Activation of a dendritic cell–T cell axis by Ad5 immune complexes creates an improved environment for replication of HIV in T cells

Matthieu Perreau,[1] Giuseppe Pantaleo,[1,2] and Eric J. Kremer[3,4]

[1]Service of Immunology and Allergy, Department of Medicine, Centre Hospitalier, Universitaire Vaudois, Lausanne, University of Lausanne, CH-1011 Lausanne, Switzerland
[2]Swiss Vaccine Research Institute, 1011 Lausanne, Switzerland
[3]Institut de Génétique Moléculaire de Montpellier, Centre National de la Recherche Scientifique 5535, 34293 Montpellier, France
[4]Université Montpellier I and II, 34967 Montpellier, France

The STEP HIV vaccine trial, which evaluated a replication–defective adenovirus type 5 (Ad5) vector vaccine, was recently stopped. The reasons for this included lack of efficacy of the vaccine and a twofold increase in the incidence of HIV acquisition among vaccinated recipients with increased Ad5–neutralizing antibody titers compared with placebo recipients. To model the events that might be occurring in vivo, the effect on dendritic cells (DCs) of Ad5 vector alone or treated with neutralizing antiserum (Ad5 immune complexes [IC]) was compared. Ad5 IC induced more notable DC maturation, as indicated by increased CD86 expression, decreased endocytosis, and production of tumor necrosis factor and type I interferons. We found that DC stimulation by Ad5 IC was mediated by the Fcγ receptor IIa and Toll–like receptor 9 interactions. DCs treated with Ad5 IC also induced significantly higher stimulation of Ad5–specific CD8 T cells equipped with cytolytic machinery. In contrast to Ad5 vectors alone, Ad5 IC caused significantly enhanced HIV infection in DC–T cell cocultures. The present results indicate that Ad5 IC activates a DC–T cell axis that, together with the possible persistence of the Ad5 vaccine in seropositive individuals, may set up a permissive environment for HIV–1 infection, which could account for the increased acquisition of HIV–1 infection among Ad5 seropositive vaccine recipients.

CORRESPONDENCE
Giuseppe Pantaleo:
giuseppe.pantaleo@chuv.ch
OR
Eric J. Kremer:
eric.kremer@igmm.cnrs.fr

The detection of high frequencies of HIV-1–specific CD4 and CD8 T cells in HIV-1–infected subjects with nonprogressive disease (1), the demonstration that CD8 T cells are key players in vivo in the control of SIV replication (2), and the association of polyfunctional CD4 and CD8 T cells with better control of virus replication (1, 3, 4) provided the rationale for developing T cell vaccine strategies that, although they are unlikely to prevent infection, may eventually control HIV replication after infection (5).

The T cell vaccines that have entered clinical evaluation include adenovirus (Ad) and poxvirus vectors (6, 7). Ad vectors were used either alone or in combination with DNA-based vaccines. Each of these approaches has induced vigorous T cell responses (8, 9) and has partially controlled SIV replication in non-human primates (6). The high seroprevalence

to some Ad serotypes in target populations remains a major issue for these vectors (7, 8).

On the basis of the encouraging preclinical and phase I/II clinical programs, a trivalent Ad5-gag/pol/nef vaccine candidate entered a phase II test-of-concept-efficacy study called STEP in December 2004 and enrolled 3,000 high-risk HIV seronegative subjects. The primary objectives of the study were to determine the effects of the vaccine on the reduction of the acquisition of infection and on the reduction of the set-point viremia. Late last year, STEP was prematurely terminated because of a lack of efficacy and because of the observation of a twofold increase in the incidence of HIV acquisition among vaccinated recipients with

The online version of this article contains supplemental material.

high Ad5 neutralizing antibodies (Abs [NAbs]) titers compared with placebo recipients (www.HVTN.org).

Several hypotheses have been proposed to explain the increased acquisition of HIV infection including the following: unique microenvironment of the mucosal compartment where the mechanisms for increased acquisition to HIV infection likely operate; generation of enhancing Abs that facilitate HIV infection; and vector-mediated activation of Ad5-specific CD4 T cells that become ideal targets for HIV infection. It is important to mention that Ads are not cleared after infection, and numerous clinical reports have demonstrated that Ads cause latent infections that are normally well controlled by the host (10). Therefore, the administration of an Ad5 vector, its persistence, and continual exposure may have an impact on both Ad-specific Abs generation and T cell responses.

In this study, we developed a series of ex vivo strategies to delineate the immunological events that may have operated among vaccine recipients with preexisting immunity to Ad5.

In particular, we investigated the effects of Ad5 immune complex (IC) exposure on DCs, Ad5-specific CD4 and CD8 T cell responses, and enhancement of HIV infection.

