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Extensive Cross-Reactivity of CD4$^+$ Adenovirus-Specific T Cells: Implications for Immunotherapy and Gene Therapy

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Adenovirus (Ad)-specific T-cell responses in healthy adult donors were investigated. Ad5, inactivated by methylene blue plus visible light, induced proliferation and gamma interferon (IFN-γ) production in peripheral blood mononuclear cells of the majority of donors. Responding T cells were CD4$^+$ and produced IFN-γ upon restimulation with infectious Ad5 and Ads of different subgroups. T-cell clones showed distinct cross-reactivity patterns recognizing Ad serotypes from either one subgroup (C), two subgroups (B and C), or three subgroups (A, B, and C). This cross-reactivity of Ad-specific T cells has relevance both for Ad-based gene therapy protocols, as well as for the feasibility of T-cell-mediated adoptive immunotherapy in recipients of an allogeneic stem cell transplantation.

Adenoviruses (Ads) rarely cause severe clinical symptoms in healthy children and adults since infections in immunocompetent individuals are usually self-limited. For this reason, Ad has been considered to be a safe vector for gene delivery and vaccination strategies, although preexisting immunity is a major limitation when vectors are being administered repeatedly (16, 47). However, Ads may cause life-threatening complications in immunocompromised children (20, 29). In recent years, the incidence of Ad infections in pediatric recipients of an allogeneic stem cell transplant (SCT) has increased remarkably (2, 8, 14, 17, 22, 39; M. J. D. van Tol et al., unpublished data). Recipients of a T-cell-depleted allogeneic graft, i.e., patients with a non-HLA-identical donor, have a higher risk of developing Ad infection, probably due to the delayed immune reconstitution in these children after SCT (17; van Tol et al., unpublished). Dissemination of the infection often leads to a fatal outcome (14, 17, 20, 21, 32).

Currently, 51 serotypes of Ad have been identified, distributed among six subgroups (A to F) (11, 20). Subgroup A, B, and C serotypes are most frequently isolated from pediatric immunocompromised hosts and are the major cause of disease (4, 14, 20). Treatment of adenoviral infections with antiviral medication by using drugs such as cidofovir and ribavirin has not been unequivocally effective (3, 5, 23, 25, 28, 31, 38). A novel therapeutic approach may be immunotherapy by means of Ad-specific lymphocytes since case reports have suggested that donor lymphocyte infusions may contribute to the clearance of an Ad infection (6, 24). The present study focuses on the feasibility of generating Ad-specific T cells from a graft donor origin with the final goal of infusing these cells into the infected SCT patient. This strategy has already successfully been pursued for other viral infections or reactivations such as cytomegalovirus or Epstein-Barr virus (13, 19, 46).

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Inactivation of Ad. Stimulation of Ad-specific T cells by using wild-type or E1$^-$ E3$^-$ recombinant Ad vectors has been reported previously (9, 14, 43, 44). In clinical practice, however, biosafety constraints require a validated inactivation procedure of the Ad used for T-cell stimulation in order to circumvent infusion of infectious or genetically modified virus into the patient. Our strategy has therefore focused on complete inactivation of purified wild-type virus by using the photosensitizer methylene blue (MB) and visible light (40). MB is already in use for routine treatment of fresh frozen plasma prior to infusion to inactivate viruses such as hepatitis C virus (33, 35, 42).

MB inactivation of Ad was previously shown to reduce viral infectivity by at least 4 logs after illumination for 10 min (40). Prolonging the illumination period revealed that infectious particles could no longer be detected after 30 min of inactivation, as determined by the lack of cytopathological effect in human epithelial cells (HEp-2) cells, indicating that MB can inactivate Ad5 by at least 7 logs (data not shown).

