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Transient Subversion of CD40 Ligand Function Diminishes Immune Responses to Adenovirus Vectors in Mouse Liver and Lung Tissues

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First-generation adenovirus vectors will have limited application in gene therapy for chronic diseases because of destructive host immune responses. Important immune effectors include $CD8^+$ T cells, which mediate target cell destruction and ablate transgene expression, and B cells, which produce neutralizing antibodies that block effective readministration of vector. Previous studies indicated that activation of $CD4^+$ T cells by virus capsid proteins is necessary for full realization of effector function of $CD8^+$ T cells and B cells. In this paper, we present a strategy for preventing $CD4^+$ T-cell activation by an adenovirus vector delivered to mouse liver and lung tissues which is based on interfering with T-cell priming via CD40 ligand-CD40 interactions. Adenovirus transgene expression was stabilized in mice genetically deficient in CD40 ligand (CD40L), and neutralizing antibody to adenovirus did not develop, allowing efficient readministration of vector. A transient blockade of T-cell activation with an antibody to CD40L infused into the animal at the time of adenovirus vector-mediated gene transfer led to stabilization of transgene expression and diminished production of neutralizing antibody, allowing readministration of vector. In vitro T-cell assays suggested that a block in the primary activation of $CD4^+$ T cells was responsible for the lack of B-cell- and cytotoxic-T-cell-dependent responses. This suggests a strategy for improving the potential of adenovirus vectors based on administration of an antibody to CD40L at the time of vector administration.

The enthusiasm for use of adenovirus vectors in gene therapy, based in part on the efficiency with which they transfer genes into nondividing cells in vivo, has been tempered by the problems of attendant host immune responses (3, 28). Expression of the transgene usually extinguishes within 2 to 3 weeks, concurrent with the development of inflammation (3, 16, 18, 25, 27–29, 33). This is caused in part by activation of $CD8^+$ T cells by neoantigens expressed in the corrected cell, leading to cytotoxic-T-cell (CTL)-dependent extinction of transgene expression (25, 27, 33). Epitope targets of CTLs appear to reside on viral structural proteins (25, 27, 28), which are expressed from E1-deleted vectors, and on some transgene products (22). Non-antigen-specific immune responses (e.g., NK cells) as well as cytokine-mediated destabilization of the vector DNA might also contribute to the loss of transgene expression. Activation of B cells by input viral capsid proteins underlies another problem with adenovirus vectors, which is production of antibodies that neutralize virions and prevent effective readministration of vector (15, 27, 32). Administration of UV-inactivated virus leads to a full humoral response without CTL effects, which is consistent with the role of exogenous viral capsid proteins in the activation of B cells and of endogenously produced antigens in the activation of primary $CD8^+$ cells (27). Chronic immune suppression with drugs such as cyclosporine and cyclophosphamide has improved the stability of adenovirus-encoded transgene expression in animal models of liver- and muscle-directed gene therapy (3, 7, 8).

Activation of $CD4^+$ T cells by viral capsid proteins is a necessary step in both CTL-mediated clearance of genetically corrected cells and production of neutralizing antibodies by B cells (30, 31). The central role of the $CD4^+$ T cell in these processes has suggested a strategy to prevent both humoral and cellular responses to adenovirus vectors through a transient blockade of $CD4^+$ T-cell activation at the time of vector administration. This strategy is based on the hypothesis that chronic immune suppression should not be necessary if the primary stimulus for activation is the input capsid protein. Depletion of $CD4^+$ T cells with a monoclonal antibody (MAb) at the time of vector administration has effectively prevented CTL and B-cell responses in murine models of liver- and lung-directed gene therapy (26, 30). Moreover, selective inhibition of the T_{H2} subset of T helper cells by administration of the cytokine interleukin 12 or gamma interferon with adenovirus vector has prevented the humoral response in mouse lung tissue (30).

Activation of T cells by antigens requires a complex program of molecular interactions between the T cell and an antigen-presenting cell, each of which could be a target for immune blockade in gene therapy. Recognition of the processed antigen by the T-cell receptor triggers the engagement of accessory molecules necessary for productive immune responses. These interactions are best characterized in the setting of T-cell-dependent activation of B cells, where binding of antigen to the T-cell receptor leads to upregulation of CD40 ligand (CD40L) on the T cell, promoting the engagement of CD40 on the cognate B cell (6). Ligation of CD40L with CD40 appears to be a proximal step in a cascade involving the interaction of CTLA4-CD28 and B7.1-B7.2 which leads to activation of B cells (11). Interfering with the distal pathway by administering CTLA4 immunoglobulin (Ig) with adenovirus vector improves

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the stability of recombinant gene expression in mouse liver tissue but does not impact neutralizing antibody or allow vector readministration.