RESULTS AND DISCUSSION
Effects of Ad5 IC on DCs

Ad5-specific memory T cells and Ad5 NAbs are an important component of Ad5 preexisting immunity (11), and interaction between Ad5 vector and NAbs certainly occurs rapidly after the administration of vectors in vivo. Ad5 IC are also well characterized in vitro (12, 13). Thus, we investigated the effects of Ad5 IC on DCs. For these purposes, we generated Ad5 IC by mixing Ad5 vectors with sera containing Ad5 NAbs. Formation of ICs was determined by assessing C1q (complement protein 1q) binding (14). To reproduce the scenario in the STEP trial, the Ad5 vectors used in the present study are also E1/E3 deleted. To preclude the possibility that the effects were caused by complement activity,
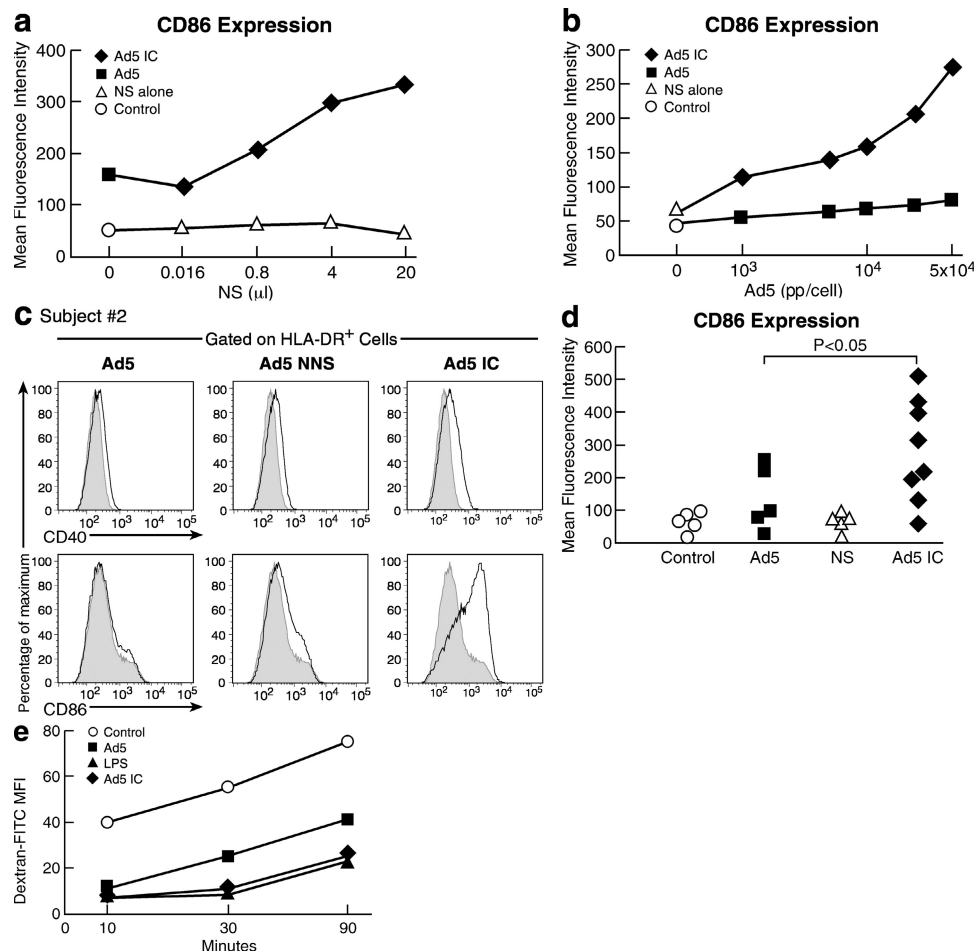

Figure 1. DCs activation and maturation. (a) Flow cytometry analysis of CD86 expression after treatment of DCs with Ad5 vector alone (filled square), Ad5 IC (formed with a fixed Ad5 dose and increasing serum volumes [Ad5 NAb titer of 512]; filled diamonds), neutralizing serum (NS) alone (open triangles) and mock-treated DCs (open circle). (b) Untreated DCs (open circle) treated with a range of Ad5 doses (filled squares) or with Ad5 IC (filled diamonds) formed with 20 µl of sera (Ad5 NS titer of 512) and various Ad5 doses and with NS alone (open triangle). (c) Flow cytometry profile of CD40 and CD86 expression in DCs treated with Ad5 vector, Ad5 vector plus NNS, or Ad5 IC. (d) Cumulative data (five DC donors and eight different sera) of CD86 expression. (e) Ag uptake after incubation of DCs with Ad5 vector, Ad5 IC, LPS, or mock treated. The assays were repeated three times with similar results.

heat-inactivated sera (30 min at 56°C) were used throughout this study. The effects of Ad5 IC on DCs were assessed initially by analyzing the induction of the expression of costimulatory molecules (CD40 and CD86). Consistent with previous studies (15), Ad5 vectors alone did not induce a substantial increase in the expression of costimulatory molecules by immature DCs (Fig. 1, a–c). The dose of Ad5 vector used, 2.5×10^4 physical particles (pp)/cell was consistent with that used in previous studies (11, 16). To determine the effects of Ad5 IC, ICs were generated by progressively increasing the volume of serum that contained high Ad5 NAbs titers (~512) in the presence of a fixed dose of Ad5 vector. The neutralizing activity of the sera was confirmed by determining the levels of Ad5 vector transduction as previously described (14) and using DCs. The percent of AdGFP-transduced DCs was inversely proportional to the volume of serum, thus demonstrating the presence of Ad5 NAbs (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20081786/DC1).