Frequency of T-cell responses to MB-inactivated Ad5. Since the use of MB-inactivated Ad5 as antigen has not been reported previously, the frequency of donors reacting against MB-inactivated Ad5 was determined in a panel of healthy adults by proliferation and gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assays. In all, 19 of 25 (76%) donors responded to MB-inactivated Ad5 by proliferation, with stimulation indices (SI) ranging from 4.5 to 234 (Fig. 1A). An analysis of this panel of healthy adults simultaneously for IFN-γ-producing cells by ELISPOT revealed that 80% of donors responded after 4 days of stimulation with MB-inactivated Ad5 (Fig. 1B). Calculated SI values for the ELISPOT results (in responding donors ranging from 4.1 to 109) correlated significantly with the SI from the proliferation assay (Pearson correlation coefficient = 0.757, $P < 0.001$) (Fig. 1C). Donors not reacting to MB-inactivated virus were also tested by proliferation against UV- or heat-inactivated Ad5, as well as non-inactivated wild-type Ad5, to determine whether the nonresponsiveness was due to the nature of the viral antigen. However, these donors were not responsive to any type of

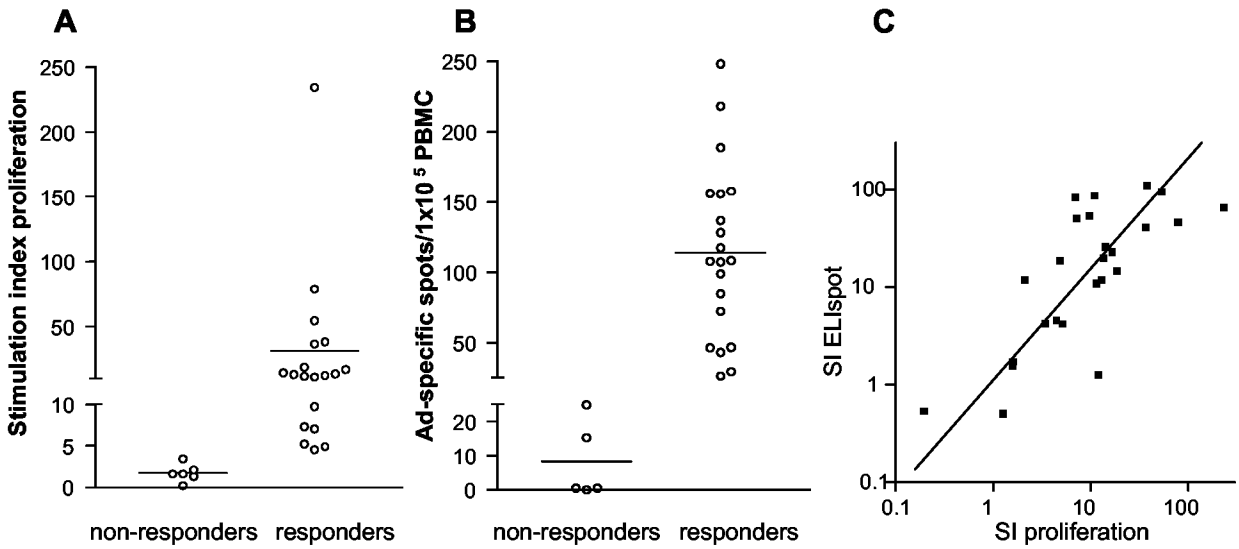

FIG. 1. Proliferation and IFN-γ responses to MB-inactivated Ad5 in healthy adults. PBMC from 25 healthy adult donors were stimulated with MB-inactivated Ad5 or were not stimulated as a control. (A) Proliferation in response to MB-inactivated Ad5 is shown as the SI for each donor. The SI is defined as proliferation (counts per minute of [^{3}H]thymidine incorporation) in response to PBMC with MB-inactivated Ad5 divided by the proliferation in response to PBMC without virus. Donors with an SI of >3 and a mean$_{Ad}$ of >(the mean$_{control}$ + 3 × the standard deviation) were considered responding donors (19 of 25). All donors responded to phytohemagglutinin stimulation (not shown). (B) IFN-γ production was determined by ELISPOT assay in the same donors. Specific spots (background spots are substracted) are shown for each donor. Donors with at least 25 specific spots/100,000 PBMC and a mean spots$_{Ad}$ value of >(mean spots$_{control}$ + 3 × the standard deviation) were considered responding donors (20 of 25). (C) SI values were calculated for both assays for comparison. The Pearson correlation coefficient is 0.757 ($P < 0.001$); the orthogonal regression line is shown ($y = 0.04 + 1.14x$).

