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Tolerance Mechanisms in Murine Autoimmune Diabetes Induced by Anti-ICAM-1/LFA-1 mAb and Anti-CD8 mAb

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A short-term administration of antibodies against ICAM-1/LFA-1 or CD8 molecules during a critical period of younger age resulted in complete protection of autoimmune diabetes in NOD mice. In this study, we attempted to elucidate the tolerance mechanisms. Transfer of splenocytes from both antibody-treated NOD mice to NOD-SCID mice failed to develop diabetes. On the other hand, when splenocytes from diabetic mice were transferred to the antibody-treated mice, 40% of both mAb-treated recipients became diabetic. *In vitro* response of T cells from these protected mice exhibited strong proliferation against syngeneic islet cells or ConA. Furthermore, semiquantitative RT-PCR analysis of cytokines showed that T cells from anti-CD8-treated mice could express IFN- γ , IL-4, IL-10 and TGF- β 1 in response to islet antigen. In contrast, T cells from anti-ICAM-1/LFA-1-treated mice expressed IFN- γ , IL-10 and TGF- β 1 but not IL-4. These results suggest that tolerance mechanisms like clonal deletion, anergy, immunoregulatory T cells or Th1 to Th2/Th3 cytokine shifting are not responsible for the tolerance induction, indicating the presence of other unrevealed mechanism responsible for the loss of capability of autoreactive T cells to infiltrate and destroy the pancreatic β -cells *in vivo*.

Type 1 diabetes (T1D, Insulin-dependent diabetes mellitus) is an organ-specific T cell-mediated autoimmune disease (5). The NOD mouse is an ideal animal model for human T1D, which shows infiltration of mononuclear cells into the islets (insulinitis) beginning at 3-4 wk of age and develops diabetes by 14 wk of age. Infiltrates, in the islets, are consists of CD4⁺ and CD8⁺ T lymphocytes, B cells, macrophages, and dendritic cells (9,4). In this model, T cells play a critical role in the development of autoimmune diabetes. Athymic NOD mice or NOD-SCID mice do not develop diabetes or insulinitis (12,8). Activation of naive T cells requires not only T cell recognition of peptide-major histocompatibility complex (MHC) complexes but also co-stimulation provided by accessory molecule interaction. Among accessory molecules, LFA-1, a member of the leukocyte integrin family, is important in cellular interactions in the immune system such as cytotoxic T cells and NK cell-mediated cytotoxicity, helper T lymphocyte responses and leukocyte adhesion (7). We have previously reported that transient blockade of ICAM-1/ LFA-1 pathway can induce peripheral tolerance against effector T cell in NOD mice (10). In addition, CD8⁺ T cells have been reported to require for the initiation of NOD autoimmune diabetes. β_2 -microglobulin-deficient NOD mice or NOD mice treated with anti-CD8 mAb in the young age do not develop insulinitis or diabetes (22,21). However, the precise tolerance mechanism induced by administration of anti-CD8 and anti-ICAM-1/LFA-1- antibodies remains unknown.

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In this study, possible immunological mechanisms involved in the induction and maintenance of tolerance by short-term administration of antibodies against ICAM-1/LFA-1 or CD8 molecules before the onset of insulinitis are characterized and discussed.

MATERIALS AND METHODS

Mice

Male and female NOD/Shi/Kbe mice were maintained in the Institute for Experimental Animals, Kobe University School of Medicine. In our NOD colony, insulinitis became noticeable in most mice at 5-7 wk of age and became much enhanced with advancing age. Overt diabetes, which began at 13 wk of age, occurred in female NOD mice. The cumulative incidence of diabetes at 30 wk of age was more than 80% for female NOD mice. All animal experiments were conducted according to the Guidelines for Animal Experiments at Kobe University School of Medicine.

Monoclonal Antibodies

Monoclonal antibodies (mAbs) against ICAM-1 (KAT-1, rat IgG2a) (14), α subunit (CD11a) of LFA-1 (KBA, rat IgG2a) (11), CD8 molecule (53-6.7, Ly-2, rat IgG2a) and β subunit (CD18) of LFA-1 (M18/2, rat IgG2a) (17) were produced as ascites in tetramethylpentadecane-treated CD1 nu/nu mice. The concentration of mAbs was measured by radial immunodiffusion (RID) kit with anti-rat IgG (The Binding Site, Birmingham, England).