A genetic deficiency of CD40L in humans with hyper-IgM syndrome (1, 2, 4, 14) and in knockout (KO) mice (17, 24) has provided insight into the biology of this molecule. The profound abnormalities in humoral responses that characterize CD40L deficiency (i.e., B-cell clonal expansion, germinal center formation, and isotype switching) are consistent with its role in B-cell regulation (6). The propensity for patients to develop opportunistic parasitic infections suggests that CD40L is primarily involved in T-cell responses. Validation of this hypothesis was recently provided by studies of mice deficient in either CD40L or CD40, which demonstrated the role of CD40L signaling in in vivo clonal expansion of antigen-specific T cells (10, 19, 21).

This study explores the potential of CD40L antibody to prevent the activation of T cells by adenovirus vectors in mouse models of liver and lung gene transfer.

MATERIALS AND METHODS

Viruses. Recombinant adenoviruses used in this study were purified on two sequential cesium gradients, desalted on gel filtration columns, and used immediately. The particle-to-PFU ratio varied from 1:20 to 1:50. H5.010CBALP expresses the human alkaline phosphatase (ALP) cDNA from a chicken β-actin promoter enhanced by sequences from the immediate early gene of cytomegalovirus (27). This minigene was placed into human Ad5 deleted of E1 (map units 1 to 9.2) with a substitution of sequence in E3b (sub360). H5.010CMV*lacZ* expresses *lacZ* from a cytomegalovirus promoter incorporated into the E1-deleted, sub360-based Ad5 virus (27).

Animal studies. Mice used in this study were C57BL/6 (6 weeks of age, females) or CD40L-deficient or normal littermates in a C57BL/6-129 chimeric background (24). Mice were instilled with the first adenovirus via the trachea (10^9 PFU in 50 μl of phosphate-buffered saline [PBS]) or via the tail vein (2×10^9 in 100 μl of PBS) on day 0 and the second virus on day 28. Some animals were harvested 3 days after each virus instillation (i.e., days 3 and 31) and necropsy was performed; lung or liver tissues were prepared for cryosections, while bronchial alveolar lavage (BAL) fluid and blood were collected for assessment of neutralizing antibody and the spleen or mediastinal lymph nodes (MLN) were harvested for immunological assays. Some animals were also administered 100 μg of antibody to CD40L (MR1 hybridoma; American Type Culture Collection) by intraperitoneal (i.p.) injection on days −3, 0, +3, and +6.

Histochemical and immunohistochemical staining. Lung and liver tissues were frozen in OCT, cryosectioned, and analyzed for ALP or β-galactosidase activity with specific histochemical stains as previously described (25, 27). In addition, liver sections were also subjected to immunohistochemical staining specific for $CD4^+$ and $CD8^+$ cells as well as major histocompatibility complex (MHC) class I expression (27).

Immunological assays. Serum and BAL were analyzed for neutralizing antibody to human Ad5 as described previously (25, 27). Lymphocytes from the spleen or MLN were restimulated in vitro with virus and used for CTL and proliferation assays as described previously (25, 27).

RESULTS

CD40L-deficient mice illustrate the necessary role of T-cell activation in host responses to adenovirus vectors. The role of CD40L-mediated signaling of T cells in cellular and humoral immune responses to adenovirus vectors was studied in mice genetically deficient in CD40L. Previous studies have demonstrated abnormalities in thymus-dependent B-cell responses in these mice (17, 24). CD40L-deficient mice and their normal littermates were intratracheally administered *lacZ*-containing E1-deleted adenovirus on day 0, to effect gene transfer to the lung, and into the peripheral circulation, to effect gene transfer to the liver. Animals were sacrificed 3 and 28 days later to assess the efficiency and stability of transgene expression, respectively. Animals were treated with an adenovirus vector containing a different reporter gene (ALP) on day 28; prior to the second vector administration, blood was analyzed for neutralizing antibodies, and tissues were harvested for analysis of reporter gene expression 3 days later (i.e., day 31). Similar results were obtained when the order of the *lacZ*- and ALP-containing viruses was reversed. Figure 1 presents representative histochemical assays of lung and liver tissues, while Tables 1 and 2 summarize the results of morphometric analyses of these tissues.

Normal littermates that were administered vectors demonstrated high-level transgene expression at day 3 in lung and liver tissues that diminished to undetectable levels by day 28 (similar to what is seen in naive C57BL/6 mice) (Fig. 1A and B and Table 1, lung; Fig. 1J and K and Table 2, liver). Substantial neutralizing antibody developed against adenovirus capsid proteins by day 28 in either BAL fluid of animals that received vector intratracheally or in blood samples of animals that were administered vector intravenously (Fig. 2). Readministration of vector on day 28 was unsuccessful as evidenced by the lack of transgene expression in the target organ 3 days later (similar to what is seen in C57BL/6 mice) (Fig. 1C and Table 1, lung; Fig. 1L and Table 2, liver). Substantially different results were obtained for the CD40L-deficient mice. Transgene expression persisted for 28 days in both lung (Fig. 1G and H and Table 1) and liver (Fig. 1P and Q and Table 2) tissues, and neutralizing antibody failed to develop (Fig. 2), resulting in efficient transgene expression following a second administration of virus in the lung (Fig. 1I and Table 1) and detectable but substantially diminished gene transfer to the liver (Fig. 1R and Table 2).

