 52 human adenovirus serotypes. While the continued development of alternative serotype and non-human Ad vectors may overcome the problem of type-specific neutralizing antibodies [2], there is still the potential problem of Ad species cross-reactive T cells. The Step Study sought to reduce HIV infection rates or HIV viral load setpoint and randomized 3000 HIV seronegative donors to placebo or three vaccinations with a gag/pol/nef-Ad5 vector [3]. Despite induction of cellular immunity in most subjects, the hazard ratio for HIV infection was actually increased in Ad5 seropositive men. This led to the suggestion that Ad5-based vaccination may cause activation or proliferation of Ad-specific T cells, thus expanding the pool of $CD4^+$ $CCR5^+$ positive cells within which HIV could establish initial infection [3, 4]. Potential mechanisms to explain an increase in Ad-specific T cells are being elucidated [4]. Perreau et al have described Ad-neutralizing antibody immune complexes which are highly immunogenic and stimulate vector-specific $CD4^+$ T cells *in vitro* that are capable of supporting HIV replication [5]. Recently Koup and colleagues found no rise in vector-specific T cells in patients receiving immunization with E1/E3/E4 deleted Ad5 vectors [6]; whether this is due to the additional E4 deletion, T cell migration or the use of lower sensitivity techniques is unclear.

Here we use high sensitivity techniques to perform a detailed immunophenotype of Ad5-specific T cells from healthy donors and demonstrate that Ad-specific memory T cells exhibit a minimally differentiated memory phenotype and can rapidly proliferate upon antigenic stimulation. Both the memory and effector/memory $CD4^+$ populations express the HIV co-receptor CCR5 and the gp120-binding integrin $\alpha_4\beta_{7,}$ and their expansion may increase the risk of HIV infection in susceptible individuals.

Methods

IFN-γ cytokine secretion assay

PBMCs were stimulated with an E1/E3 deleted replication defective Ad5 vector (AdNTR, [7] 10^4 particles/cell, 16hrs), and Ad-specific T cells were detected using the IFN-γ cytokine secretion assay [8] according to the manufacturers instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). As a negative control cells were mock infected (16hr) or as a positive control stimulated with staphylococcus enterotoxin B (SEB, Sigma, Pool, UK) (1 μg/ml, 3hrs). A donor was considered positive if the if the virus stimulated sample was ≥ 0.01 % of the mock, numbers represent the percentage of CD4+ T cells secreting IFN-γ in response to Ad5 minus the % background (mock). For enrichment prior to analysis cells were positively selected using an autoMACS separator (Miltenyi Biotec).

Flow cytometry

A LSR II flow cytometer (BD Bioscience, San Jose, CA, USA) was used and data analyzed using FlowJo software (Tree Star, Ashland, OR, USA). The following antibodies were used according to manufacturers instructions: anti- CD3/pacific blue, CD4/pacific orange, CD8/allophycocyanin(APC)-H7, CD27/fluorescein isothiocyanate (FITC), CD28/ECD, CR7/phycoerythrin(PE)-Cy7(PE-Cy7), CCR5/PE-Cy7, CD62L/APC, CD45RA/ECD or Alexa Fluor700(AF700), CD45RO/AF700,

CD49d/APC, CD57/FITC, integrin β7 unconjugated, donkey anti-rat FITC or PE (BD Biosciences), CD45RA/AF700, CD45RO/AF700 (Biolegend, San Diego, CA, USA)

T cell proliferation

The IFN-γ cytokine secretion assay was used to determine day 0 frequency of Ad-specific T cells. At the same time PBMCs were labelled with 2.5μM CFSE (carboxyfluorescein succinimidyl ester; Invitrogen) for 15 mins, washed and stimulated as above. Total cell number was counted using a haemocytometer, and cells were immunophenotyped using the antibodies above following 3 and 7 days culture. To derive Ad-specific T cell frequency the percentage $CD3^+$ $CD4^+$ $CFSE^{Dim}$ was determined by flow cytometry and then multiplied by total cell number; these are expressed as fold expansion from the day 0 starting frequency.