adenoviral stimulation, indicating that the MB inactivation of the virus was not responsible for the lack of response (data not shown). Unfortunately, sera from these donors were unavailable to test for previous Ad infections by serology. Since MB-inactivated Ad induced good proliferative responses of peripheral blood mononuclear cells (PBMC), our results confirm previous data that suggested that T-cell responses are directed to structural proteins (14, 34, 43, 45). The frequency of responders obtained with MB-inactivated Ad5 was comparable to frequencies that have been described for purified E1$^-$ E3$^-$ Ad5 (64%) (9) or for Ad2 lysate from HEp-2 cells (>90%) (14), indicating that MB-inactivated Ad is a valid source for antigenic stimulation (36). This high response rate in adult donors indicates that the generation of Ad-specific T cells would be a feasible option for the majority of transplant recipients at risk for developing a disseminated Ad infection.

Increased Ad-specific IFN-γ production and reduced alloreactivity after restimulation. If Ad-specific T-cell cultures are to be infused in patients, alloreactive, potentially graft-versus-host-disease-causing T cells should be eliminated. One way to reach this goal is a prolonged period of repetitive stimulation with specific antigen in culture. Furthermore, cells generated against MB-inactivated virus should be reactive against cells infected with infectious virus to be able to combat an ongoing infection in a patient. To this end, bulk cultures were initiated with PBMC from donors responding to MB-inactivated Ad5 and restimulated at day 12; interleukin-2 was added from day 15. At day 28, T cells proliferated specifically against autologous PBMC with MB-inactivated Ad5, as well as with active Ad5 (Fig. 2A), showing that T cells specific for structural proteins do indeed respond to cells with infectious Ad.

Intracellular cytokine staining combined with immunophenotyping revealed a strong enrichment of CD4$^+$ T cells producing IFN-γ upon restimulation with Ad5 (median, 5% IFN-γ$^+$ cells in the CD3$^+$ CD4$^+$ subset; range, 1.2 to 30.1%; $n = 8$) (Fig. 2B). CD8$^+$ cells specifically producing IFN-γ were not detected by using intracellular stainings, either by using MB-inactivated Ad or infectious Ad for stimulation (data not shown). Accordingly, other groups have demonstrated CD4$^+$-T-cell responses against Ad, either in proliferation assays (9, 14, 18) or in IFN-γ ELISPOT assays (36). In the same cultures, alloreactive responses tested after 28 days of culture were strongly reduced compared to the alloreactive responses at the initiation of culture (Fig. 2C).

Cross-reactivity of Ad5-specific bulk cultures and T-cell clones. A complicating factor in generating T cells for immunotherapy of Ad infection is that 51 serotypes of Ad have been described to date. It is currently not possible to predict which serotype will lead to infection in individual patients. Therefore, it is of interest to know whether T cells generated by stimulation with Ad5 are also able to recognize other serotypes of Ad, since Ad strains belonging to subgroups A, B, and C have been reported to cause severe infections in immunocompromised hosts (4, 14, 20).

