# Mechanisms of Drug-induced Lupus II. T Cells Overexpressing Lymphocyte Function-associated Antigen 1 Become Autoreactive and Cause a Lupuslike Disease in Syngeneic Mice

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## Abstract

**Current theories propose that systemic lupus erythematosus develops when genetically predisposed individuals are exposed to certain environmental agents, although how these agents trigger lupus is uncertain. Some of these agents, such as procainamide, hydralazine, and UV light inhibit T cell DNA methylation, increase lymphocyte function-associated antigen 1 (LFA-1) (CD11a/CD18) expression, and induce autoreactivity in vitro, and adoptive transfer of T cells that are made autoreactive by this mechanism causes a lupuslike disease. The mechanism by which these cells cause autoimmunity is unknown. In this report, we present evidence that LFA-1 overexpression is sufficient to induce autoimmunity. LFA-1 overexpression was induced on cloned murine Th2 cells by transfection, resulting in autoreactivity. Adoptive transfer of the transfected, autoreactive cells into syngeneic recipients caused a lupuslike disease with anti–DNA antibodies, an immune complex glomerulonephritis and pulmonary alveolitis, similar to that caused by cells treated with procainamide. These results indicate that agents or events which modify T cell DNA methylation may induce autoimmunity by causing T cell LFA-1 overexpression. Since T cells from patients with active lupus have hypomethylated DNA and overexpressed LFA-1, this mechanism could be important in the development of human autoimmunity. (*J. Clin. Invest.* 1996. 97:2866–2871.) Key words: DNA modification methylases • glomerulonephritis • pulmonary diseases • antinuclear antibodies • autoimmunity**

## Introduction

Procainamide (Pca)[1], hydralazine, and UV light can trigger human lupus (1, 2). These agents also inhibit T cell DNA methylation, and polyclonal as well as cloned human and murine CD4+ T cells become autoreactive after treatment with these and other DNA hypomethylating agents (3–7). Adoptive transfer of T cells made autoreactive by this mechanism causes a lupuslike disease in unirradiated syngeneic recipients (6, 7), suggesting a mechanism by which these agents might induce lupus. However, how these agents modify T cells to make them pathogenic is unknown.

The autoreactivity correlates with lymphocyte function-associated antigen 1 (LFA-1) (CD11a/CD18) overexpression, and concentrations of anti–CD11a insufficient to affect antigen reactivity will completely inhibit the autoreactive response (8). This suggests that LFA-1 overexpression contributes to the autoreactivity, and inhibiting function of the additional molecules reverses it (8). Cloned human T cells transfected with a CD18 cDNA also overexpress CD11a/CD18 and become autoreactive (5), further supporting the relationship between LFA-1 overexpression and T cell autoreactivity. These observations raise the possibility that LFA-1 overexpression might also contribute to the development of autoimmunity induced by hypomethylated T cells. However, inhibiting T cell DNA methylation probably affects expression of multiple genes, and the ability of the hypomethylated cells to induce autoimmunity may require altered expression of more than one gene. In this report, we examined the role of LFA-1 overexpression in autoimmunity by stably transfecting a cloned murine T cell line with a CD18 cDNA construct. We then asked if the transfected cells become autoreactive and induce a disease similar to that caused by treating the same cells with Pca, a DNA methylation inhibitor.

## Methods

*Mice and peritoneal macrophage (Mø) isolation.* Young (6–8 wk of age) female AKR (H-2$^k$), C3H (H-2$^k$), DBA/2 (H-2$^d$), and SJL (H-2$^s$) mice were obtained from The Jackson Laboratories (Bar Harbor, ME) and maintained in a specific pathogen-free environment. Peritoneal Mø were obtained by i.p. thioglycollate (Becton Dickinson and Co., Cockeysville, MA) injection and harvested 3 d later as previously described (6, 7).

*T cell culture.* D10.G4.1 cells (9), obtained from the American Type Culture Collection (Rockville, MD), were cultured as previously described (6, 7). Because of a report that the D10.G4.1 cell line may contain an autoreactive subset (10), the D10 cells were subcloned by limiting dilution at ≤ 0.2 cells/well, and a nonautoreactive subclone selected for use in these studies. All cells used for cytofluorographic or functional analysis were studied 6 d after challenge to avoid changes in LFA-1 expression due to restimulation.