Transient blockade of CD40 ligand with antibody prevents primary T-cell activation and prolongs transgene expression. The encouraging results obtained for the CD40L-deficient mice provided a basis for developing an adjunct gene therapy with adenovirus vectors based on pharmacological inhibition of CD40L signaling. We further speculated that the capsid proteins of the input virus are the primary source of antigen for $CD4^+$ T-cell activation, thereby restricting the time of costimulatory blockade to a short interval when vector is administered.

Experiments in C57BL/6 mice not treated with antibody or treated with radioisotype-labeled control antibody demonstrated high-level transgene expression at day 3 in lung (Fig. 1A and Table 1) and liver (Fig. 1J and Table 2) tissues that diminished to undetectable levels by day 28 (Fig. 1B and Table 1, lung; Fig. 1K and Table 2, liver). Gene transfer experiments were also performed in C57BL/6 animals injected i.p. with MAb to CD40L on days −3, 0, +3, and +6 relative to the initial vector administration. Studies in murine lung tissue demonstrated some stabilization of transgene expression in CD40L MAb-treated animals for at least 60 days (Fig. 1D and E show examples of histochemistry after 3 and 28 days, respectively; the results of morphometric analysis through day 60 are in Table 1): the number of airways showing transgene in >25% of epithelial cells declined from 72% on day 3 to 36% on day 60 (Table 1). Transgene expression was also stabilized in the livers of animals treated with CD40L MAb (Fig. 1M and N for 3 and 28 days, respectively), in which transgene-expressing hepatocytes persisted for 28 days with less than a 50% reduction in number (i.e., from 89 to 47%) (Table 2); similar levels of transgene expression persisted through day 60, which was the latest time point evaluated (data not shown).

Recipient animals were analyzed for antigen-specific activation of $CD4^+$ and $CD8^+$ T cells by both in vitro and in vivo assays. The effect of CD40L blockade on $CD4^+$ T cells was studied in proliferation assays of lymphocytes stimulated with UV-inactivated adenovirus (Table 3). Previous studies have shown a predominant T_{H1} response in these assays characterized by the secretion of gamma interferon and interleukin 2 from lymphocytes derived from regional sites (i.e., spleen for liver gene transfer and MLN lymphocytes for lung gene transfer [25, 27, 31]). Activation of adenovirus-specific T cells was

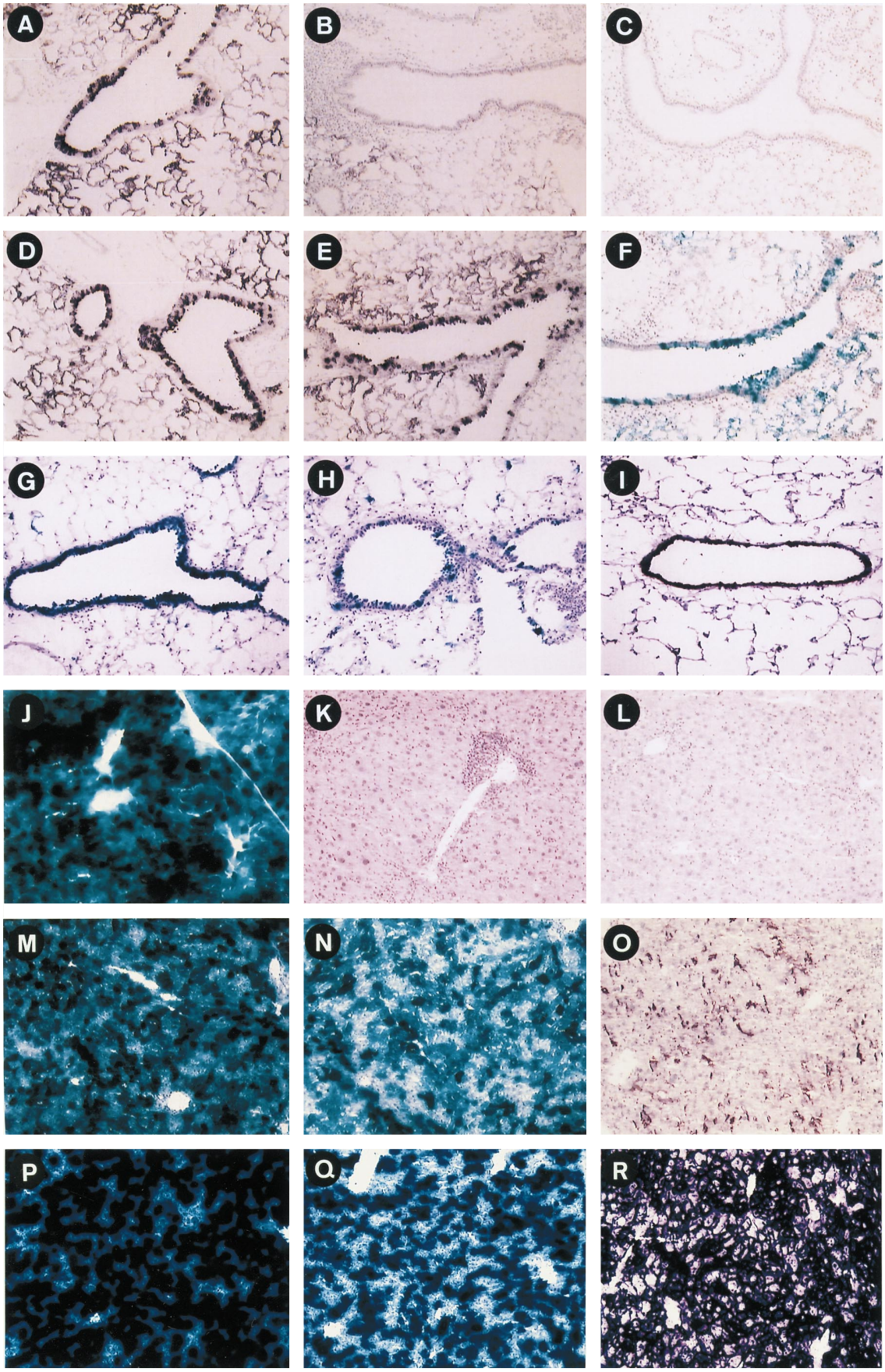
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FIG. 1. Histochemical analysis of lung and liver tissues. Mice were instilled with vector 1 (10^9 PFU) intratracheally (rows 1 to 3) or (2×10^9 PFU) intravenously (rows 4 to 6) and killed 3 (first column) and 28 (second column) days later for analysis of transgene expression in lung or liver tissue by a histochemical assay. Some animals were instilled with a second virus (vector 2) on day 28 and killed 3 days later (day 31; third column) for analysis of transgene expression. Rows 1 and 2: vector 1, ALP; vector 2, *lacZ*. Rows 3 to 6: vector 1, *lacZ*; vector 2, ALP. Lung-directed gene therapy model: first row, C57BL/6 mice; second row, C57BL/6 mice treated with CD40L antibody; third row, CD40L-deficient mice. Liver-directed gene therapy model: fourth row, C57BL/6 mice; fifth row, C57BL/6 mice treated with CD40L antibody; sixth row, CD40L-deficient mice. Magnification, ×95.