Cells from 28-day T-cell cultures generated against MB-inactivated Ad5 were tested against a panel of Ad serotypes, including strains belonging to subgroup C (Ad1, Ad5, and Ad6), subgroup B (Ad3, Ad7, Ad11, Ad34, and Ad35), and subgroup A (Ad12, Ad18, and Ad31). Cells proliferated in response to Ad5, as well as in response to other serotypes belonging to the same subgroup (C), which have extensive homology with Ad5. However, proliferation was also observed

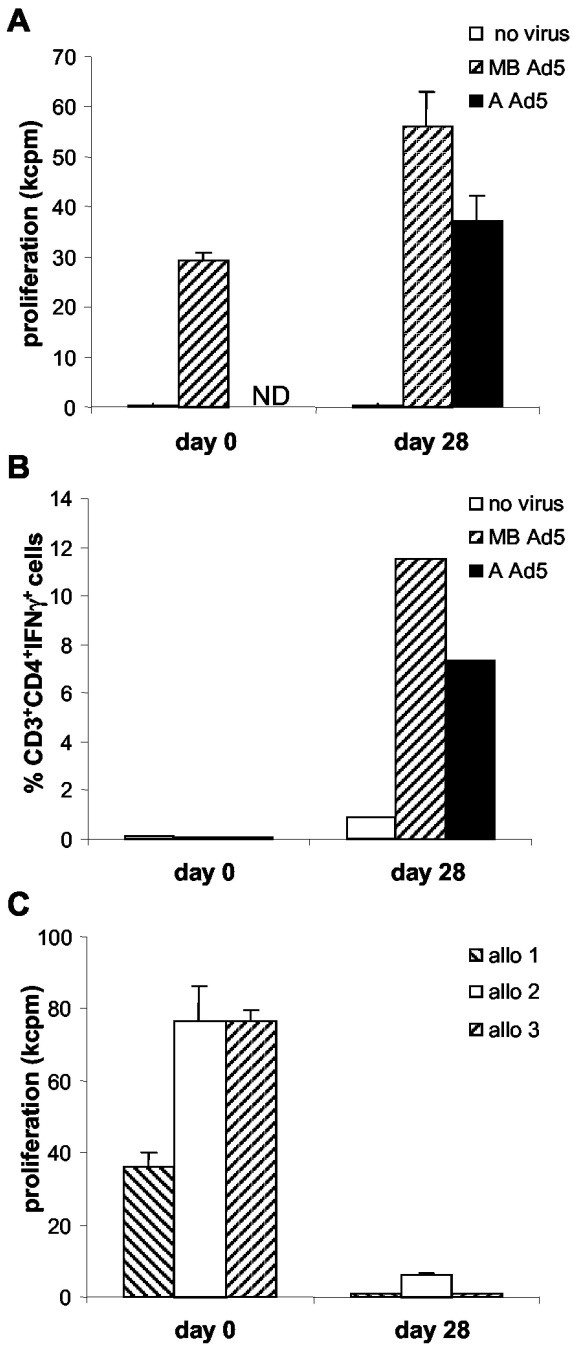

FIG. 2. Proliferation of T cells, frequency of IFN-γ-producing $CD4^+$ T cells, and alloreactivity after 28 days of culture. PBMC of healthy adult donors were stimulated with MB-inactivated Ad5 (multiplicity of infection [MOI] = 10) at day 0 and restimulated at day 12. At day 28, the specificity of the cultures was assessed and compared to the response at the initiation of culture. (A) Proliferation against irradiated autologous PBMC with MB-inactivated Ad5 (MB Ad5) and active Ad5 (A Ad5) at MOI = 100 or without virus was measured in a 6-day proliferation assay. The means and standard errors of the mean of triplicate wells are shown. ND, not done. (B) The percentage of IFN-γ-producing T cells at day 28 was assessed by intracellular FACS staining after an 18-h stimulation with irradiated autologous PBMC with MB-inactivated Ad5 and active Ad5 at an MOI of 100 or without virus. Brefeldin A was added after 1 h of coculture. The percentages IFN-γ^+ cells within the $CD3^+$ $CD4^+$ subset prior to culture (day 0) and after 28 days of culture are shown. IFN-γ^+ cells were not detected in the $CD3^+$ $CD8^+$ subset (data not shown). (C) Alloreactivity was tested in a 6-day proliferation assay against irradiated allogeneic PBMC of three donors. The means and standard errors of the mean of triplicate wells are shown. All data are representative for experiments with six different healthy adults.

in response to the less homologous serotypes from subgroup B and subgroup A in several bulk cultures (an example is shown in Fig. 3A). These results suggest that Ad serotypes belonging to different subgroups share T-cell epitopes.