*Generation of the pSub2-CD18 construct and transfection into D10 cells.* The mammalian expression vector pSub2, which contains a neomycin-resistant cassette, was kindly contributed by Dr. Mike

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*Received for publication 19 January 1996 and accepted in revised form 22 March 1996.*

1. *Abbreviations used in this paper:* LFA-1, lymphocyte function-associated antigen 1; Mø, macrophage; Pca, procainamide; RFI, relative fluorescence intensity.

The Journal of Clinical Investigation
Volume 97, Number 12, June 1996, 2866–2871

Clarke. The pSub2 polylinker site was cleaved with EcoRV, and EcoR1(Not1) adapters (GIBCO BRL, Gaithersburg, MD) ligated to the blunt ends. A full-length cDNA-encoding murine CD18, kindly contributed by Dr. Raymond Wilson (Baylor College of Medicine, Houston, TX), was excised with EcoR1 and cloned into the EcoR1 sites generated by the adapters, and the constructs subcloned. The cDNA orientation in the subclones was determined by digestion with BglII, and a plasmid containing the complete cDNA in the sense direction was selected. The construct was linearized by digestion with ScaI and D10 cells transfected by electroporation (250 V and 960 μF), using previously published protocols (5). Stable transfectants were selected and maintained by culturing in media containing 250 μg/ml Geneticin (G418) (GIBCO BRL). Where indicated, transfectants were subcloned by limiting dilution at ≤ 0.2 cells/well. Control pSub2 transfectants were generated using the native vector (lacking the CD18 insert) and the same linearization, transfection, and selection protocols.

*Cytotoxicity and proliferation assays.* Proliferation assays were performed as described (6, 7), using irradiated (3,000 R) peritoneal Mø as antigen presenting cells. For cytotoxic responses, peritoneal Mø were labeled with $^{51}$Cr and used as targets as previously described (6, 7). All proliferation and cytotoxicity experiments were performed in quadruplicate, and results are presented as the mean±SEM. Where indicated, mAb to I-A$^d$ (Becton Dickinson), H-2K$^k$ (PharMingen, San Diego CA), I-A$^k$ (PharMingen), or CD11a (M17/4.2; American Type Culture Collection [and grown according to their directions]) were added to the cultures. In some experiments, the anti-CD11a was affinity purified from culture supernatant using protein G–sepharose, and quantitated by ELISA (6). These mAbs were used at concentrations equal to or greater than those recommended by the manufacturer to completely saturate the cells.

*Flow cytometric analysis.* Cultured T cells were stained with anti–CD4-FITC (Becton Dickinson, and Co.) or anti-CD11a (LFA-1 α)