documented initially at day 7 in non-antibody-treated animals that were administered vector into the lung or liver; it increased progressively over the ensuing 14 days. T-cell activation was substantially inhibited in both models by coadministration of CD40L antibody; the greatest inhibition was observed when vector was administered into the lung.

Activation of $CD8^+$ T cells by vector-infected cells was analyzed in chromium release assays using MHC H-2-compatible target cells infected with adenovirus. As shown previously, specific lysis was demonstrated with lymphocytes harvested from C57BL/6 recipients on day 7 which were stimulated in vitro by adenovirus-infected antigen-presenting cells and incubated with adenovirus-infected targets (Fig. 3); no lysis was demonstrated when incubation was with mock-infected targets. Lymphocytes harvested from animals treated with CD40L antibody also demonstrated CTL activity to adenovirus-infected cells; however, the extent of lysis was consistently lower than that obtained with control animals (i.e., vector without CD40L antibody). The necessity to amplify CTL by in vitro stimulation prior to the cytolytic assay may obscure more significant differences in CTL activation that occurred following the primary exposure in vivo.

The primary effect of CD40L inhibition on the activation of adenovirus-specific $CD4^+$ T cells was further evaluated in vivo by immunocytochemical techniques. These experiments were restricted to the model of liver-directed gene transfer because of technical limitations of immunofluorescence in lung tissue sections. On day 14, liver tissues were analyzed by double immunofluorescence for infiltration of $CD4^+$ and $CD8^+$ T cells (Fig. 4). Non-antibody-treated animals showed a typical mixed-lymphocyte infiltrate that was dominated by $CD4^+$ T cells (Fig. 4B) and associated with substantial upregulation of MHC class I on the basolateral surfaces of hepatocytes (Fig. 4A). Previous studies have suggested that secretion of gamma interferon from T_{H1}-activated $CD4^+$ T cells contributes to the increase in MHC class I which may sensitize the hepatocytes to CTL-mediated elimination (31). Animals treated with antibody to CD40L still mobilized a mixed-lymphocyte infiltrate; however, the proportion of $CD4^+$ T cells was substantially lower (Fig. 4D) and the increase in MHC class I was substantially blunted (Fig. 4C). The specificity of the immunofluorescence assays was demonstrated in mock-infected animals (Fig. 4E and F).

CD40L antibody diminishes formation of blocking antibody to vector. We studied the impact of a transient blockade of CD40L signaling at the time of vector administration on the production of neutralizing antibody and efficiency of repeated vector administration. Animals who received vector on day 0 with or without CD40L MAb were treated with an adenovirus vector containing a different reporter gene on day 28; prior to administration of the second vector, blood was analyzed for neutralizing antibodies, and tissues were harvested for analysis of reporter gene expression 3 days later (i.e., day 31).