The recognition pattern of different Ad serotypes was further investigated at the clonal level. To this end, T-cell clones were generated by limiting dilution from 28-day bulk cultures and resulting clones were tested for specificity by using uninfected and Ad5-infected autologous Epstein-Barr virus-transformed B cells (BLCL). Fluorescence-activated cell sorting (FACS) analysis showed that Ad-specific T-cell clones were $CD4^+$, IFN-γ-producing cells, a finding that is in agreement with the phenotype of the responding cells from bulk cultures (data not shown).

In all, 11 independent T-cell clones from four different donors were tested for cross-reactivity against Ad belonging to subgroup C (Ad1, Ad5, and Ad6), subgroup B (Ad3, Ad7, Ad11, Ad34, and Ad35), and subgroup A (Ad12, Ad18, and Ad31). Proliferation assays showed three different recognition patterns. Two clones were reactive against subgroup C viruses only (Fig. 3B). Four clones recognized both subgroup C and subgroup B viruses (Fig. 3C). Finally, five clones recognized Ad from all three subgroups tested, showing the broadest reactivity profile (Fig. 3D). Ad5-specific clones used either HLA-DR or HLA-DP as a restriction element for antigen recognition, as assessed by the addition of blocking antibodies to HLA-DR and HLA-DP (Fig. 3B to D). There was no correlation between the restriction element (HLA-DR or HLA-DP) and the cross-reactivity pattern. Although reactivity of Ad-specific T-cell cultures to other serotypes has been reported previously (14, 44), this is the first report in which extensive cross-reactivity of Ad-specific $CD4^+$ T cells is described at the clonal level, indicating that different Ad strains harbor shared antigenic epitopes.

In general, human T-cell recognition of epitopes that are conserved between different but related subtypes of viruses has been described for enteroviruses, herpesviruses, flaviviruses, and influenza A viruses (1, 7, 26, 30) and is not unique for Ad. Recently, it has been proposed that exposure of individuals to consecutive infections with different strains of a virus will result in repeated cycles of stimulation and expansion of those T cells that recognize shared epitopes (15). In this way, consecutive infections with different strains of Ad could explain the observed cross-reactivity. Such cross-reactivity implies the presence of sequence homology in structural proteins between the Ad serotypes from different subgroups. For instance, the hexon protein, which is the major structural protein and comprises ca. 95% of the capsid, is ca. 80% homologous between subgroups A, B, and C (10). Further characterization of the cross-reactive T-cell epitopes will demonstrate whether these shared epitopes are completely identical between Ad strains or whether amino acid differences exist that have no crucial effect on HLA-binding or T-cell receptor recognition.