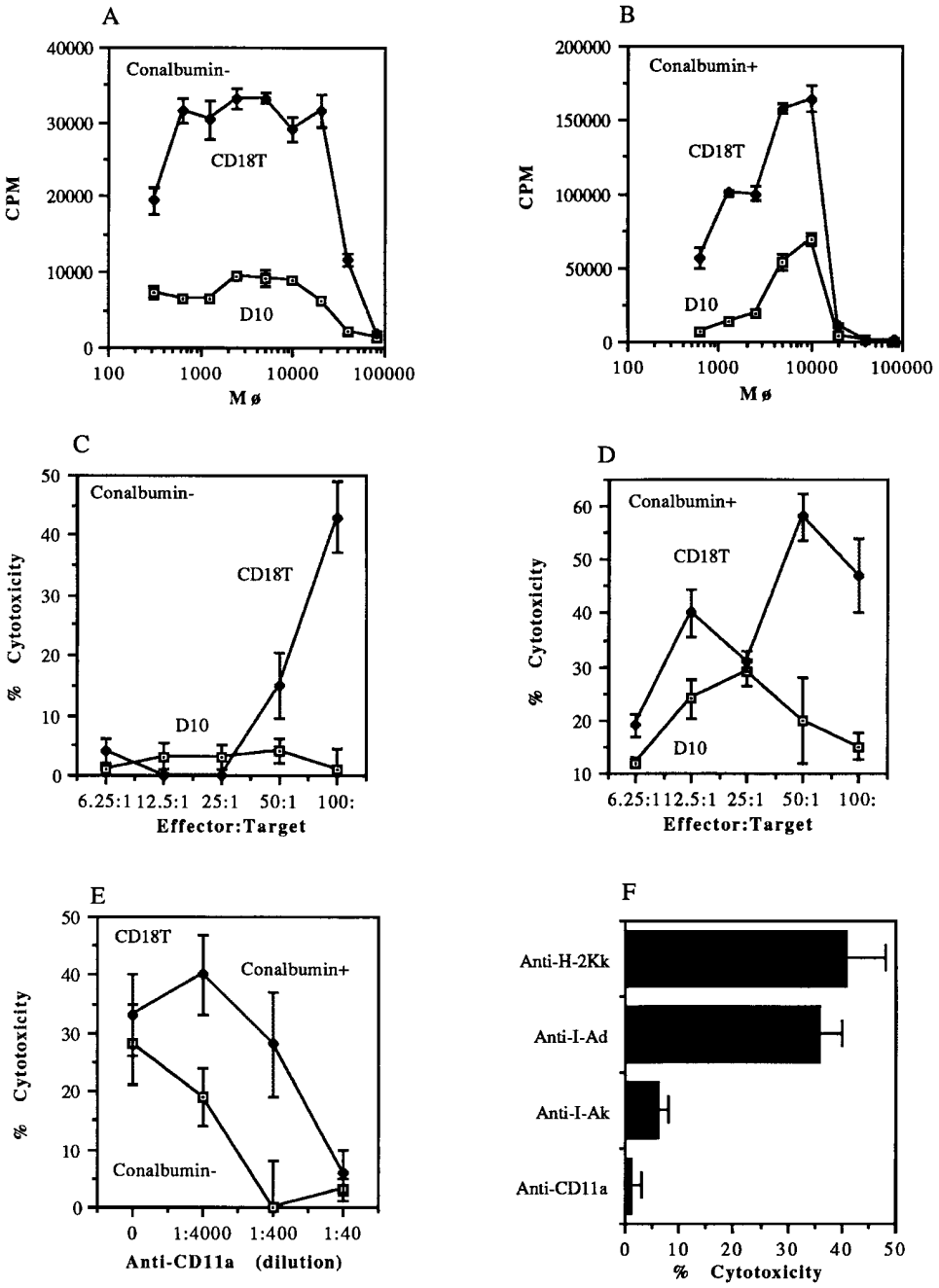


*Figure 1.* (*A*) 20,000 CD18-transfected (*CD18T*, *closed diamonds*) and -untransfected (*D10*, *open squares*) D10 cells were cultured with the indicated number of irradiated AKR peritoneal Mø. Proliferation was measured 4 d later by $^3$H-labeled TdR incorporation. (*B*) 20,000 CD18T and D10 cells were cultured with the indicated number of irradiated AKR peritoneal Mø and conalbumin. Proliferation was determined by $^3$H-labeled TdR incorporation as in *A*. (*C*) ~ 5,000 $^{51}$Cr-labeled AKR peritoneal Mø were incubated with CD18T or D10 cells at the indicated effector/target ratios. $^{51}$Cr release was measured 18 h later. (*D*) ~ 5000 $^{51}$Cr-labeled AKR peritoneal Mø were incubated with CD18T or D10 cells at the indicated effector/target ratios, in the presence of conalbumin, and $^{51}$Cr release measured as in *C*. (*E*) 125,000 CD18T cells and ~ 2500 $^{51}$Cr-labeled AKR Mø were cultured with (*closed diamonds, conalbumin+*) or without (*open squares, conalbumin-*) conalbumin. The indicated dilutions of anti-CD11a were added, and $^{51}$Cr release was measured as in *C*. (*F*) 125,000 CD18T cells were cultured with ~ 2500 $^{51}$Cr-labeled AKR Mø without added antigen as in *E*. The indicated antibodies were added at a final concentration of 10 μg/ml, and $^{51}$Cr release was measured as in *C*. In this experiment, the cytotoxicity in control cultures without added antibodies was 47±4%.

(M17/4.2) and goat anti–mouse Ig-FITC (Coulter Corp., Hialeah, FL) as previously described (6, 7), and analyzed on a Coulter ELITE flow cytometer. The results are presented in graphic form as plots of cell count vs fluorescence intensity in arbitrary units, and in numeric form as relative fluorescence intensity (RFI).