Up-regulation of CD86 expression was observed 48 h after treatment of immature DCs with Ad5 IC, and the up-regulation paralleled the increase in serum volume and thus in Ad5 IC formation (Fig. 1 a). Similarly, up-regulation of CD86 was also observed using a fixed volume of serum and increasing the amount of Ad5 vector (Fig. 1 b). No significant increase in the expression of CD86 was observed in the presence of serum or with Ad5 vector alone (Fig. 1, a–d). Significantly ($P < 0.05$), increased expression of CD40 and CD86 were observed in the presence of Ad5 IC and not in the presence of Ad5 vector alone or after treatment of DCs with Ad5 plus a nonneutralizing serum (Fig. 1, c and d).

To test the functional maturation of DCs induced by Ad5 IC, we assayed their ability to take up antigens (Ags). Immature DCs efficiently capture Ags primarily through macropinocytosis and mannose receptor-mediated endocytosis, but they lose this function during maturation (17). Immature DCs and DCs treated with Ad5 vector alone, with Ad5 IC, or with LPS were incubated with FITC-labeled dextran, and the level of Ag uptake was evaluated as a function of time (Fig. 1 e). Consistent with functional immaturity, mock-treated immature DCs maintained their ability to internalize the dextran. DCs incubated with Ad5 vector alone induced modest DC maturation and, indeed, their ability to Ag uptake was partially preserved. Consistent with the induction of maturation, Ag uptake by DCs treated with Ad5 IC or LPS was lost.

To better define the effects of Ad5 IC on activation and maturation of DCs, we determined the cytokine profile of DCs treated with Ad5 vector, sera ± NAbs, Ad5 incubated with a nonneutralizing serum (NNS) or Ad5 IC. The panel of cytokines investigated included TNF-α, IL-12p70, IL-10, IFN-I, IFN-γ, and IL-1β. DCs treated with Ad5 vector, Ad5/NNS, or neutralizing serum, did not induce the release of any significant levels of the cytokines tested (Fig. 2, a and b; and not depicted). In contrast, when assaying Ad5 IC our results were consistent with those found when testing the effects of Ad5 IC on the induction of the expression of costimulatory molecules (Fig. 1). Progressive increments in secretion

of TNF-α and IFN-I from DCs were observed either at a fixed dose of Ad5 vector together with increasing volume of serum or vice versa (Fig. 2, a and b). No secretion of the other investigated cytokines was observed (unpublished data). We then investigated the relationship between the NAb titers and the levels of cytokines secretion. Interestingly, we found a significant correlation ($P < 0.05$) between the levels of TNF-α and IFN-I secreted and the Ad5 NAb titers (Fig. 2, c and d). Collectively, these results indicate that Ad5 IC induced greater activation and maturation of DCs and, more importantly, the anti-Ad5 NAb titer was a key determinant in the induction of DCs activation.

Ad5 IC signal through the Fcγ receptors (FcγRs)

According to previous studies, cross-linking of the FcγRs by immobilized Igs induces expression of costimulatory molecules (18). To better define the role of FcγRs, DCs were treated with Ad5 IC and the induction of TNF-α and IFN-I secretion was determined in the presence or absence of blocking Abs against FcγRI, IIa, and III. The three FcγRs were differently expressed on DCs with the FcγRIIa the most expressed (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20081786/DC1). We found that blocking FcγRI, III, and particularly IIa reduced the secretion of TNF-α and IFN-I (Fig. 3 a). These data demonstrate that FcγRs are
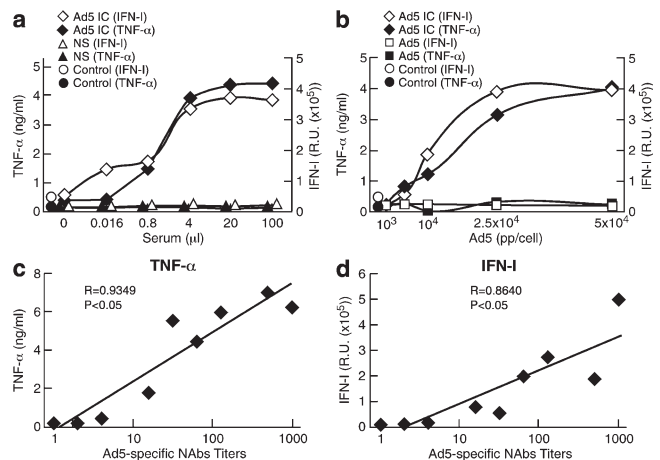

Figure 2. Secretion of proinflammatory cytokines from DCs.
(a) DCs were treated with Ad5 IC (TNF-α, filled diamonds; IFN-I, open diamonds) formed with a fixed dose of Ad5 vector and a range of serum volumes (Ad5 NS titer was 512), NS alone (TNF-α, filled triangles; IFN-I, open triangles). Culture supernatants were collected and analyzed for the presence of TNF-α by ELISA and IFN-I. (b) DCs were also treated with Ad5 vector alone (TNF-α and IFN-I, filled and open squares, respectively) or with Ad5 IC (TNF-α and IFN-I, filled and open diamonds, respectively) formed with 20 µl of serum (Ad5 NAb titer was 512) and untreated DCs (IFN-I and TNF-α, open and filled circles, respectively). Representative data of one out of nine subjects are shown. (c and d) Correlation between levels of TNF-α and IFN-I secretion and Ad5 NAb titers. Each point on the line results from the mean value of several pooled sera analyzed. At least 28 sera were analyzed for TNF-α or IFN-I in duplicate. Statistical analysis was performed using Spearman's rank correlation.

involved in delivering an Ad5 IC–derived activation signal to DCs. To further exclude the effects of unknown serum components, we used purified Igs (14) to generate Ad5 IC and confirmed the efficient induction of DC activation (unpublished data). The purified Igs were predominantly IgG (IgM and IgA were a minor fraction), and IgG$_1$ and IgG$_2$ were the predominant isotypes (unpublished data).

