Brief Definitive Report

Induction of Experimental Autoimmune Myocarditis in Mice Lacking CD3 or CD8 Molecules

By Josef M. Penninger, Nikolaus Neu,* Emma Timms, Valerie A. Wallace, Dow-Rhoon Koh, Kenji Kishihara, Christian Pummerer,* and Tak W. Mak

*From the Amgen Research Institute, Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, M4X 1K9 Toronto, Ontario, Canada; and the *Department of Pediatrics, University of Innsbruck, A-6020 Innsbruck, Austria*

Summary

Experimental induction of most autoimmune diseases appears to depend on the activation of $CD4^+$ T helper cells, while $CD8^+$ lymphocytes may have a role in disease progression. To study the role of $CD4^+$ and $CD8^+$ T cell subsets in T cell-dependent autoimmunity, mice lacking CD4 or CD8 molecules after gene targeting were injected with cardiac myosin to induce organ specific autoimmune myocarditis. Mice homozygous for the CD8 mutation ($CD8^{-/-}$) developed significantly more severe disease as compared to $CD4^{+/-}CD8^{+/-}$ controls. Surprisingly, $CD4^{-/-}$ mice developed autoimmune myocarditis with infiltration of $TCR\alpha\beta^+CD4^-CD8^-$ T cells in the heart tissue and appearance of autoantibodies. These data demonstrate that the lack of $CD4^+$ or $CD8^+$ T cells has no significant influence on the initiation of autoimmune myocarditis. $CD4^+$ and $CD8^+$ cells regulate disease severity and these results may explain the occurrence of autoimmunity in CD4 immunodeficiencies.

In mice, autoimmune diseases arise either spontaneously or can be experimentally induced by injection of the specific autoantigen. Experimental induction of most autoimmune disease requires $CD4^+$ T helper lymphocytes responsive to the self-antigen presented on major histocompatibility complex (MHC) class II molecules (reviewed in reference 1). In virtually all experimental and genetic animal models of autoimmunity, administration of monoclonal antibodies (mAb) against CD4 molecules blocks disease (reviewed in references 2–5). Thus, $CD4^+$ T helper cells are postulated to be crucial for the initiation and development of T cell dependent autoimmunity and clinical trials targeting the $CD4^+$ cell lineage to treat various human autoimmune diseases are currently underway (2–5). Besides $CD4^+$ cells, MHC class I restricted $CD8^+$ lymphocytes can be found in autoimmune lesions (1–5). The significance and role of $CD8^+$ lymphocytes in the development of autoimmunity, especially in experimentally induced autoimmune diseases, is controversial.

Coxsackie virus (CB3)-induced myocarditis and dilated cardiomyopathy are the most frequent cardiac complications in young patients, and several clinical and experimental studies suggest that later stages of this disease are mediated by an autoimmune response (6–8). The human disease can be mimicked in mice by inoculation of CB3-virus, which in the mouse model probably leads to an autoimmune response to cardiac myosin exposed after virus-mediated myocyte damage (9–11). This hypothesis is supported by the fact that myocarditis can be induced with purified cardiac myosin, i.e., in the absence of virus (12). Cardiac myosin-induced autoimmune myocarditis is strictly organ-specific and cannot be induced with other isoforms of muscle myosin (12). Experimental induction of autoimmune myocarditis is MHC class II haplotype dependent and can be inhibited by treatment with mAbs against MHC class II or CD4 molecules (13). Adoptive transfer studies in SCID mice have also demonstrated that autoantigen-induced myocarditis is mediated by $CD4^+$ cells (14). By contrast, immunodepletion studies suggested that $CD8^+$ cells have a pathogenic role in later phases of the disease (13).

Mice rendered CD4 or CD8 deficient as a result of gene targeting (15, 16) are ideal models to study the role of $CD4^+$ and $CD8^+$ T cell subsets in the development of autoimmune diseases. To investigate whether $CD4^{-/-}$, $CD8^{-/-}$ or control $CD4^{+/-}CD8^{+/-}$ mice are susceptible to antigen-induced myocarditis, these mice were injected with purified cardiac myosin (12). We show that both $CD4^{-/-}$ and $CD8^{-/-}$ mice develop autoimmune myocarditis suggesting that the initiation of antigen-induced autoimmunity does not depend on $CD4^+$ or $CD8^+$ T cells.