*DNA isolation and Southern analysis.* DNA was purified from D10 cells and transfectants as previously described (5), and Southern analysis performed using previously published protocols (5). Briefly, 30 μg of genomic DNA from control or transfected D10 cells were digested with NotI (Boehringer Mannheim, Germany) or EcoRI, fractionated through agarose gels, and transferred to nylon filters (MagnaGraph; Micro Separations, Inc., Westborough, MA). The filters were hybridized with digoxigenen-labeled (Genius system; Boehringer Mannheim) CD18 or M13, excised from pSub2 with EcoRI. Signals were detected by chemiluminescent methods according to the manufacturer's directions.

*ELISA assays.* Total serum IgG and IgM concentrations were measured using previously published protocols (6, 7). Anti–ssDNA and anti–dsDNA antibody titers were determined by coating Immulon 4 plates with purified ssDNA (Sigma Chemical Co., St. Louis, MO) or dsDNA (cesium chloride-purified $KS^+$-SV2CAT plasmid) as described (7). Horseradish peroxidase-conjugated goat anti-mouse polyvalent (IgG, IgM, IgA) antibody (Sigma Chemical Co.) was used as the secondary antibody before developing with Sigma Fast tablets. Controls included identical determinations performed in the presence of 2.5 μg/ml purified dsDNA or ssDNA as specific inhibitor. Results were calculated relative to the controls with specific inhibitor and are presented as the mean of quadruplicate determinations on sera from individual mice. Positive controls included pooled serum from ≥ 6-mo-old female NZB/W mice. Results from the anti–dsDNA ELISA were confirmed using *Crithidia luciliae* (Quantafluor, Chaska, MN), performed by the Immunopathology Laboratory at the University of Michigan Hospital (Ann Arbor, MI) using an FITC-conjugated goat anti–mouse IgG (Coulter Corp.) and scored on a 0–4+ scale. Defining a positive *Crithidia* test as ≥ 2+ gave excellent (< 10% error) correlation with positive (OD ≥ 0.3) ELISA tests.

*Tissue fixation and histologic analysis.* Tissues were fixed in formalin, and then sectioned and stained as described (6). Frozen kidney sections were reacted with FITC-conjugated antisera specific for IgG using standard procedures.

*Statistical analysis.* The difference between means was tested using Student's two-tailed *t* test.

## Results

*Establishment of CD18 transfectants.* Initial experiments asked whether CD18 transfection induces autoreactivity in murine T cells, similar to human T cells (5). D10, a cloned conalbumin-reactive murine Th2 line originally isolated from AKR ($Ia^k$) mice (9) and previously shown to induce autoimmunity after treatment with DNA methyltransferase inhibitors (7), was first subcloned by limiting dilution, and then an antigen-reactive subclone was selected. A full-length cDNA-encoding murine CD18 was cloned into the mammalian expression vector pSub2, linearized by digestion with ScaI, transfected into the D10 subclone by electroporation, and stable transfectants selected using G418. LFA-1 expression was compared on the transfectants and untransfected D10 cells by flow cytometry. While initially there was some heterogeneity in LFA-1 staining intensity, a uniform population overexpressing LFA-1 grew out over time (RFI 13.5 for control D10, and 30.8 for the transfectants). Incorporation of the full-length CD18 cDNA was confirmed by Southern analysis (see Fig. 2).

*Characterization of CD18-transfectant autoreactivity.* Fig. 1, *A* and *B* compares the proliferative responses of control and transfected cells to syngeneic Mø without and with antigen. The transfected cells respond to syngeneic Mø without antigen and demonstrate an enhanced response to antigen-presenting Mø, similar to transfected human cells (5). These results were confirmed using the killing of syngeneic Mø (Figs. 1, *C* and *D*). Fig. 1 *E* demonstrates that the autoreactive response of the transfectants is more sensitive to inhibition with anti-CD11a than is the antigen response, similar to drug-treated and transfected human T cells (5, 8). Fig. 1 *F* demonstrates that the autoreactive response is inhibited by antibodies to syngeneic