Ad5 neutralizing Ab titre

The level of neutralizing Ab in donor serum was quantitated using an Ad5 vector expressing β-galactosidase as previously reported [9, 10].

Results

Ad5-specific $CD4^+$ T cells within healthy adult donors exhibit a unique minimally differentiated memory phenotype.

The frequency of Ad5-specific T cells in peripheral blood was measured using IFN-γ cytokine secretion assay following stimulation with a replication deficient Ad5 vector. Fig. 1a shows that Ad-specific CD4 T cells were observed in all 12 donors, with a mean frequency of 0.14% of the CD4+ repertoire (range 0.01-0.46 %). An example of cytokine secretion in one donor is shown in Fig. 1b. Donor serum was screened for Ad5 specific neutralizing antibodies, all donors were seropositive, no correlation (Spearman rank correlation test P=0.73) was seen between frequency of Ad5 specific T cells and neutralising Ab titre (data not shown) consistent with the recent report of O'Brien et al [11]. IFN-γ secreting cells were selectively enriched by magnetic selection and a detailed immunophenotype was determined through the use of multicolour flow cytometry (Fig. 1c). In all 5 donors studied, IFN-γ-secreting Ad5-specific $CD4^+$ T cells largely expressed the chemokine receptor CCR7 which is representative of a central memory phenotype, and expression of CD27 and CD28 was also retained on most cells [12, 13]. Interestingly, however, over 60% of cells also highly expressed the CD45RA isoform which is usually associated with naïve $CD4^+$ T cells or 'revertant' effector memory (EMRA) populations [14] (Fig. 1d). As these populations had been defined on the basis of their expression of IFN-□ in response to short-term stimulation with adenovirus, they clearly do not represent a naïve T cell subset. However, expression of the co-stimulation/survival receptors CD27 and CD28 argues strongly against their representing typical EMRA subpopulations.

To further characterize their unique phenotype, Ad5-specific T cells were stained for CD45RO and LFA-1 which are found only upon antigen-experienced T cells, the secondary lymphoid homing marker CD62L which is expressed on naïve and central memory cells but not effector cells, and CD57 which is found on terminally differentiated effector cells. Ad5-specific T cells displayed a consistent $CD45RO^+$ and $LFA\text{-}1^+$ phenotype, defining them as true antigen-experienced subsets. CD57 expression was not observed, and staining with CD62L showed a heterogonous pattern (Fig. 1e). Thus Ad5-specific memory CD4+ T cells in the peripheral blood appear to be maintained in a minimally activated antigen experienced memory state and have not yet undergone down regulation of CD45RA

expression. The phenotype was markedly different to the SEB stimulated or total CD4 cells (see supplementary digital content 1) and is in contrast to memory CD4+ CMV specific T cells which display a fully differentiated memory phenotype [15-18]. We hypothesized that if Ad5-specific T cells comprised a unique early central memory phenotype rather than an RA+ effector phenotype then the cells should have high proliferative capacity and would down-regulate expression of CD45RA upon prolonged culture. To characterize the proliferative response, Ad5 stimulated cultures from 4 donors were monitored using CFSE labelling and immunophenotypic analysis over 7 days. During culture it was observed that the proliferative population lost expression of CD45RA, and also underwent downregulation of CCR7 and CD28 (Fig. 1f), a transition characteristic of a switch to effector phenotype.

Ad5 specific CD4 T cells have high proliferative capacity and express CCR5 and $\alpha_4\beta_7$ in resting and proliferated states.