Materials and Methods

Mice. To generate mice in an MHC haplotype susceptible to cardiac myosin-induced autoimmune disease, CD4 or CD8 gene deficient mice (15, 16) were backcrossed into a B10.Br strain ($H\text{-}2^k$) ($CD4^{-/-}$ = 4th backcross; $CD8^{-/-}$ = 5th backcross). As

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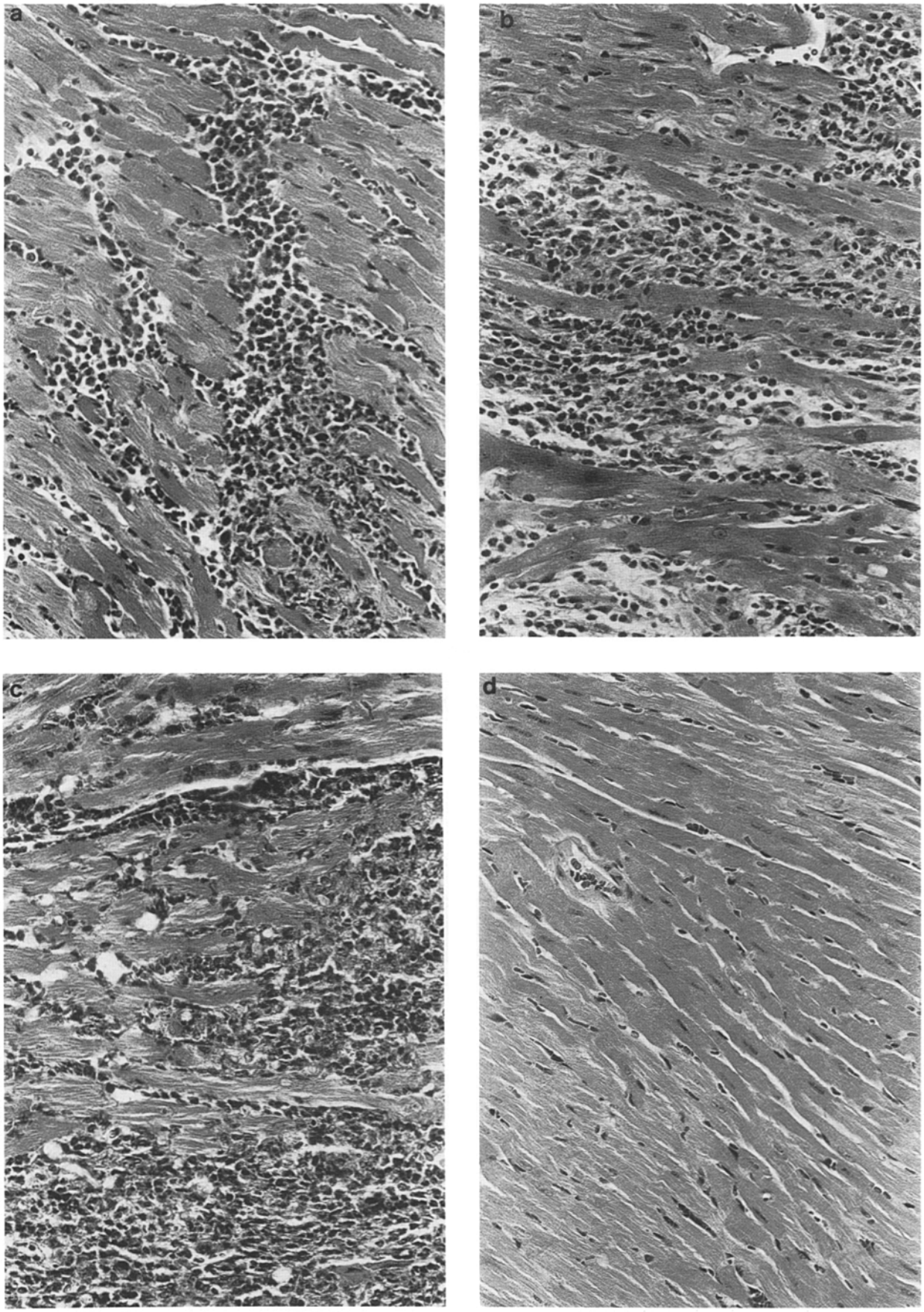
a
b
c
d

a control, male $CD8^{-/-}$ (B10.Br; 5th backcross) were bred with female $CD4^{-/-}$ (B10.Br; 4th backcross) mice of the same breeding nucleus to generate mice heterozygous at both loci ($CD4^{+/-}$ $CD8^{+/-}$). It should be noted that $CD4^{-/-}$ or $CD8^{-/-}$ throughout the text refers to $CD4^{-/-}CD8^{+/+}$ or $CD4^{+/+}CD8^{-/-}$ genotypes. Care of animals was in accordance with guidelines of the Canadian Research Council.