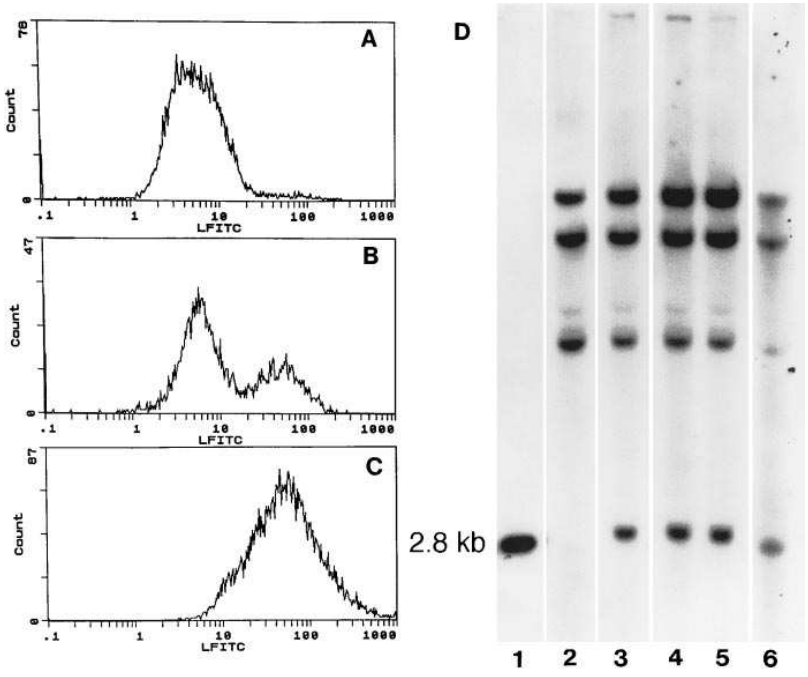


*Figure 2.* (*A*) Untreated D10 cells stained with anti–murine CD11a and goat anti–mouse FITC, and then analyzed by cytofluorography. The x-axis represents fluorescence intensity on an arbitrary scale, and the y-axis represents cell number. (*B*) D10 cells cultured with 50 μM Pca for 6 d and then stained as in *A*. LFA-1 overexpression is seen on a subset. (*C*) Representative CD18-transfected D10 subclone, stained as in *A*. (*D*) DNA from the pSub2-CD18 construct (lane *1*), untransfected D10 cells (lane *2*), or subcloned CD18 transfectants (lanes *3–6*) was digested with EcoR1, fractionated by agarose gel electrophoresis, transferred to nylon membranes, and then hybridized with a digoxygenin-labeled full-length CD18 cDNA. The full-length CD18 cDNA in the CD18 transfectant and the pSub2-CD18 construct is identified by the 2.8-kb mark. Untransfected D10 cells (lane *2*) lack the 2.8-kb band.

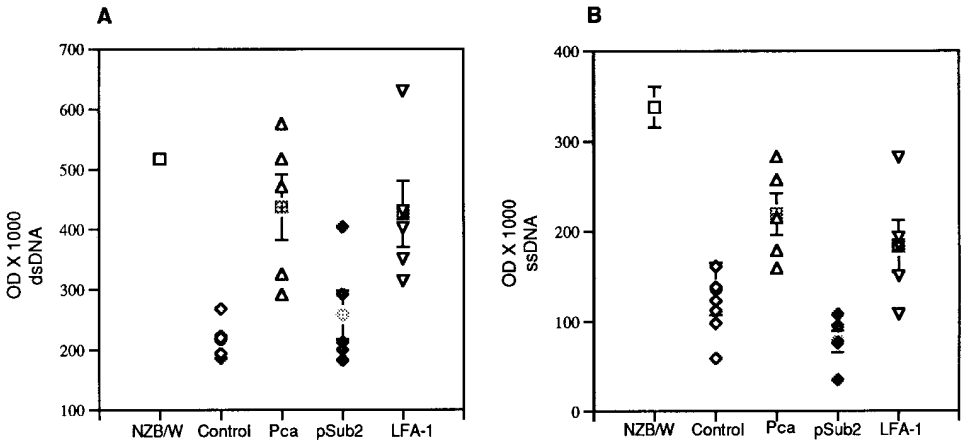


*Figure 3.* Sera were obtained from 6-mo-old female New Zealand Black/White (*NZB/W*) mice or from mice receiving untreated (*Control*), 50 μM Pca-treated (*Pca*), pSub2-transfected (*pSub2*), or CD18-transfected (*LFA-1*) D10 cells. Anti–dsDNA (*A*) or ssDNA (*B*) antibodies were measured by ELISA. Mice receiving the CD18-transfected cells made significantly more anti–dsDNA ($P < 0.05$) and ssDNA ($P < 0.01$) than mice receiving the pSub2-transfected cells. Similarly, Pca-treated cells induced more anti–dsDNA ($P < 0.005$) and more anti-ssDNA ($P < 0.005$) than untreated cells.