To investigate whether Ad-specific T cells could act as targets for HIV infection, we measured expression of the HIV co-receptor CCR5 and integrin $\alpha_4\beta_{7,}$ which can bind HIV gp120 [19]. Expression was determined after short term *ex vivo* stimulation using the IFN-γ cytokine secretion assay or after several days in culture in combination with CFSE labelling. Fig. 2a shows that Ad5-specific memory $CD4^+$ T cells express high levels of CCR5 and $\alpha_4\beta_{7,}$ maintained through 7 days. In addition, they demonstrate high proliferative potential: the T cells expand rapidly following antigen stimulation, resulting in a mean 320 fold expansion over 7 days (n=6, range 120-464) (Fig. 2b,c).

Discussion

It is established that adenoviruses are widespread in the human population and that a significant proportion of individuals will have pre-existing immunity especially to the more prevalent serotypes such as Ad5 [20]. Neutralizing antibodies may limit the efficiency of gene delivery by Ad vectors, however this is likely to be overcome by the use of alternate serotype or non-human adenovirus vectors, as neutralizing antibodies are serotype specific. In contrast the immunodominant antigen targeted by cellular immunity is the highly conserved hexon protein, leading to a broadly cross-reactive memory T cell repertoire [21]. The majority of studies to date have found CD4 Ad-specific T cells to be more numerous than CD8s [22, 23] and have shown them capable of cytotoxicity, proliferation [24] and secretion of Th1 cytokines such as IFN-γ, TNF-α IL-2 [25]. Feuchtinger and colleagues described hexon-specific T cells isolated for adoptive immunotherapy by a similar IFN-γ secretion technique as used in this study to be composed of $CD45RA^+$ $CD27^+$, $CD62L^+$ cells [26]. However these populations did not express CCR7, leading the authors to describe them as an intermediate effector memory phenotype. By examining the phenotype in further detail we show that short-term reactivated Ad5 specific T cells display a unique early memory phenotype. Cells co-express CD45 RA and RO as well as CCR7, their ability to secrete cytokine clearly indicates they are not naïve cells, and the expression of the co-stimulatory receptor CD27 and CD28 indicated they are not a CD4+ equivalent of the CD8+ EMRA populations seen following chronic exposure to viruses such as CMV. These cells had a very high proliferative capacity and increased on average 320 fold in 7 days, following antigenic stimulation. Importantly the CD4 T cells in both memory and proliferating states express the HIV co-receptor CCR5, indicating they are potential target for HIV infection. Integrin $\alpha_4\beta_{7,}$ which has been shown to bind gp120 and may be an alternative co-receptor for HIV [19] was also highly expressed on the Ad-specific T cells. The importance of $\alpha_4\beta_7$ as a co-receptor in HIV lifecycle is unclear [27]; the interaction may alternatively serve to induce activation of LFA-1

enhancing virus replication and cell to cell spread [19]. It has recently been shown that simian immunodeficiency virus preferentially targets minimally differentiated CD4+ T cells that express integrin $\alpha_4\beta_7$ [28].

These results add to the debate concerning the use of adenovirus vectors for vaccination against HIV. Ad5-specific memory CD4 T cells are found in the majority of healthy adults [21-24, 26, 29] and reside in a unique minimally differentiated memory phenotype with extremely high proliferative capacity. This subset expresses the requisite receptors for HIV infection and may represent a potential reservoir of HIV-susceptible cells following primary infection.