The most impressive results were obtained in the model of lung-directed gene therapy. The development of neutralizing antibody in BAL fluid following lung-directed gene transfer of vector was inhibited 20-fold in animals administered CD40L antibody (Fig. 2). Gene transfer with the second vector was unsuccessful in non-antibody-treated animals (Fig. 1C) or animals treated with isotype-labeled control MAb (data not shown), as evidenced by the complete absence of transgene expression 3 days after vector readministration. This contrasts with animals treated with CD40L antibody during the first vector administration, in whom gene transfer was accomplished following a second administration of vector (Fig. 1F). The level of transgene expression accomplished following a second vector administration was independent of the time interval between the two vector treatments; transgene expression was detected in >25% of airway epithelial cells of 30, 35, and 36% of airways when the second vector was administered 30,

TABLE 1. Quantitative analysis of mouse lung tissues for efficiency of transgene expression[a]

Treatment group	% Transgene/ airway[b]	% of total airways		
		Day 3	Day 28	Day 31
Control	0	8	95	100
	1–25	16	5	0
	>25	76	0	0
CD40L KO	0	12	35	27
	1–25	13	35	38
	>25	75	30	45
CD40L Ab (expt I)	0	10	20	35
	1–25	18	38	35
	>25	72	42	30
		Day 3	Day 42	Day 45
CD40L Ab (expt II)	0	9	25	30
	1–25	21	35	35
	>25	70	40	35
		Day 3	Day 60	Day 63
CD40L Ab (expt III)	0	7	27	32
	1–25	20	37	32
	>25	73	36	36

[a] Data were quantified after examining a total of 100 airways from three mice for the presence of transgene-containing respiratory epithelial cells.

[b] 0, no transgene expression; 1–25, transgene expression in 1 to 25% of epithelial cells; >25, transgene expression in >25% of epithelial cells.

TABLE 2. Morphometric analysis of mouse liver tissue for efficiency of transgene expression[a]

Treatment group	% Transgene expression		
	Day 3	Day 28	Day 31
Control	90.5 ± 2.6	0	0
CD40L Ab	89.3 ± 3.1	46.7 ± 4.8	8.2 ± 4.2
CD40L KO	92.3 ± 4.0	60.4 ± 2.8	85.9 ± 3.4

[a] Data were quantified with a Quantimet 500+ (Leica) by analyzing a total of 15 lobes from three mice for *lacZ*-expressing hepatocytes on days 3 and 28 and for ALP-expressing hepatocytes at day 31. Data are presented as the means ± standard deviations.

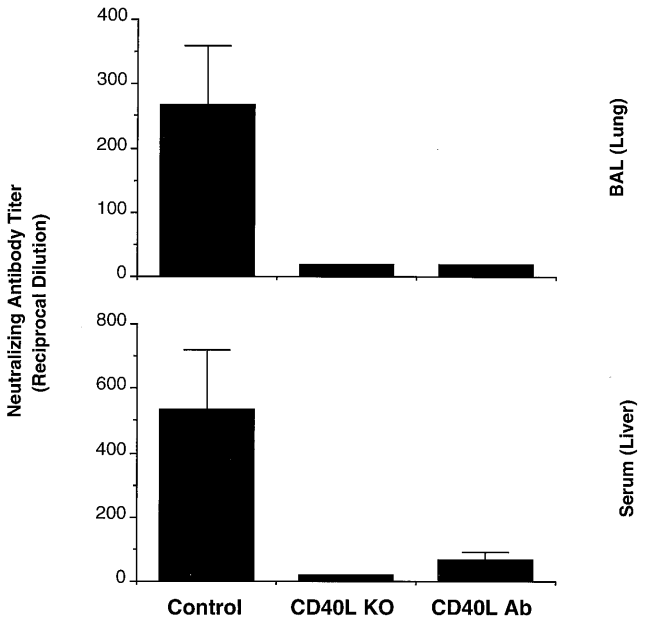

FIG. 2. Antiviral neutralizing antibodies. BAL (lung experiment) and serum (liver experiment) samples were obtained from animals 28 days after primary exposure to adenovirus and were analyzed for neutralizing antibodies to Ad5. Experiments involved C57BL/6 mice (control), CD40L-deficient mice (CD40L KO), and C57BL/6 mice treated with CD40L antibody (CD40L Ab). Data are presented as the mean neutralizing antibody titers of three samples ± standard deviations.

42, and 90 days, respectively, after the original vector and CD40L antibody treatment. This is somewhat lower than the number of airways that express transgene in a naive animal treated with vector (i.e., 75%) (Table 1). Antibody to CD40L partially blocked the production of neutralizing antibody in serum following intravenous infusion of virus (Fig. 2). This was sufficient to enable some gene transfer to the liver with the second vector (8% of hepatocytes) (Fig. 1O), which did not occur in the absence of CD40L antibody (Fig. 1L), but is substantially reduced from that achieved following a primary administration of vector to a naive animal (89%) (Fig. 1J).