class II, but not syngeneic class I or allogeneic class II, MHC molecules. This specificity is similar to that seen in human and murine T cells treated with DNA methylation inhibitors (3, 6), and argues that the autoreactive cells are responding to determinants on self I-A molecules. This was further tested by comparing the responses to allogeneic Mø. The CD18 transfectants lysed H-$2^k$ (AKR and C3H) Mø more efficiently than the same number of H-$2^s$ (SJL) or H-$2^d$ (DBA/2) Mø (40±1.0% vs 24.5±0.5%, $P < 0.01$, H-$2^k$ vs H-$2^d$ and H-$2^s$), suggesting preferential lysis of Mø-bearing self-Ia.

To confirm the relationship between CD18 transfection, LFA-1 overexpression, and autoreactivity, the transfected cells were resubcloned. The CD18-transfected subclones expressed amounts of LFA-1 similar to that induced by Pca (Fig. 2, *A–C*) and incorporated a full-length CD18 cDNA (Fig. 2 *D*). To exclude the possibility that the pSub2 vector causes autoreactivity or autoimmunity, D10 cells were also transfected with the linearized pSub2 vector without the CD18 cDNA insert, transfectants selected with G418, and the cells subcloned. Incorporation of the vector was confirmed by Southern analysis as before, using the bacterial M13 origin of replication sequence as a probe (not shown). The control pSub2 transfectants expressed amounts of LFA-1 equivalent to control D10 cells (RFI 32 for untransfected D10 and RFI 28 for the pSub2 transfectants). The responses of the two transfected lines were then compared. Using proliferation assays, the CD18 subclone was