Signaling through FcγRs may be caused by either direct cross-linking or by downstream events of receptor-mediated internalization. To discriminate between these two possibilities, DCs were cultured in wells coated with purified Ad5-specific Abs. No significant secretion of TNF-α, IL-12p70, IL-10, IFN-I, IFN-γ, or IL-1β was observed (unpublished data), thus suggesting that cross-linking of FcγRs is not an activation signal sufficient to induce the release of cytokines in DCs despite the fact that it induces expression of costimulatory molecules. Therefore, these results suggested that down-

Figure 3. Ad5 IC deliver the activation signal to DCs through FcγRs and TLR9. (a) DCs were treated with Ad5 IC in the presence of anti-FcγRI, IIa, and III blocking Abs. Culture supernatants were analyzed for the secretion of TNF-α and IFN-I. Results are expressed as the percent of the TNF-α or IFN-I secretion levels in the cultures stimulated with Ad5 IC in the presence or absence of FcγRs blocking Abs. (b) DCs were treated with Ad5 vector, helper-dependent vector, Ad5 IC, or HD IC . Supernatants were analyzed for the presence of TNF-α and IFN-I. Results are expressed as the percent of the TNF-α and IFN-I secretion levels versus controls (treatment of DCs with Ad5 IC). (c) DCs were stimulated with Ad5 IC (NAb titer of 512) and LPS (TLR4 agonist) or CpG in the presence or absence of TLR9 inhibitor. At the end of the stimulation period, supernatants were analyzed for the presence of TNF-α and IFN-I. Results are expressed as the percent of the cytokine levels measured in culture treated with TLR9 inhibitor versus those found in control cultures (absence of inhibitors). All of the experiments in Fig. 3 were performed twice in triplicate. Error bars represent SEM.

stream events of receptor-mediated Ad5 IC internalization were involved in the delivery of activation signals.

Internalized Ad5 IC deliver activation signals
On the basis of the observations detailed in the previous section, it is possible that FcγR-mediated Ad5 IC internalization preferentially delivers some Ad components (proteins, virus-associated RNA, or the double-stranded DNA genome) to distinct subcellular compartments. To test this hypothesis, we incubated DCs with a helper-dependent Ad5 vector alone or complexed with sera containing NAbs (helper-dependent [HD] IC). Although the genomes of helper-dependent vectors are deleted in all viral genes, the virions contain the same core and capsid proteins and virus–associated RNAs (19). We found that neither the helper-dependent vector alone nor the HD IC promoted the release of TNF-α or IFN-I (Fig. 3 b), suggesting that the genome of the E1/E3-deleted Ad5 vector harbors critical signals for complete DC activation and maturation.

In this regard, nucleic acids of viral or mammalian origin can be recognized by intracellular pathogen recognition receptors. For example, human lupus Ab–DNA complexes activate DCs through the interaction between FcγRII and TLR9 (20). Furthermore, several potential immunoregulatory sequences (IRSs) can inhibit the release of IFN-I after stimulation with TLR9 and TLR7 agonists (21). Among these, IRS 869 mediated the most potent inhibition of IFN-I secretion in TLR9-stimulated plasmacytoid DC (21). We therefore asked whether IRS 869 could suppress TNF-α and/or IFN-I production induced by Ad5 IC. We found that IRS 869 suppressed both TNF-α and IFN-I production after stimulation with Ad5 IC (>90% inhibition; Fig. 3 c). LPS, which stimulates DCs through the cooperation of CD14 with TLR4 (22), and CpG (TLR9 agonist) (21) were used as controls. IRS 869 did not inhibit TNF-α or IFN-I production by DCs stimulated with LPS, whereas it inhibited cytokine production of DCs stimulated with CpG (Fig. 3 c).

Consistent with previous studies (23, 24), our data suggest that Ad5 IC are internalized via FcγRIIa and are likely delivered to intracellular compartments where the vector genome interacts with TLR9, which in turn leads to activation of DCs. Importantly, these data argue against a major role for Ad capsid proteins and RNA.