The experiments described above evaluated the effect of transient inhibition of CD40L on the performance of adenovirus vectors. This strategy presumes that the recipient animal regains normal T-cell function in vivo soon after initial vector administration. The transient nature of our immune blockade was confirmed in animals evaluated for primary response to vector 28 days after treatment with the regimen of CD40L MAb; at that time, the animals were capable of a full spectrum of host responses to the vector, including activation of CTLs and formation of neutralizing antibody similar to what is seen in a naive animal after vector treatment (data not shown).

DISCUSSION

The necessary role of $CD4^+$ T cells in both the humoral and cellular immune responses to adenovirus vector suggested strategies to improve the utility of this vector system based on pharmacological inhibition of T-cell activation. Recent studies of the biology of antigen-specific T-cell activation suggested that CD40L- or CD40-mediated signaling may be an appropriate target for intervention. Initial characterization of mice deficient in either CD40L or CD40 suggested a primary defect in B-cell activation and germinal-cell formation believed to be due to a defect in B-cell signaling through CD40 (12, 17, 24). Consistent with this is the hyper-IgM syndrome found in humans who are genetically deficient in CD40L (1, 2, 4, 14). Recent studies in the mouse models indicate, however, that CD40L deficiency leads to a primary defect in T-cell activation (10, 19, 21). It is unclear whether this is a direct signaling effect from CD40L or an indirect effect mediated by downstream signaling events. The role of CD40L in T-cell priming helps explain manifestations of the hyper-IgM syndrome that are most consistent with T-cell dysregulation and suggests that interfering with CD40L signaling may prevent both humoral and cellular immune responses to adenovirus vectors.

The hypothesis on which the strategy of immune ablation was derived is that the $CD4^+$ T-cell activation necessary for CTL and B-cell responses is primarily in response to capsid proteins of the administered virus, which are presented in an MHC class II-dependent manner. Evidence in support of this hypothesis includes the full spectrum of $CD4^+$ T-cell primary responses to a UV-inactivated vector in C57BL/6 mice (i.e., in vitro proliferation assays and appearance of neutralizing antibody) which are absent when vector is administered to mice deficient in MHC class II (25, 27). The corollary of this hypothesis is that inhibition of this process may be necessary only for a limited period after vector administration during a time when exogenous antigens are presented. The success of transient depletion of $CD4^+$ T cells with antibody in the setting of adenovirus vectors directed to liver and lung tissues provides further support for the strategy (26, 30).

Gene therapy studies in mice genetically deficient in CD40L illustrated the importance of this molecule in T-cell responses to adenovirus vectors. Adenovirus-encoded transgene expression was stabilized in lung and liver tissues, and the animals failed to produce neutralizing antibody, allowing readministration of vector at levels similar to that seen in a naive mouse. A

TABLE 3. Inhibition of adenovirus-specific T-helper response by antibodies to CD40L[a]

Tissue	Day	Treatment group	[^{3}H]thymidine incorporation (mean cpm ± SD)	
			Unstimulated	Stimulated
Lung	7	Control	384 ± 44	4,288 ± 125
		CD40L Ab	287 ± 40	358 ± 18
	14	Control	379 ± 56	8,278 ± 431
		CD40L Ab	323 ± 28	769 ± 65
	21	Control	245 ± 17	12,992 ± 1,024
		CD40L Ab	336 ± 26	3,349 ± 120
Liver	7	Control	316 ± 38	6,321 ± 225
		CD40L Ab	287 ± 16	1,191 ± 104
	21	Control	264 ± 32	14,028 ± 344
		CD40L Ab	289 ± 54	5,951 ± 257

[a] Lymphocytes of MLNs (for lung experiment) or splenocytes (for liver experiment) from infected mice were incubated in medium (unstimulated) or restimulated with UV-inactivated virus (stimulated) for 24 h. Supernatants were tested on HT-2 cells for proliferation, and [^{3}H]thymidine incorporation was measured 72 h later.