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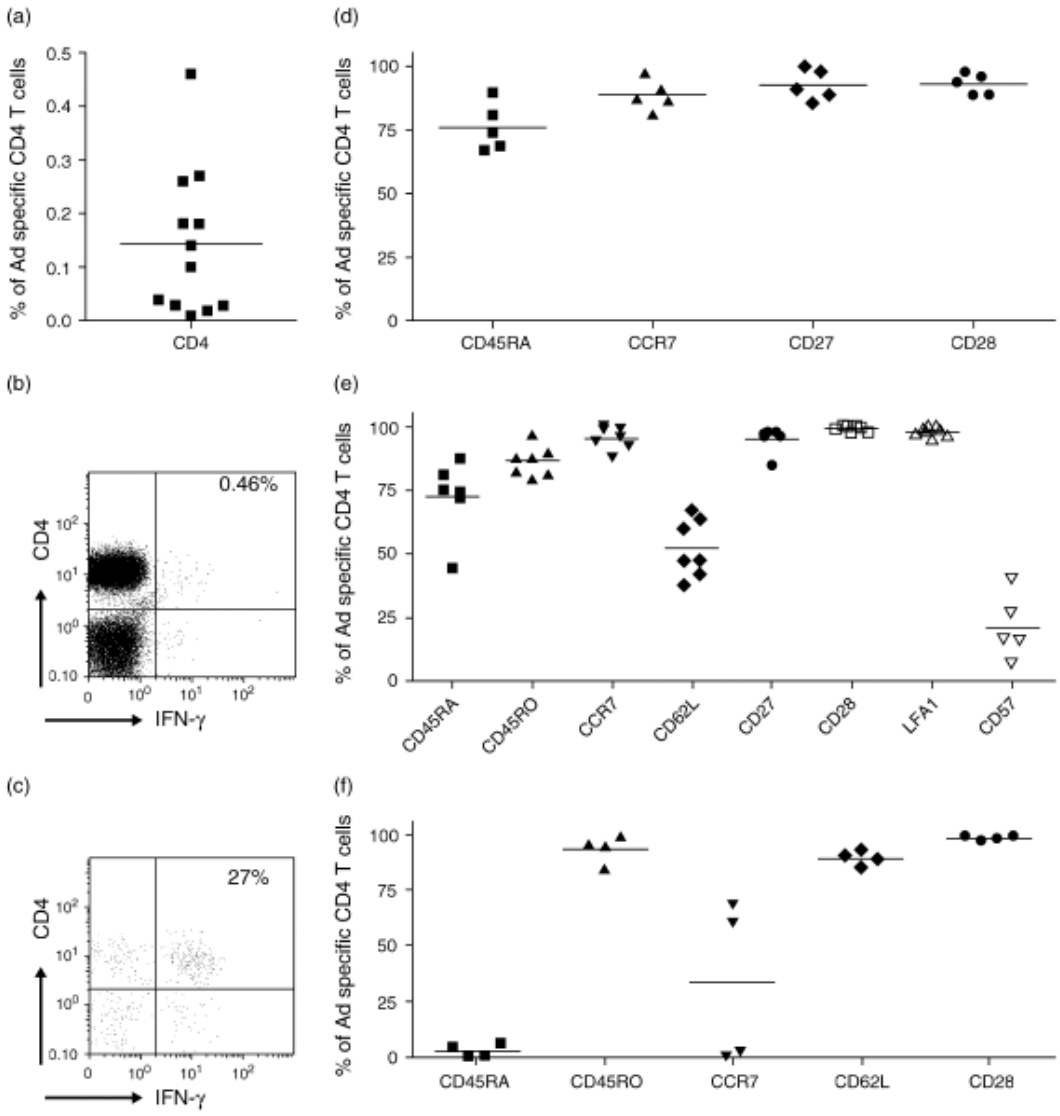

Fig. 1 Ad5-specific CD4+ T cells display an RA+ minimally differentiated phenotype and become effector cells upon proliferation. (a) Frequency of CD4+ Ad5-specific measured by IFN-γ cytokine secretion in response to stimulation with Ad-NTR (n=12). Example of IFN-γ secretion assay pre (b) and post (c) selection. (d) Classical memory phenotype marker expression on CD3+ CD4+ Ad-specific T cells post IFN-γ cytokine selection (n=5). (e) Extended phenotype marker expression on CD3+ CD4+ Ad-specific T cells post IFN-γ cytokine selection (n=7). (f) Phenotype marker expression on CFSEDim, CD3+ CD4+ Ad5-specific T cells 7 days post stimulation with Ad-NTR (n=4).