Ad5 IC stimulate/activate Ad5–specific CD4 and CD8 T cells
To investigate the effects of Ad5 IC on the stimulation/activation of Ad5-specific CD4 and CD8 T cells in Ad5 seropositive subjects, we developed an ex vivo stimulation assay of Ad5-specifc T cells that may mimic those operating in vivo. DCs were generated from five subjects with high (>1,000) Ad5 NAb titers. Immature DCs were incubated with Ad5 vector alone or with Ad5 IC for 24 h. Two sera were used to generate Ad5 ICs for each DC population, autologous serum (from the donor-providing DCs) and heterologous serum with high (4,096) Ad5 NAb titer. At the end of the incubation period, Ad5 vector or Ad5 IC–treated DCs were used to stimulate Ad5-specific CD4 and CD8 T cells
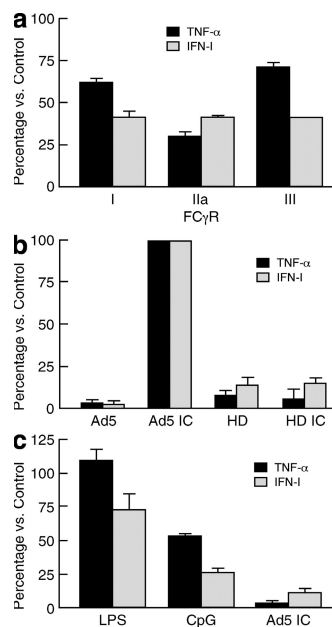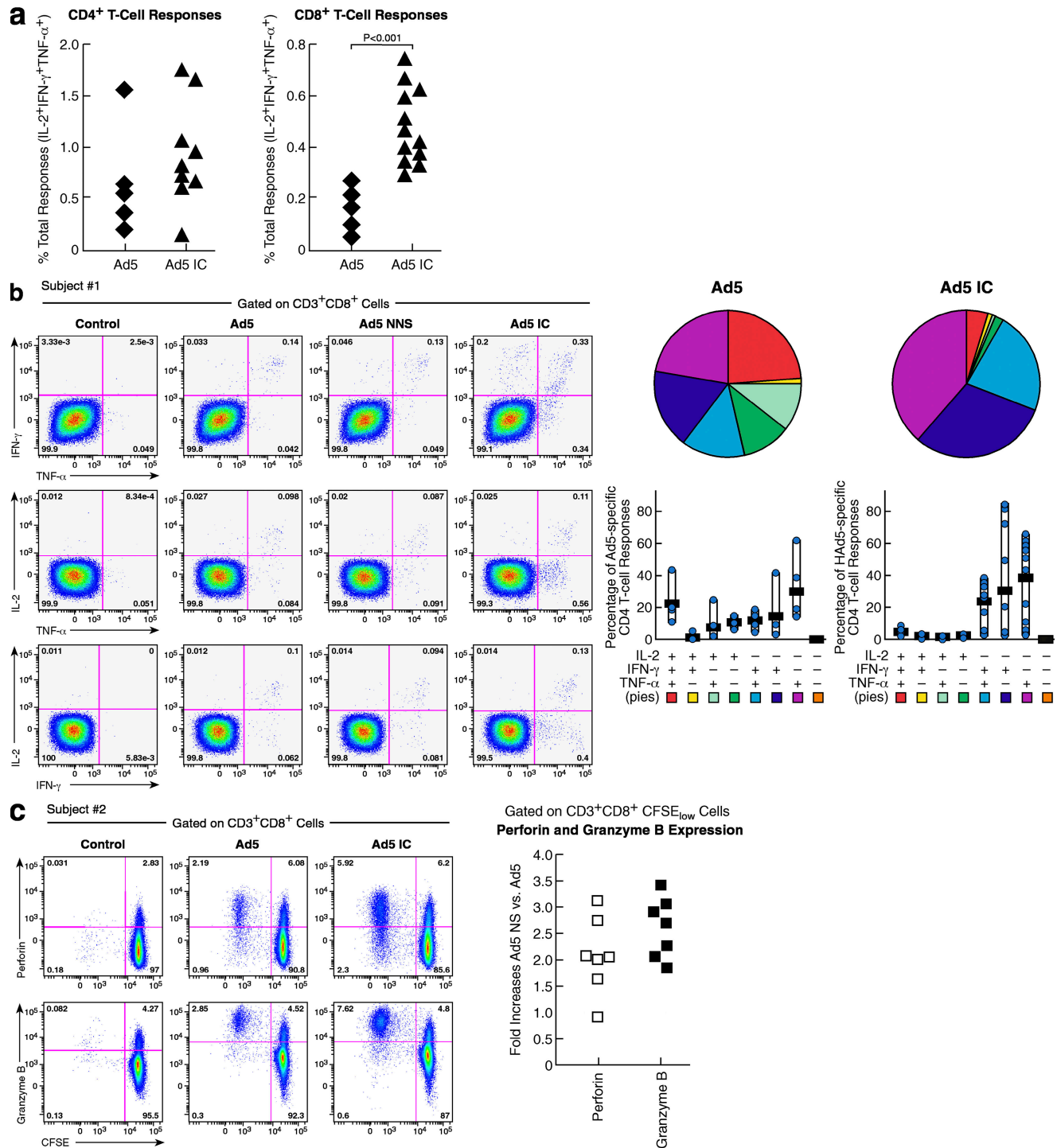
Figure 4. Ad5 vector and Ad5 IC stimulate Ad5–specific CD4 and CD8 T cells. (a) Cumulative data on the percentage of the total cytokine response (IL-2 + TNF-α + IFN-γ) of Ad5-specific CD4 and CD8 T cells after stimulation with Ad5 vector or Ad5 IC–treated DCs. The data were generated from five Ad5 seropositive subjects with Ad5 Ab titers >1,000. Blood mononuclear cells were stimulated with DCs treated with either autologous or heterologous neutralizing sera. (b) Flow cytometry profiles of CD8 T cells secreting IL-2, TNF-α, and IFN-γ after stimulation with Ad5 vector, Ad5 + NNS, or Ad5 IC–treated DCs from a representative subject. All the possible combinations of the responses are shown on the x axis, and the functionally distinct cell populations within the responding CD8 T cell populations are shown on the y axis. Responses are grouped and color-coded on the basis of the number of functions. In the pie chart, each slice corresponds to the fraction of CD8 T cells with a given number of functions within the responding CD8 T cell populations. Bars correspond to the fractions of functionally distinct T cell populations within the total CD8 T cells. (c) Perforin and granzyme B expression in proliferating CD8 T cells. Analysis of perforin and granzyme B expression was determined on gated CFSE-low proliferating CD3+CD8+ T cells after stimulation with DCs treated with Ad5 or Ad5 IC. Cumulative data are also shown.