Immunization Procedure. Cardiac myosin was purified from mice as described (17). 7-wk-old mice were immunized twice at a 7-d interval (subcutaneously) with 150 μg of cardiac myosin emulsified in Freund's complete adjuvant (FCA) or with FCA alone (12). 21 d after the first immunization, mice were sacrificed and analyzed histologically for infiltration of the heart muscle and serologically for the presence of IgG auto-Abs against cardiac myosin (10, 12). For histological analysis, hearts were fixed in formaldehyde and processed for hematoxylin and eosin staining. IgG auto-Ab titers were determined by ELISA-technique using the cardiac myosin isoform as test antigen (12, 17). Specificity of myosin IgG auto-Abs against the cardiac isoform has been demonstrated previously (17).

Immunocytometry. For immunoperoxidase staining (13), cryostat sections were fixed in acetone (10 min) and endogenous peroxidase reactivity blocked with NaN_3 and H_2O_2. After incubation with mAbs (30 min), sections were washed in tris-buffered saline (TBS, pH 7.4) and further incubated with peroxidase-conjugated rabbit anti-rat Ig (0.25 mg/ml; DAKOPATTS, Copenhagen, Denmark) or unconjugated rabbit anti-hamster IgG (0.1 mg/ml; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by peroxidase conjugated swine anti-rabbit Ig (0.25 mg/ml; DAKOPATTS). All sections were developed for 10 min with 0.06% diaminobenzidine and 0.01% H_2O_2 and counter-stained with hematoxylin.

The following mAbs were used: Mac-1 (rat IgG2b; clone M1/70); GK1.5 (anti-CD4; rat IgG2b); 53-6.72 (anti-CD8α/Lyt2; rat IgG2a); KT3 (anti-CD3; rat IgG); GL-3 (anti-pan TCR-$\gamma\delta$; hamster IgG); H57-597.2.1 (anti-pan TCR$\alpha\beta$; hamster IgG). All mAbs were derived from American Type Culture Center, Rockville, MD) and used as supernatants.

Results and Discussion

$CD4^{-/-}$, $CD8^{-/-}$, or control $CD4^{+/-}CD8^{+/-}$ mice were injected with purified cardiac myosin in FCA (12). In comparison to $CD4^{+/-}CD8^{+/-}$ mice, $CD8^{-/-}$ animals developed a significantly more severe disease as assessed by the histological extent of heart infiltration (Table 1 and Fig. 1, *a* and *b*) suggesting that $CD8^+$ lymphocytes do not only act as cytotoxic effector cells in autoimmunity but may also regulate the severity of disease (18). A similar regulatory role of $CD8^+$ T cells has been previously described in experimental autoimmune encephalitis (EAE), i.e., increased frequency of relapses in $CD8^{-/-}$ mice and resistance to a second induction of EAE in mice depleted of $CD8^+$ cell using mAbs (19, 20). More surprisingly, $CD4^{-/-}$ animals injected with the autoantigen also developed autoimmune myocarditis (Fig. 1 *c*). Incidence and severity of autoimmune myocarditis in $CD4^{-/-}$ mice were comparable to $CD4^{+/-}CD8^{+/-}$ control mice (Table 1).

Table 1. *Incidence and Histological Severity of Cardiac Myosin-induced Myocarditis in $CD4^{-/-}$, $CD8^{-/-}$, and $CD4^{+/-}CD8^{+/-}$ Mice*

		Myocarditis	
Genotype	Immunization	Incidence (positive animals/ total)	Severity in positive animals
$CD4^{+/-}8^{+/-}$	Cardiac myosin	5/12	1.8 ± 0.8
$CD4^{+/-}8^{+/-}$	FAC only	0/6	–
$CD4^{-/-}$	Cardiac myosin	3/6	1.0 ± 0.0
$CD4^{-/-}$	FCA	0/7	–
$CD8^{-/-}$	Cardiac myosin	4/5	3.3 ± 1.0
$CD8^{-/-}$	FCA	0.8	–

Incidence and disease severity were scored histologically on hematoxin and eosin stained heart sections in a double blind test by two independent investigators. Histological grading of severity is (12, 17): 0 = no infiltration in heart muscle; 1 = up to 5% of histological section is infiltrated; 2 = 5–10%; 3 = 10–20%; 4 = >20%. Disease severity was compared between different groups injected with cardiac myosin using a Student's *t* test: $CD4^{+/-}CD8^{+/-}$ vs. $CD4^{-/-}$, no significant difference ($p > 0.05$); $CD4^{+/-}CD8^{+/-}$ vs. $CD8^{-/-}$ and $CD4^{-/-}$vs. $CD8^{-/-}$, significantly different ($p < 0.05$). The incidence of disease in both $CD4^{-/-}$ and $CD8^{-/-}$ animals did not differ significantly from $CD4^{+/-}CD8^{+/-}$ controls (χ^2-test, $p > 0.05$). One representative experiment is shown. The results were confirmed in two additional experiments.