***In vivo* antibody treatment**

Female NOD mice were injected intraperitoneally (i.p.) with 100 μ g of anti-ICAM-1 and 100 μ g of LFA-1 mAbs for 6 consecutive days at 2 wk of age. In another experiment, female NOD mice were injected i.p. with 500 μ g of anti-CD8 mAbs twice a week for 2 wk from 2 wk of age. Urinary and blood glucose were monitored for the development of diabetes of mAb-treated mice.

Histology

Mice were sacrificed for histological examination at 30 wk of age. The pancreases, which were removed from mice, were fixed in 10% formalin solution. Paraffin-embedded 5 μ m-thin sections were stained with hematoxylin and eosin (H-E) and the severity of insulinitis was assessed by using the following criteria: 0, normal islet; 1, periinsulinitis; 2, mononuclear islet cell infiltration in < 25%; 3, 25-50% infiltration; 4, >50% infiltration of islets.

Adoptive transfer of diabetes to NOD-SCID mice

Splenocytes were isolated from 22-wk-old female NOD mice that had been treated with mAbs at 2 wk of age. 6-8-wk-old female NOD-SCID recipients received intravenous injections of 3×10^7 splenocytes/mouse. All recipients were monitored for the development of diabetes until six wk after the transfer.

T cell proliferation against islet antigens

Splenocytes were prepared from 12-wk-old female NOD mice that had been treated with anti-ICAM-1/LFA-1 mAb or anti-CD8 mAb at 2 wk of age. Red blood cells were removed from the splenocyte preparations by lysis with 0.015 M Tris-0.83% NH_4Cl and T cells were then isolated by deleting with goat anti-mouse IgG-coated magnet beads (Polysciences Inc., Warrington, PA). These splenic T cells (2×10^5 /well) were cultured with 20 mitomycin-C-treated NOD islets isolated from 7-8-wk-old male NOD mice in 200 μ l of RPMI 1640 containing 0.5% NOD-SCID serum, 5×10^{-5} M 2-ME, 50 U/ml penicillin, and 50 μ g/ml streptomycin (Flow, Mclean, VA). These cells were also cultured with 1 μ g/ml of concanavalinA (ConA, Sigma, Japan). After 72 h-culture, 1 μ Ci ^3H -thymidine (20-30 Ci/mmol; Amersham, Tokyo,

Japan) was added at 16 h before harvesting. Cells were then harvested by Packard FilterMate harvester (Packard, Meriden, CT) and ^3H -thymidine incorporation was measured on TopCount Microplate scintillation counter (Packard). Values are indicated as means of stimulation index (mean cpm in the presence of antigen/mean cpm without antigen) $\pm$ standard deviation of three individual experiments.

Transfer of diabetogenic splenocytes into mAb-treated mice

Splenocytes were isolated from acutely diabetic female NOD mice. 8-10-wk-old mAb-treated recipients received intravenous injections of 6×10^7 splenocytes/mouse. All recipients were monitored for the development of diabetes until 35 wk after the transfer and were sacrificed for histological examination.

Reverse transcription-PCR analysis of antigen-stimulated T cells

mRNA was extracted from T cells proliferated against syngeneic NOD islets with a Micro-Fast Track mRNA isolation kit (Invitrogen, NV, Leek, the Netherlands) and 0.1 μg of mRNA was reverse-transcribed with a cDNA cycle kit (Invitrogen), using oligo-dT primers and AMV reverse transcriptase to generate cDNA to use as a template in PCR amplifications. The PCR analysis was carried out using cDNA samples for analysis of a single cytokine, 20 μM of each primer, and 1.25 U of Ex Taq polymerase (Takara, Shuzo, Shiga, Japan) in a 50 μl final volume. Samples were amplified with an initial 3 min denaturation at 94°C , followed by 40 cycles of 1 min at 94°C , 1 min at 56°C , and 2 min at 72°C , with 10 min at 72°C on the last cycle in a Gene Amp PCR System 9700 (Perkin-Elmer/Cetus Corp., Norwalk, CT). The upstream and downstream primers were for IFN- γ , for IL-4, for IL-10, for TGF- β 1 and for cyclophilin. The sequences of the specific oligonucleotide primer pairs, 5' and 3', are shown in Table I. PCR products were analyzed by electrophoresis in 1.2% agarose gel containing 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide.