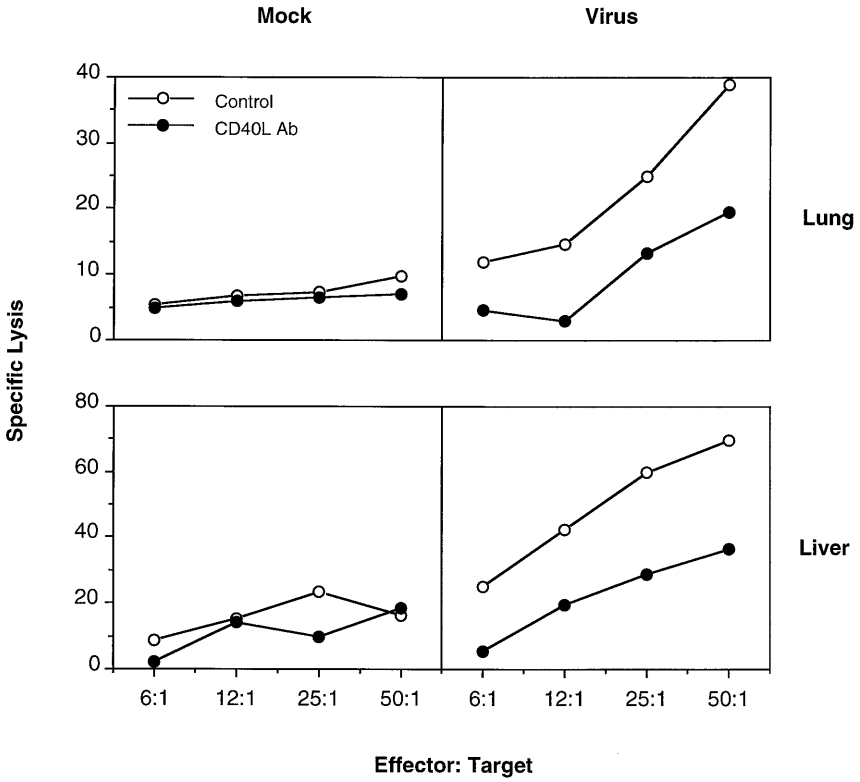

FIG. 3. CTL responses. Lymphocytes harvested from control C57BL/6 mice (open circles) and C57BL/6 mice treated with antibody to CD40L (closed circles) 7 days after administration of virus were restimulated in vitro for 5 days and tested for specific lysis on mock-infected (Mock) or virus-infected (Virus) C57SV cells in a 6-h ^{51}Cr release assay. The percentage of specific lysis is expressed as a function of different effector-to-target ratios (6:1, 12:1, 25:1, and 50:1). Splenocytes and MLN cells were used for lung and liver experiments, respectively.

therapeutic strategy to pharmacologically block T-cell activation via CD40L-CD40 interactions was developed using an antibody to CD40L that interferes with its binding to CD40 without activating its own signaling pathways. Administration of this antibody with thymus-dependent antigens in BALB/c mice prevents the development of germinal centers, primary antibody responses, and B-cell memory (9). Successful treatment of autoimmune diseases has been achieved in animal models with this antibody (5).

Transient inhibition of CD40L with antibody blocked $CD4^+$ T-cell priming in the lung model of gene therapy and diminished $CD4^+$ T- and B-cell effector responses; the persistent transgene expression and efficiency of vector readministration into the lung were essentially identical in animals genetically deficient in CD40L and in those transiently inhibited with CD40L antibody. This was not the case in the liver model of gene transfer, in which the expression of transgene was equally prolonged, but the blockade of neutralizing antibody and efficiency of vector readministration were more impressive in the CD40L KO mice than in the animals transiently depleted with MAb to CD40L. This suggests that the partial effect obtained with CD40L antibody is probably due to the incomplete blockade achieved with the antibody rather than redundant pathways of T-cell activation. Vector infused into the peripheral circulation is deposited to a minor extent in the spleen, where professional antigen-presenting cells are positioned to efficiently process large quantities of antigen for activation of $CD4^+$ T cells and B cells.

Of importance to the application of these data is the premise that pharmacological inhibition of $CD4^+$ T-cell activation is necessary for only a restricted period of time after vector administration. This means that chronic immune suppression is not necessary to sustain transgene expression and prevent neutralizing antibodies from forming. Our data support this premise in that transgene expression and efficient readministration of vector continues for at least 60 days, which is far beyond the period that the CD40L antibody is active. Previous studies by Durie et al. indicate that repeated injection of similar quantities of this antibody every 4 days is necessary to chronically suppress CD40L function (6).

Blockade of $CD4^+$ T-cell activation with a CD4-depleting antibody has yielded encouraging results in previous studies of adenovirus vector delivery to mouse liver and lung tissues (26, 30). However, previous attempts to interfere more specifically with the actual activation of $CD4^+$ T cells by vector have been less successful. Coadministration of interleukin 12 with vector blocked T_{H2} activation, leading to complete elimination of neutralizing antibody in lung tissue and partial inhibition of antibody when vector was delivered to the liver (26, 30); these benefits were realized at the expense of full, if not accentuated, T_{H1} responses. As expected, transgene expression was not prolonged but vector was effectively readministered in the lung model. Kay et al. administered an inhibitor of the CD28-CTLA4 pathway (i.e., CTLA4 Ig) in a murine model of adenovirus vector delivery to the liver (13). This soluble receptor blocked T-cell activation and prolonged transgene expression beyond the period of CTLA4 Ig administration. In contrast to our studies, there was no effect on neutralizing antibody and vector was not successfully readministered. This could be due to incomplete inhibition of B7-mediated signaling or to the fact