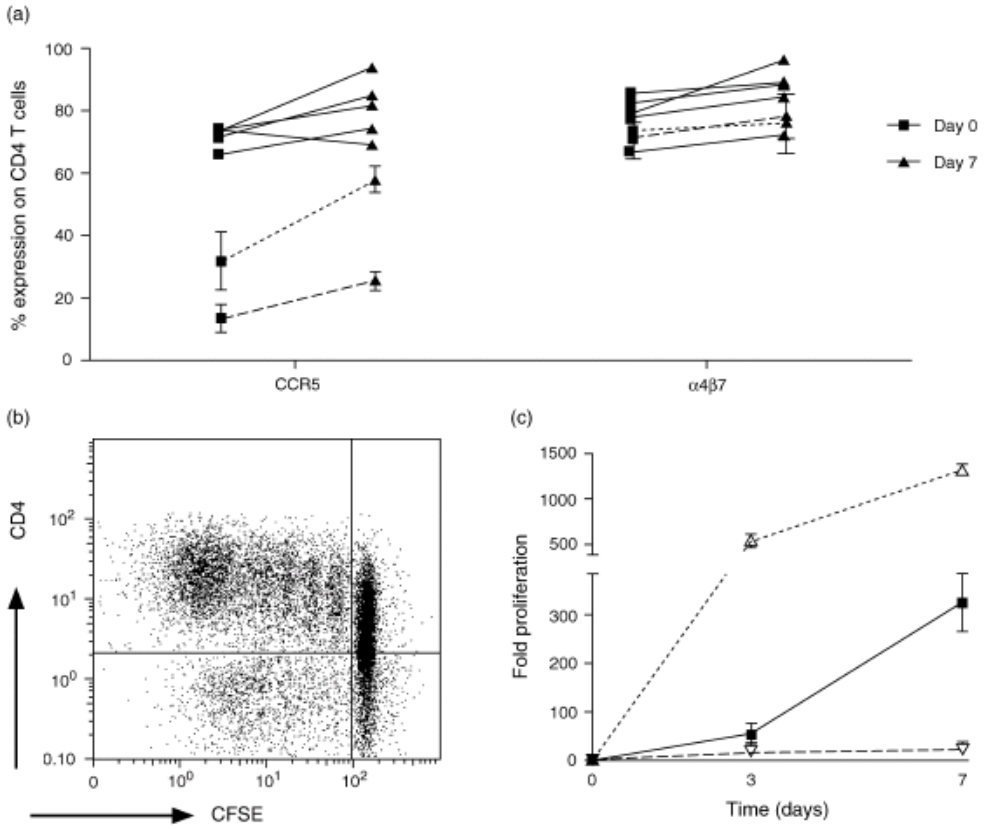

Fig. 2 Ad5-specific CD4 cells express CCR5 and α4β7 in memory and effector phenotypes and have a high proliferative capacity. (a) CCR5, α4 and β7 integrin expression (dual positive for both sub-units) on; CD3+ CD4+ Ad-specific T cells day 0 and day 7 post stimulation with Ad-NTR (solid lines), mean of total CD3+CD4+ T cells in mock stimulated cultures (dashed line) and mean of CD3+CD4+CFSEDIM in the SEB stimulated culture (dotted line). Error bars represent one standard deviation, n=5 (b) Example of proliferation as measured by CFSE labelling, 7 days post stimulation with Ad-NTR. (c) Fold proliferation of CD4 T cells stimulated with Ad-NTR (solid line), mock stimulated (dashed line) or stimulated with SEB (dotted line); percentage of CD3+, CD4+, CFSEDim cells was measured by flow cytometry and multiplied by total cell counts to derive Ad-specific T cell numbers; these are expressed as fold expansion from day 0 starting frequency determined by IFN-γ secretion assay. Error bars represent one standard deviation, n=5.

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