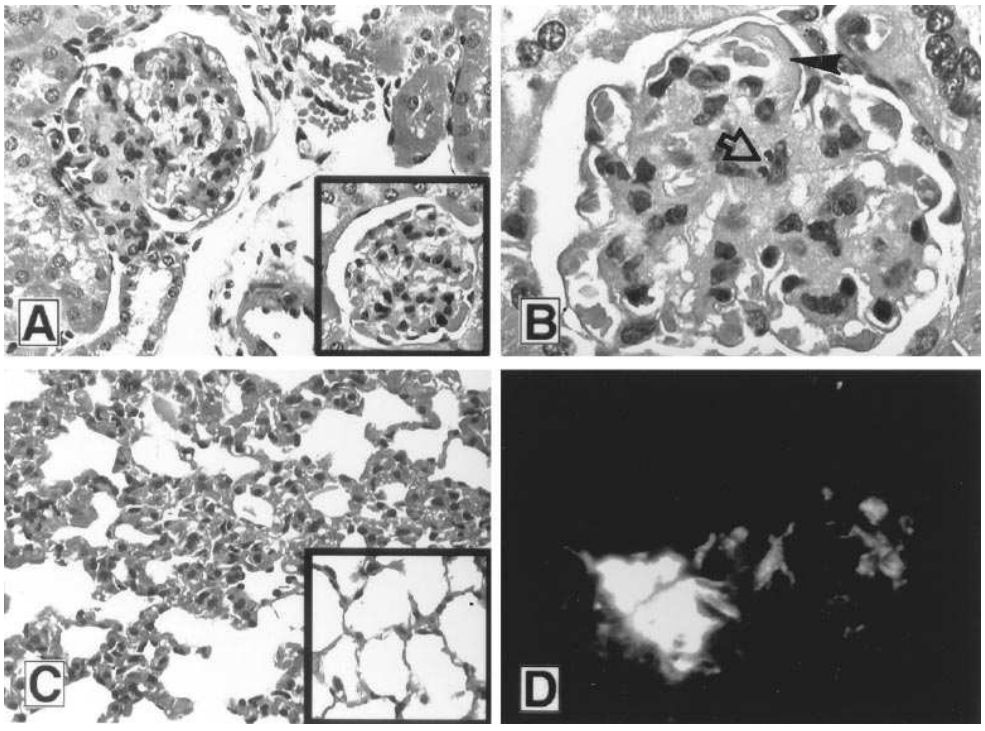


*Figure 4.* (*A*) Hematoxylin and eosin stain of a representative kidney section from mice receiving CD18-transfected D10 cells. Note hypercellularity, increased mesangial matrix, and obliteration of glomerular capillary loops (× 100). The inset shows a representative glomerulus from mice receiving pSub2-transfected cells. The glomerulus is normal (× 100). (*B*) Wire loop lesion (*closed arrow*) in the glomerulus of a mouse receiving CD18 transfected cells. Karyorrhectic nuclear debris is also seen (*open arrow*). (Hematoxylin and eosin stain, 250X). (*C*) Hematoxylin and eosin stains of lung sections from mice receiving CD18-transfected cells. Note widening of the alveolar septae caused by an alveolitis with interstitial infiltration by acute and chronic inflammatory cells (× 100). (*Inset*): A representative lung section from mice receiving pSub2-transfected D10 cells, showing normal architecture (× 100). (*D*) IgG immunofluorescent stain of a glomerulus from a mouse receiving CD18-transfected D10 cells. There is 3+ deposition of IgG in the glomeruli (× 250).

significantly more autoreactive than the pSub2 subclone and approximately equal in autoreactivity to Pca-treated cells (CD18: 26,650±1,984 CPM; pSub2: 3,971±356 CPM; 50 μM Pca 23,628±6,551 CPM; untreated D10: 3,052±665 CPM; $P <$ 0.001, CD18 vs pSub2, mean±SEM of quadruplicate determinations using 20,000 T cells and 20,000 irradiated AKR peritoneal Mø without added antigen). The CD18 subclone was also significantly more autoreactive than the pSub2 subclone using syngeneic Mø killing assays (22±7% vs 0±6% lysis, CD18 vs pSub2, $P < 0.05$, mean±SEM of quadruplicate determinations at an effector/target ratio of 25:1). Together, these results indicate that transfection of murine T cells with a CD18 cDNA construct induces LFA-1 overexpression and autoreactivity that is similar to that seen in drug-treated human and murine T cells and transfected human T cells.

*Induction of autoimmunity by CD18-transfected cells.* Unirradiated syngeneic (AKR) female mice were then given six i.v. injections of $5 \times 10^6$ untreated, Pca-treated, pSub2 transfected, or subcloned pSub2-CD18-transfected D10 cells, administered every 2 wk. 4 wk after the last injection, the mice were killed and evidence for autoimmunity sought. Fig. 3, *A* and *B* compare the anti–dsDNA and anti–ssDNA responses in these four groups. Control and pSub2-transfected D10 cells did not induce significant amounts of either autoantibody. In contrast, both Pca-treated and CD18-transfected D10 cells induced significant amounts of antibody to dsDNA and ssDNA relative to their respective controls. Furthermore, both Pca-treated and CD18-transfected cells induced essentially identical amounts of both autoantibodies. To exclude the possibility that the autoantibodies observed were due to polyclonal B cell activation rather than selective induction of anti–DNA-specific B cells, total IgG and IgM were measured in these four groups. As reported previously (6, 7), no significant differences were found.

Fig. 4 shows representative histologic and immunofluorescent pictures of tissues from mice receiving these cells. Untreated and pSub2-transfected cells caused no histologic abnormalities. In contrast, CD18-treated cells induced an immune complex-mediated proliferative glomerulonephritis and pulmonary alveolitis similar to that induced by Pca-treated cells (7). This supports the contention that LFA-1 overexpression contributes significantly to the disease process. However, in contrast to mice receiving Pca-treated cells, no liver disease was seen in mice receiving CD18-transfected cells (not shown), while the mice receiving Pca-treated cells again developed a disease resembling primary biliary cirrhosis (previously shown in reference 7). The reason for this is uncertain, but the observation suggests that Pca may have additional, and as yet unidentified, effects on other genes in D10 cells.