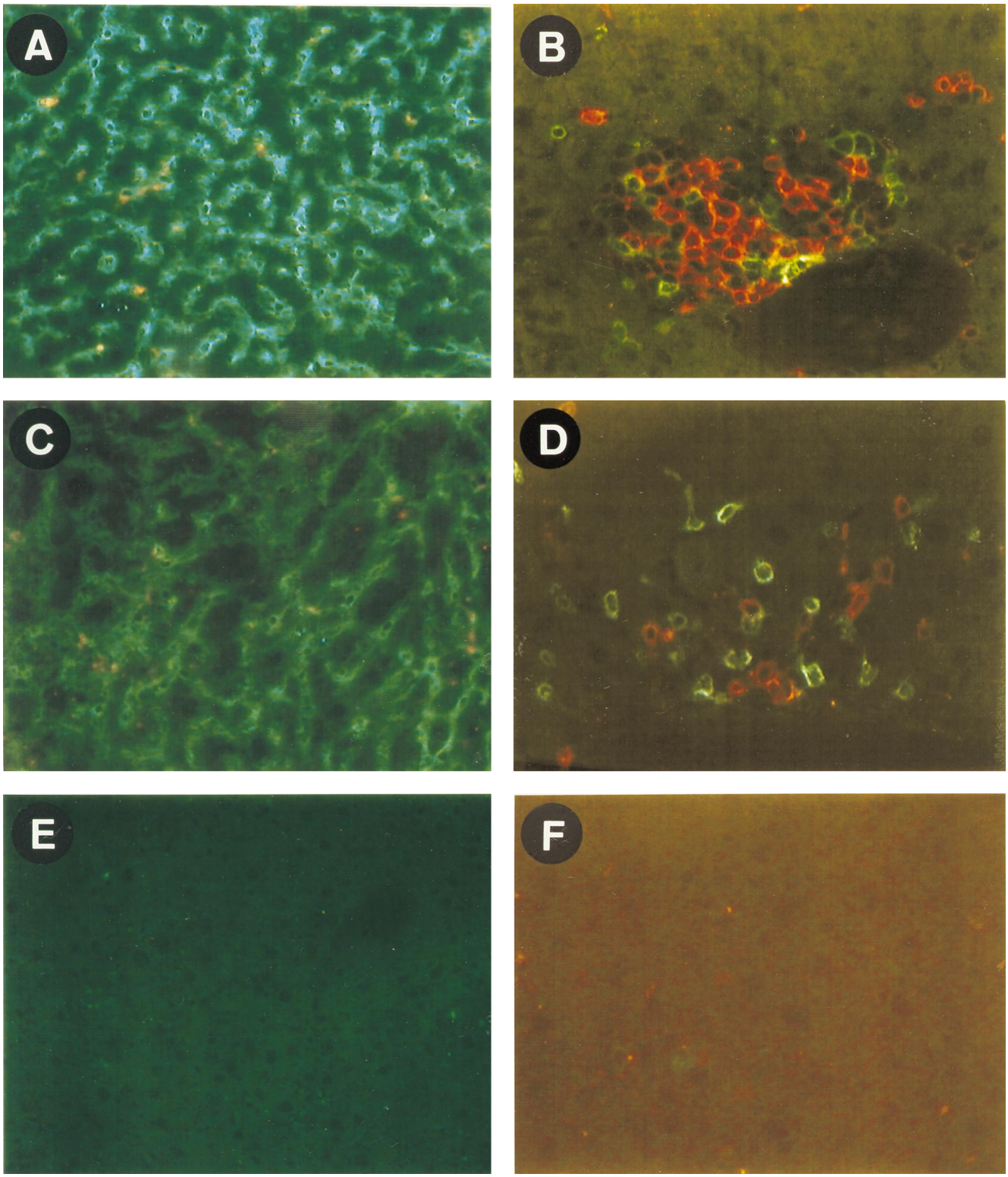

FIG. 4. MHC class I expression and $CD4^+$ and $CD8^+$ cell infiltration in mouse liver. Liver tissues harvested from *lacZ*-virus-infected C57BL/6 mice on day 14 (A and B), *lacZ*-virus-infected C57BL/6 mice treated with CD40L antibody on day 14 (C and D), and naive C57BL/6 mice (E and F) were stained for MHC class I expression (A, C, and E) and $CD4^+$ and $CD8^+$ cell infiltration (B, D, and F) by immunofluorescence as described in the text. $CD4^+$ cells were labeled with rhodamine (red), while $CD8^+$ cells were labeled with fluorescein isothiocyanate (green). Magnifications, ×178 (A, C, and E) and ×356 (B, D, and F).

that CTLA4 and CD28 provide opposing signals for T-cell activation. Studies in KO mice indicate that CD28 is a stimulator of T cells while CTLA4 is a negative regulator of T cells, suggesting that the inhibition of B7-mediated signaling with CTLA4 Ig may yield complex and partial results (20, 23).

In summary, these studies suggest that transient interference with CD40L signaling of T cells may diminish destructive host immune responses to adenovirus vectors. The effects obtained with antibody inhibition of CD40L were less complete than those observed in the genetic KO mice, suggesting that more efficient pharmacological approaches should be considered. The utility of this approach for human gene therapy will require further investigation, including the development of humanized CD40L antibodies and testing in appropriate models.

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