Table 2 summarizes immunoperoxidase staining of cells infiltrating the heart tissue of animals that had developed autoimmune disease. In all mice analyzed, the cellular infiltrate was composed of macrophages (Mac-1^+, 70–80%) and $CD3^+$ T lymphocytes (16–24%). In $CD4^{+/-}CD8^{+/-}$ control mice almost all (>95%) infiltrating T cells were TCR$\alpha\beta^+$ and expressed either CD4 or CD8 accessory molecules. The same results have been previously obtained in normal B10.Br and other mouse strains (data not shown and reference 13). In $CD8^{-/-}$ mice, virtually all infiltrating TCR$\alpha\beta^+$ cells coexpressed CD4 molecules. Strikingly, in $CD4^{-/-}$ mice <30% of TCR$\alpha\beta^+$ lymphocytes infiltrating the heart expressed CD8 molecules. These data show that most TCR$\alpha\beta^+$ lymphocytes present in autoimmune lesions of $CD4^{-/-}$ mice have a $CD4^-CD8^-$ phenotype. TCR$\gamma\delta^+$ cells could be found in hearts of $CD4^{+/-}CD8^{+/-}$, $CD8^{-/-}$, and $CD4^{-/-}$ mice in a low frequency (Table 2).

The antibody response to a T cell-dependent antigen,

Figure 1. Cellular infiltrates in hearts of $CD4^{+/-}CD8^{+/-}$ (*a*), $CD8^{-/-}$ (*b*), and $CD4^{-/-}$ mice (*c*) injected with cardiac myosin. In all cases, the infiltrates of mononuclear cells are diffuse and interstitial. (*d*) shows the normal morphology of heart muscle from a $CD4^{-/-}$ mouse injected with FCA alone. It should be noted that heart areas are shown to illustrate infiltration and that the infiltrations shown to not correspond to histological grading of the whole heart as described in Table 1. Hearts were taken from mice 21 d after first injection of cardiac myosin (12), fixed in formaldehyde, and processed for conventional histology (hematoxin and eosin staining). ×80.

Table 2. *Phenotype of Cells Infiltrating the Hearts of $CD4^{+/-}CD8^{+/-}$, $CD4^{-/-}$, and $CD8^{-/-}$ Mice*

Phenotype	$CD4^{+/-}CD8^{+/-}$	$CD4^{-/-}$	$CD8^{-/-}$
Mac-1	78.4	74.3	69.6
CD3	16.1	17.7	23.7
TCR$\alpha\beta$	16.2	**14.1**	20.1
TCR$\gamma\delta$	1.3	3.0	3.4
CD4	8.2	0	20.9
CD8	7.1	**3.9**	0

Heart sections were stained with indicated mAbs followed by peroxidase labeled second stage Abs (13). In each instance, at least 1000–2500 infiltrating cells were counted. Numbers indicate percentages of positive cells among total infiltrating cells. Percentages are shown for one animal representative of each haplotype. Phenotypes and percentages of infiltrating cells, expecially the prevalence of $CD4^-CD8^-TCR\alpha\beta^+$ lymphocytes in the heart infiltrate of $CD4^{-/-}$ mice, were confirmed in more animals (not shown). Unspecific background staining was in all instances <1% of infiltrating cells.

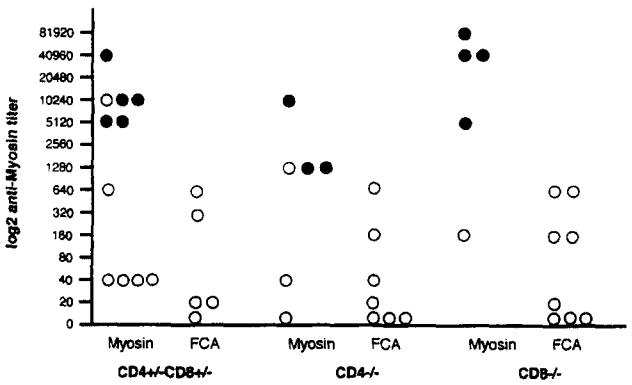

Figure 2. Myosin IgG autoantibody titers in $CD4^{+/-}CD8^{+/-}$, $CD8^{-/-}$, and $CD4^{-/-}$ mice injected with cardiac myosin in FCA or with FCA alone. (*Filled circles*) Individual mice with myocarditis. (*Open circles*) Mice without disease. Sera were collected from mice 21 d after initial cardiac myosin injection and myosin IgG auto-Ab titers were determined by ELISA-technique (17).