Statistical analysis

Statistical analysis of insulinitis score were made by the non-parametric Mann-Whitney U Test. Data are represented as mean $\pm$ SD.

Table I. Specific Oligonucleotide Primer Sequences for RT-PCR.

PCR product		Primer sequences
IFN- γ	Sense	5' TGAACGCTACACACTGCATCTTGG 3'
	Antisense	5' CGACTCCTTTTCCGCTTCCTGAG 3'
IL-4	Sense	5' ATGGGTCTCAACCCCCAGCTA 3'
	Antisense	5' GCTCTTTAGGCTTCCAGGAAGTC 3'
IL-10	Sense	5' AGCTGGACAACATACTGCTAACC 3'
	Antisense	5' TCATTCATGGCCTTGTAAGACAC 3'
TGF- β 1	Sense	5' AGGAGACGGAATACAGGGCTTTCG 3'
	Antisense	5' ATCCACTTCCAACCCAGGTCCTTC 3'
Cyclophilin	Sense	5' GACAGCAGAAAACCTTTCGAGC 3'
	Antisense	5' TCCAGCCATTCAGTCTTGG 3'

RESULTS

Administration of anti-ICAM-1/LFA-1 mAb and anti-CD8 mAb to NOD mice

15 mice were injected with anti-ICAM-1/LFA-1 mAb for 6 consecutive days at 2 wk of age. Another 15 mice were injected with anti-CD8 mAb twice a wk for two wk from 2 wk of age. Anti-CD18 mAb-treated mice were used as control. At the end of 40 wk of age, none of

both group of mAb-treated mice suffered from diabetes, while 86.7% (13 of 15) in control mice became diabetic (Fig. 1A).

Histological examinations revealed that both mAb-treatment completely prevented the development of insulinitis, while 90% islets of control mice showed significant insulinitis (Table II).

Table II. Pancreatic insulinitis in mAb-treated mice.

mAb	n	Insulinitis score at 30 wk					Mean $\pm$ SD ¹
		0	1	2	3	4	
anti-CD18	5	10%	13%	20%	25%	32%	2.58 $\pm$ 0.28
anti-ICAM-1/LFA-1	5	100%	0	0	0	0	0*
anti-CD8	5	100%	0	0	0	0	0*

Five nondiabetic mice of each group were sacrificed for histological examination at 30 weeks of age. At least 20 islets per mouse were examined for the evaluation of the degree of insulinitis as described in materials and methods.

¹ Statistical significance of insulinitis score (Mann-Whitney U Test) were performed between anti-CD18 group and anti-ICAM-1/LFA-1 or anti-CD8 group. * $p=0.0053$