from autologous blood mononuclear cells. We then assessed secretion of IL-2, IFN-γ, and TNF-α (6 h after stimulation) using polychromatic flow cytometry. No significant difference (P > 0.05) was observed in the percent of total, IL-2, IFN-γ, and TNF-α–secreting Ad5-specific CD4 T cells in blood cells stimulated with Ad5 vector– or Ad5 IC–treated DCs (Fig. 4 a). In contrast, a highly significant (P < 0.001) approximately threefold difference was found in the percentage of total cytokine-secreting Ad5-specific CD8 T cells stimulated with Ad5 IC–treated DCs compared with Ad5 vector–treated DCs (Fig. 4 a). Furthermore, major changes in the cytokine profile of Ad5-specific CD8 (Fig. 4 b), but not CD4, T cells (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20081786/DC1) were observed between the two stimulation conditions. In particular, we detected a significant (P < 0.05) increase in the dual IFN-γ⁺/TNF-α⁺ (Fig. 4 b) and a significant (P < 0.05) decrease in the IL-2–secreting (IL-2⁺/IFN-γ⁺/TNF-α⁺ IL-2⁺/TNF-α⁺ and single IL-2⁺; Fig. 4 b) CD8 T cell populations. With regard to Ad5-specific CD4 T cells, the cytokine profile was modestly different between Ad5 vector alone and Ad5 IC–treated DCs These results indicate that Ad5 IC induced stimulation of higher frequencies of Ad5-specific memory CD8 T cells with an effector cytokine profile.

We next determined the effects of Ad5 IC on the proliferation capacity of Ad5-specific memory T cells and consistently observed a trend toward increase proliferation (about twofold) of CD4 and CD8 T cells stimulated with Ad5 IC–treated DCs, although these differences did not reach statistical significance (P > 0.05; Fig. S4, available at http://www.jem.org/cgi/content/full/jem.20081786/DC1). We also determined the cytotoxic profile of the proliferating (CFSE low) Ad5-specific CD8 T cells based on the expression of perforin and granzyme B. The proportion of Ad5-specific CD8 T cells expressing perforin and granzyme B was ∼2.5-fold greater (P < 0.05) when Ad5 IC–treated DCs were used as stimuli (Fig. 4 c).

Collectively these results indicate that Ad5 IC induced substantial anti–Ad5 vector effector CD8 T cells and a trend toward a larger expansion of Ad5-specifc T cells. The more effective stimulation of Ad5-specific CD8 T cells likely results from the capacity of DCs to efficiently process and present exogenous Ags, such as IC, to CD8 T cells via cross-presentation (24).

Ad5 IC enhances HIV infection

It was critical to investigate whether the increased T cell activation induced by Ad5 IC enhanced HIV infection. To address this issue we used an ex vivo model that likely mimics the cellular interactions that occurred during the STEP trial. We stimulated DCs with Ad5 vector or Ad5 IC and then incubated these cells with autologous blood mononuclear cells from four subjects to reactivate memory Ad5-specific T cells. Autologous or heterologous (4,096 titer) NS sera were used to generate Ad5 IC. The cocultures were then incubated with HIV-1$_{LAV}$. Ex vivo HIV infection and propagation was
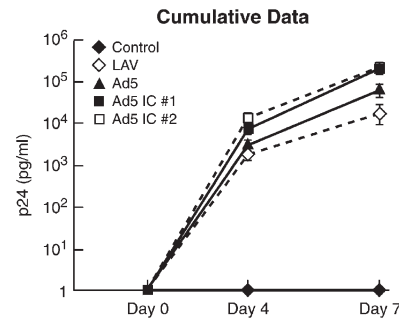

Figure 5. Ad5 IC stimulates HIV infection in vitro. Mean p24 levels at day 0, 4, and 7 in blood mononuclear cells from donors were cultured with Ad5 or Ad5 IC–treated autologous DCs. Uninfected cultures and HIV infection in cultures in which blood mononuclear cells were mixed with untreated DCs were used as controls. Four donors were studied. Data represent the mean of triplicate determinations.

assessed by measuring p24 in the culture supernatants. It is important to mention that DCs, through the formation of conjugates with CD4 T cells, facilitate productive HIV-1 infection in cell cultures even in the absence of polyclonal or Ag-specific activation of T cells (25). Indeed, we observed productive HIV-1 infection in cell cultures containing mock-treated DCs mixed with autologous blood mononuclear cells (Fig. 5). Nonetheless, we consistently observed approximately threefold higher levels of HIV replication in the cell cultures containing blood mononuclear cells stimulated with DCs treated with Ad5 IC as compared with Ad5 vector or cultured with DCs alone (Fig. 5). The mean p24 level at day 4 was 3,230 pg/ml in the culture of blood mononuclear cells stimulated with DCs treated with Ad5 vector alone versus 10,800 pg/ml in the culture of blood mononuclear cells stimulated with DCs treated with Ad5 IC (P < 0.05). At day 7, the mean p24 level was 64,400 pg/ml in the culture of blood mononuclear cells stimulated with DCs treated with Ad5 vector alone versus 207,600 pg/ml in the culture of blood mononuclear cells stimulated with DCs treated with Ad5 IC (P < 0.001). Therefore, Ad5 IC likely induced greater activation of T cells that translated into enhanced levels of HIV replication in tissue culture.