SRBC, is markedly reduced in $CD4^{-/-}$ mice (15). Since mice with myocarditis develop high titers of IgG autoantibodies (auto-Ab) against cardiac myosin (10, 21, 22), we analyzed whether auto-Abs were present in $CD4^{-/-}$ or $CD8^{-/-}$ mice. Fig. 2 shows titers of anti-myosin IgG auto-Abs in mice injected with cardiac myosin or FCA alone. All $CD4^{+/-}CD8^{+/-}$ and $CD8^{-/-}$ mice with cellular heart infiltrate developed high titers of IgG auto-Abs, and high auto-Ab titers correlated with the severity of cellular infiltration. Surprisingly, experimental autoimmune myocarditis in $CD4^{-/-}$ animals was accompanied by the appearance of IgG auto-Abs (Fig. 2). Mice injected with FCA alone did not show any heart infiltration (Table 1 and Fig. 1 *d*) or high titers of auto-Abs (Fig. 2).

Development of specific IgG auto-Abs against cardiac myosin in the absence of help by $CD4^+$ T cells suggests that cells other than $CD4^+$ lymphocytes are functional that can induce immunoglobulin-class switching and myosin specific IgG auto-Ab production. Alternatively, IgG auto-Ab production in myocarditis may not require T cell help. Since, however, experimental autoimmune myocarditis is T cell dependent (13, 14) and auto-Ab titers are abrogated after treatment with anti-CD4 and anti-MHC class II mAbs (14), the production of IgG auto-Abs appears to depend on T helper cells. Few examples of autoimmunity have been described that may be mediated by $CD4^-CD8^-TCR\alpha\beta^+$ (23) or $CD4^-CD8^-TCR\gamma\delta^+$ cells (24) and vast numbers $CD4^-CD8^-TCR\alpha\beta^+$ lymphocytes arise in autoimmune *gld/gld* or *lpr/lpr* mice (reviewed in 25). Approximately 10% of peripheral T cells in $CD4^{-/-}$ mice have a $CD4^-CD8^-TCR\alpha\beta^+$ phenotype (15) and it has been demonstrated that these cells can provide MHC class II restricted help for T cell dependent antibody production (Rahemtulla, A., T. M. Kuendig, A. Narendran, M. F. Bachmann, M. Julius, P. S. Ohashi, C. J. Paige, R. M. Zinkernagel, and T. W. Mak; manuscript submitted for publication). It should be noted that $CD4^-CD8^-TCR\alpha\beta^+$ T cells in $CD4^{-/-}$ mice display a phenotype previously described for various normal mouse strains (26–29) suggesting that these cells constitute a T cell population that is also present in normal mice but that is expanded in $CD4^{-/-}$ mice.

Our data demonstrate that T cell-dependent and organ-specific autoimmune diseases, i.e., autoimmune myocarditis, can develop in mice lacking $CD4^+$ or $CD8^+$ T cells. Both T cell subsets, however, participate in disease propagation in which $CD4^{-/-}$ mice develop a mild, but $CD8^{-/-}$ mice a more severe form of disease. In $CD4^{-/-}$ mice, most T cells infiltrating the heart muscle have a $TCR\alpha\beta^+CD4^-CD8^-$ phenotype, a cell population is probably also responsible for initiation of autoimmunity in these mice. The same population is probably also responsible for the development of EAE in $CD4^{-/-}$ mice (Koh, D. R., A. Ho, A. Rahemtulla, and T. K. Mak, manuscript submitted for publication). These data caution against attempted treatments of autoimmune diseases with mAbs against CD4 or CD8 molecules (2–5) since other than $CD4^+$ or $CD8^+$, cell populations have the functional capability to induce organ-specific autoimmunity and might explain the occurrence of autoimmunity in patients with genetic or acquired immunodeficiency syndromes, e.g., the high incidence of non-viral myocarditis in AIDS patients (30).

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Address correspondence to Tak W. Mak, Amgen Research Institute, Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, 500 Sherbourne Street, M4X 1K9 Toronto, Ontario, Canada.

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