## Discussion

These results confirm that LFA-1 overexpression is important in the T cell autoreactivity induced in vitro by DNA methylation inhibitors. Earlier experiments in which human CD4+ T cell clones were transfected with CD18 also suggested that LFA-1 overexpression contributes to T cell autoreactivity. However, the interpretation of the previous results was limited by the experimental design in which the transfectants were selected for autoreactivity. In the present study, the CD18 transfectants were selected for drug resistance rather than autoreactivity, indicating that the autoreactivity observed in the first experiments was not an artifact of the selection technique. The present study also addressed the specificity of the autoreactivity. The antibody inhibition studies imply that the autoreactivity observed in the LFA-1–transfected cells is directed at Ia molecules. These results are supported by experiments indicating that transfected cells preferentially recognize self $Ia^k$-bearing Mø. We propose that the LFA-1 overexpression may overstabilize the normally low affinity interaction between the TCR and Ia molecules without appropriate antigen in the binding cleft. This is consistent with earlier reports that LFA-1 is important for responses in which TCR-Ia interactions are of low affinity (11, 12). Alternatively, the additional LFA-1 molecules may transmit an augmented activation signal (13).

The cells overexpressing LFA-1 also induced a lupuslike disease in vivo. The degree of LFA-1 overexpression and autoreactivity were similar to Pca-treated cells, and both Pca-treated and CD18-transfected cells induced similar amounts of autoantibody and similar kidney and lung lesions. This argues that the primary mechanism by which Pca induces autoreactivity in vitro and autoimmunity in vivo is by LFA-1 overexpression. However, DNA methylation inhibitors do not modify LFA-1 gene expression in all the treated cells (8), while all the subcloned transfectants overexpress LFA-1. In addition, the autoreactivity induced by DNA methylation inhibitors is self-limited (3), while the transfected cells express stable autoreactivity. Finally, the CD18 transfectants did not induce liver disease. These observations suggest that Pca-treated cells are more potent in inducing autoimmunity, and support the contention that Pca may have effects on additional, and as yet unidentified, gene products which contribute to the disease process(es).

It is likely that in both the Pca and transfectant models, a response of autoreactive T cells to self-Ia determinants in vivo contributes to the disease process. Chronic graft-vs-host disease, in which semiallogeneic CD4+ T cells also respond to host Ia molecules in vivo, causes a similar disease (14, 15). It is possible that in both models, interactions between the MHC-responsive cells and Ia-bearing host Mø results in Mø death by apoptosis (16), as well as the release of cytokines like IL-4 and IL-6. In the proper setting, like lymph nodes or the spleen, phagocytosis and presentation of apoptotic nuclear fragments, together with the release of cytokines promoting B cell differentiation, could contribute to autoantibody formation.

Alternatively, it has been suggested that hypomethylated DNA released from lysed cells might contribute to the development of autoimmunity by directly activating B cells (17). However, it seems unlikely that this mechanism plays a major role in this model, since similar diseases were induced by both hypomethylated and transfected cells, and it would not be expected that the transfection causes DNA hypomethylation. To confirm this, DNA was isolated from both Pca-treated and CD18-transfected D10 cells, and total deoxycytosine and deoxymethylcytosine content was measured by reverse phase HPLC (4, 18). In CD18-transfected D10 cells, deoxymethylcytosine represented 3.55±0.35% of the total deoxycytosine content, compared to 2.14±0.16% in Pca-treated D10 (mean±SEM of duplicate determinations, $P < 0.05$) and 3.20±0.16% in untreated D10. These results, together with earlier studies demonstrating that heat-killed, hypomethylated T cells do not induce autoimmunity (6), suggest that hypomethylated DNA fragments do not play a major pathologic role in this system.

Finally, these results may have relevance to drug-induced and idiopathic human lupus. LFA-1 overexpression, induced

by modifying T cell DNA methylation, may be the mechanism by which Pca induces a lupuslike disease. In addition, patients with active idiopathic lupus have hypomethylated T cell DNA (18, 19), similar to that caused by DNA methylation inhibitors, as well as an autoreactive T cell subset which overexpresses LFA-1 (8, 20). Our results indicate that altered LFA-1 expression, induced by DNA hypomethylation, could also contribute to the pathogenesis of idiopathic human lupus.

## Acknowledgments

The authors thank Ms. Beverly Saunders for her help in preparing this manuscript, Drs. Michael Clarke and Raymond Wilson for contributing important materials, and Dr. Richard Miller for reviewing this manuscript.

This work was supported by United States Public Health Service grants AR42525, AR08309, and AR20557, and grants from the Veterans Administration and the Arthritis Foundation.

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