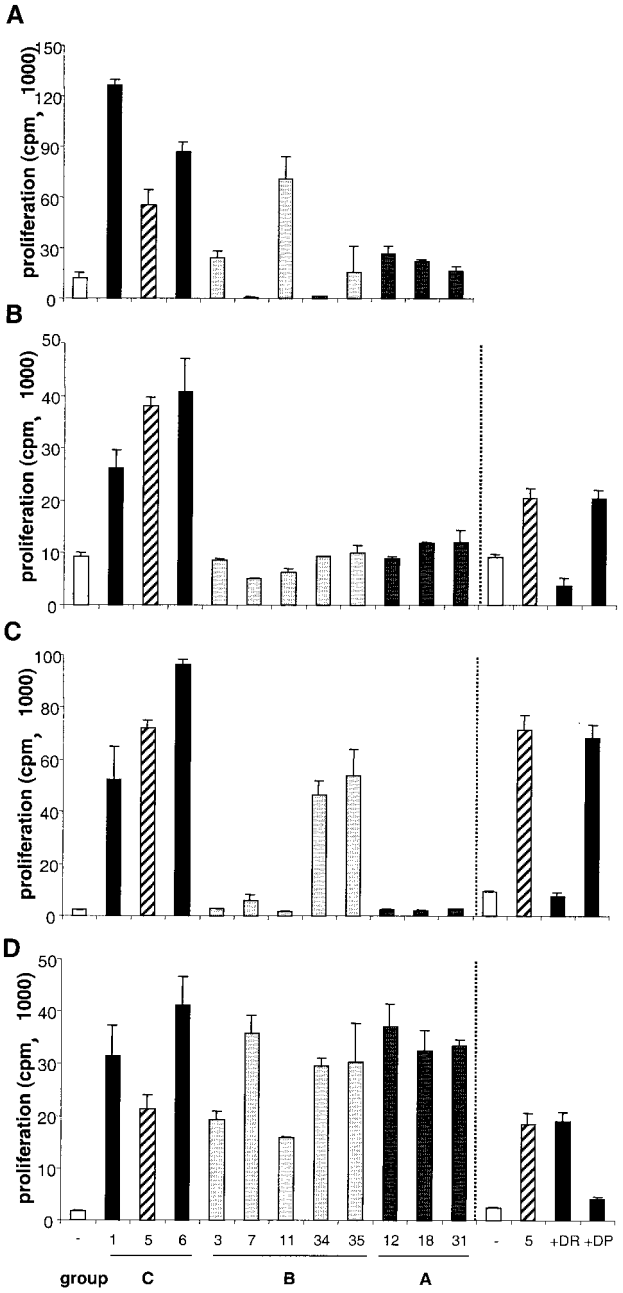

FIG. 3. Cross-reactivity of T cells from Ad5-induced bulk cultures and $CD4^+$-T-cell clones. (A) Twenty-eight-day T-cell cultures were tested in a 6-day proliferation assay for reactivity against irradiated autologous PBMC without virus (−) or PBMC infected with Ad strains from subgroups C (Ad1, Ad5, and Ad6), B (Ad3, Ad7, Ad11, Ad34, and Ad35), and A (Ad12, Ad18, and Ad31). The response pattern of a representative donor out of four donors tested is shown. (B to D) $CD4^+$-T-cell clones, obtained by limiting dilution, were stimulated with irradiated autologous BLCL without virus (−) or infected with Ad strains from subgroups C (Ad1, Ad5, and Ad6), B (Ad3, Ad7, Ad11, Ad34, and Ad35), and A (Ad12, Ad18, and Ad31) and tested in a 4-day proliferation assay. Panel B represents a clone with restricted recognition (only subgroup C). Panel C represents a clone with a broader reactivity against both subgroup C and B serotypes. Panel D depicts a clone which recognizes Ad from all subgroups tested (subgroup A, B, and C). Blocking antibodies to HLA-DR and HLA-DP were added to BLCL infected with Ad5 (hatched bars) to determine the restriction element (separate experiment, dashed line; anti-HLA-DQ and anti-class I was also added and did not block responses).

Preexisting immunity limits the application of Ad5 both in gene therapy protocols and in vaccine delivery. The neutralizing antibody response to the Ad-based vector may be circumvented efficaciously by subsequent use of vectors containing hexons from different serotypes (37, 48). Preexisting cellular immunity, however, may not be as easily circumvented by this hexon gene switch strategy due to the extensive cross-reactivity of T cells described here.

In conclusion, our results show that Ad-reactive T cells can be cultured after stimulation with MB-inactivated Ad5. In order to control Ad infections in patients, these T cells should probably be infused preemptively when Ad-DNA is detected for the first time in plasma by PCR, indicating dissemination of the infection (12, 27, 41). The high degree of cross-reactivity allows the use of these cells in immunocompromised patients irrespective of the serotype of the Ad strain that infects a particular patient and may thus be a valuable tool to decrease the mortality rate in this pediatric patient group.

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