Our study advances our understanding of the likely immunological events operating after Ad5 vector–based vaccination and suggests a series of events that could explain the increase in acquisition of HIV infection in Ad5 seropositive subjects in the STEP trial. During Ad5 vector–mediated gene transfer, the vectors likely infect several target cells, including professional Ag-presenting cells like DCs. Infection of these latter cells will eventually activate memory Ad5-specific CD4 and CD8 T cells and hopefully generate an HIV-specific T cell response. However, the presence of high Ad5 NAbs titers is associated with the formation of Ad5 IC. Compared with Ad5 vector alone, Ad5 IC induced "hyperactivation" and maturation of DCs, as indicated by the increased expression of costimulatory molecules, the loss of the capacity of Ag uptake, and the secretion of TNF-α and IFN-I. The activation

signal to DCs mediated by Ad5 IC results in part from the cooperation between FcγRs and TLR9.

The survival signals and clonal expansion of memory T cells mediated by IFN-I (26, 27) may explain the effect of Ad5 IC on the increase of Ad5-specifc memory effector CD8 T cells, likely through cross-priming and the trend toward larger expansions of both Ad5-specific memory CD4 and CD8 T cells. The reactivation of Ad5-specific memory T cells likely prevents effective generation of the primary immune response against the vector-encoded HIV Ags through two nonexclusive mechanisms: one, an unfavorable cytokine environment resulting from the inflammatory response caused by the Ad5 IC formation; and two, the killing of DCs. Because DCs will express not only HIV but also Ad5 Ags (28), they become a target of the reactivated Ad5-specific CD8 T cells, thus resulting also in the reduction of the DC pool presenting HIV Ags. In support of this hypothesis, preliminary data from the STEP trial indicate that the percent of responders to HIV proteins (measured by IFN-γ ELISpot) was less (∼25%) in subjects with Ad5 NAb titers >200, and the magnitude of the HIV-specific T cell response (frequency of IFN-γ–secreting cells in blood) was also reduced by ∼50% (www.HVTN.org). Finally, increased survival of activated memory Ad5-specific CD4 T cells mediated by IFN-I (26) may transiently enlarge this pool of memory CD4 T cells that effectively support HIV replication and spreading, thus facilitating susceptibility to HIV infection. Our results also provide new insights into the type of experimental strategies and assays that can be instrumental for the preclinical assessment of the safety of other viral vector-based vaccines in addition to Ad5.

In conclusion, we have shown that ICs containing E1/E3-deleted Ad5 vectors and NAbs cause a pattern of innate/adaptive inflammatory activation that, together with a possible persistence of the Ad5 vector, may provide the basis for a chronic permissive environment for HIV-1 infection, thus helping to explain the increased acquisition of HIV-1 infection among the Ad5 seropositive vaccine recipients in the STEP trial. Moreover, our results also suggest that the delivery/formulation of Ags through ICs may result in a powerful immunization strategy to stimulate T cells.

MATERIALS AND METHODS

Blood mononuclear cell and DC isolation. Blood mononuclear cell isolation (from 14 healthy subjects) and monocyte-derived DC generation were performed as previously described (15).

Ad vectors. Two E1/E3-deleted Ad5 vectors (Adβgal and AdGFP) have been previously described (15). Adβgal harbors a *lacZ* expression cassette and was used in T cell stimulation, Ag uptake, Fc receptor blocking, in vitro HIV infection, and costimulatory molecule experiments. AdGFP harbors a GFP expression cassette and was used for the assessment of NAb titers, in DC transduction assays, and in costimulatory molecule experiments. The helper-dependent Ad5 vector (19) is deleted in all viral genes and contains a *lacZ* expression cassette (provided by M. Castro, Gene Therapeutics Research Institute, Cedars Sina Medical Center, University of California, Los Angeles, Los Angeles, CA). Ad5 vector titers were measured in virus pp/cell as described by Mittereder et al. (29).

NAb titers. The neutralizing activity of a panel of sera (*n* = 76) was determined by transduction inhibition assays (14) using the 911 cell line. With regard to the DC transduction assay, immature DCs (2.5×10^5 cells) were resuspended in medium containing 2.5×10^4 pp/cell and 0.016–20 μl of serum, incubated for 48 h, and then analyzed by flow cytometry.

Cytokine secretion. DCs from 14 donors were incubated with AdGFP, Ad5 IC, or 50 ng/ml LPS (*Escherichia coli* 0127 B5; Sigma-Aldrich) for 48 h. Supernatants were collected and analyzed for the presence of TNF-α, IL-12p70, IL-1β, IFN-γ, and IL-10 by ELISA (BD). IFN-I quantification was performed as previously described (15) using cells that contain a luciferase expression cassette under the control of IFN-I–inducible promoter. The NAb titers and number of sera analyzed for each titer were the following: 0, 1; 4, 3; 8, 2; 16, 5; 32, 1; 64, 6; 128, 4; 512, 2; and titer 1,024, 4 sera.

Ag uptake. Ag uptake was determined using FITC-labeled dextran (MW 10,000; Sigma-Aldrich) as previously described (15). In brief, immature DCs were incubated with Adβgal, Ad5 IC, or 50 ng/ml LPS for 48 h. DCs were then washed and incubated with 1 mg/ml FITC-labeled dextran for 10, 30, or 90 min. Control DCs were incubated at 4°C. Assays were performed twice.

FcR Abs. The following monoclonal Abs were used for functional assays: CD16 (FcγRIII; 3G8; BD), CD32a (FcγRIIa IV.3), CD32 (FcγRII AT10), and CD64 (FcγRI; 10.1) (20).

Expression of costimulatory molecules. DCs were cultured either alone or with Adβgal vector or Adβgal mixed with 20 μl of neutralizing serum and 50 ng/ml LPS for 48 h. The expression of costimulatory molecules was assessed on HLA-DR–positive cells using an HLA-DR Alexa Fluor 700–conjugated Ab (LN3; eBioscience). Five DC donors and eight sera were used.

Intracellular cytokine staining. DCs were first treated for 24 h with Ad5 vector alone or with Ad5 IC. Cell cultures were then extensively washed, mixed with autologous blood mononuclear cells (10^6 cells), and cultured for 6 h in 1 ml of complete media containing 1 μl/ml GolgiPlug (BD). Intracellular cytokine staining was performed as previously described (30). The Abs used were the following: CD4-APC-H7, CD8-PerCP-Cy5.5, CD3-ECD, IFN-γ–AF700, IL-2–PE, and TNF-α–PE-CY7. Data were acquired on an LSRII three laser (488, 633, and 405 nm; BD) and analyzed using FlowJo and SPICE 4.1.5 software (M. Roederer, Vaccine Research Center, NIAID, Bethesda, MA).

Ex vivo proliferation assay. DCs were first treated for 24 h with Ad5 vector alone or with Ad5 IC. Cell cultures were then extensively washed, mixed with autologous blood mononuclear cells, and cultured for 6 d. Blood mononuclear cells proliferation was assessed using CFSE (Invitrogen) as previously described (9, 30). Cells were stimulated with Ad5 IC formed with autologous NS and heterologous serum. Stimulation with 100 ng/ml *Staphylococcus aureus* enterotoxin B was used as a positive control. All sera tested had Ad5 NAb titers >1,000. The percent of proliferating CD4+ and CD8+ T cells, i.e., CFSE-low cells, was determined in the CD3+ cell population. The criteria for scoring as positive the proliferating cell cultures included both CFSE-low cells >1% after subtracting the percent of CFSE-low cells in unstimulated cell cultures and stimulation index >3. The stimulation index was calculated by the fold increase between stimulated versus unstimulated cell cultures. Expression of perforin and granzyme B expression on CFSE-low proliferating CD8 T cells was performed using APC-conjugated anti-perforin (BioLegend) and AF700-conjugated anti-granzyme B (BD).

HIV infection in vitro. DCs were first treated for 24 h with Ad5 vector alone or with Ad5 IC. Cell cultures were then extensively washed, mixed with autologous blood mononuclear cells, and cultured for 48 h. Cell cultures of 10^6 cells were then inoculated with 30 pg HIV-1$_{LAV}$ for 3 h. At the end of the incubation, cells were washed, replated at 10^6 cells per well, and HIV replication was assessed by measuring p24 in the culture supernatant.

Mock-infected cultures were used as a negative control. HIV infection was also determined in cultures in which blood mononuclear cells were mixed with untreated DCs. p24 measures were performed in triplicates and measurements were performed at days 0, 4 and 7 using an ELISA (Immunogenetics).

TLR inhibitors. Phosphorothioate-protected IRS 869 and control oligonucleotide (ODN; Sigma-Aldrich) were resuspended to make a final concentration of 500 µM. TLR9 immunostimulatory sequence 2216 was purchased from InvivoGen. In brief, 2.5×10^5 immature DCs were seeded in 500 µl in 48-well plates and treated with IRS 869 or control ODN (final concentration of 5 µM) for 2 h. Immunostimulatory sequence 2216 (also at 5 µM) or Ad5 IC prepared using 20-µl serum (NAbs titer >1,000) for 15 min at room temperature were then incubated with the DCs for ~18 h at 37°C in 5% CO_2. The supernatants were collected at the indicated times and tested for TNF-α or IFN-I.

Statistical analysis. Statistical significance (p-values) was calculated by using a Student's t test for the comparisons, a Spearman rank test for the correlations, and a Wilcoxon rank test for the SPICE analyses. A two-tail p-value of <0.05 was considered significant. The analyses of multiple comparisons have been taken into account for the calculation of statistical significance.

Online supplemental material. The four figures show data on Ad5 vector transduction efficiency, expression of different FcγRs on DCs, and effects of Ad5 IC stimulation on CD4 T cells and T cell proliferation. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20081786/DC1.

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