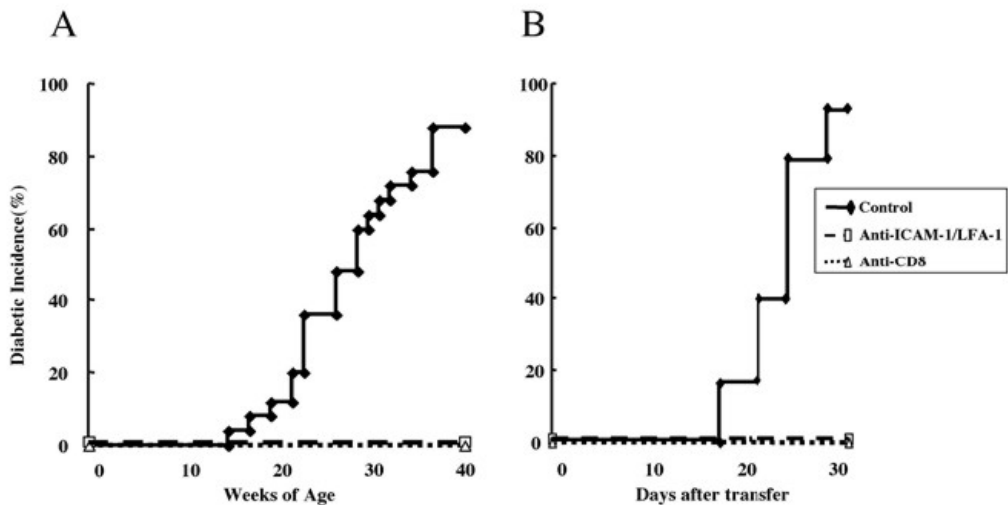


Figure 1. A. Incidence of diabetes in anti-ICAM-1/LFA-1 and anti-CD8 mAb-treated NOD mice at 2wk of age. Treatment with each 100 μ g of anti-ICAM-1 and LFA-1 mAbs ($\square$) was for 6 consecutive days and treatment with 500 μ g of anti-CD8 mAbs (Δ) was twice a wk for 2 wk. Control mice were treated with anti-CD18 mAb ($\blacklozenge$). Overt diabetes was monitored by checking urinary and blood glucose until 30 wk of age. B. Transfer activity of mAb-treated NOD mice. Donor splenocytes were isolated from 22-wk-old female NOD mice that had been treated with anti-ICAM-1/LFA-1 mAb ($\square$), anti-CD8 mAb (Δ) and control anti-CD18 mAb ($\blacklozenge$) from 2 wk of age. Donor cells were transferred to 6-8-wk-old female NOD-SCID recipients; five in each group received intravenous injections of 3×10^7 splenocytes/mouse. Recipients were monitored for the development of diabetes until 6 wk after transfer.

Transfer of splenocytes from anti-ICAM-1/LFA-1mAb-treated and anti-CD8 mAb-treated NOD mice

To evaluate the effector activity to destroy pancreatic β cells, splenocytes from 22-wk-old NOD mice that had been treated with anti-ICAM-1/LFA-1 mAb and anti-CD8

mAb, were transferred to NOD-SCID mice. Both splenocytes from anti-ICAM-1/LFA-1 mAb and from anti-CD8 mAb treated mice failed to develop diabetes (Fig.1B), indicating that the diabetogenic effector cells are deleted or inactivated even at 20 wk after cessation of the mAb treatment in tolerarized mice.

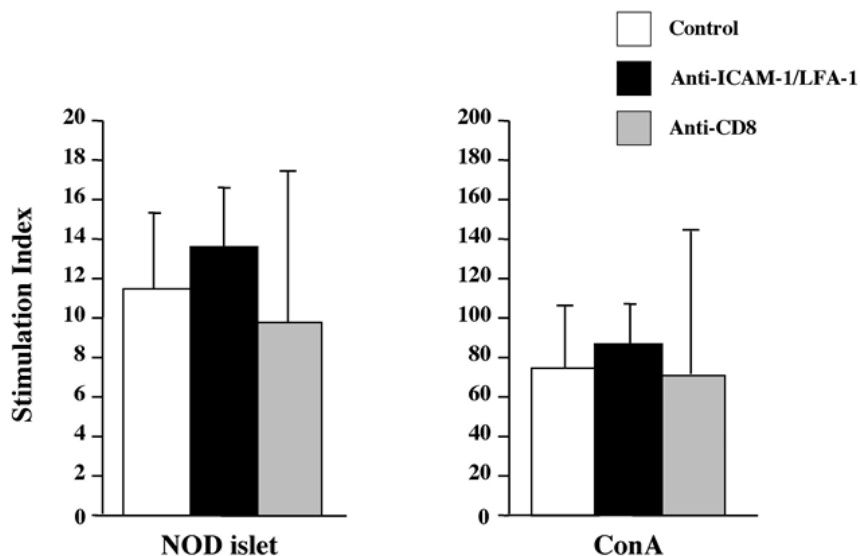


Figure 2. Proliferative T cell response to syngeneic islet cell antigens or Con-A. Splenic T cells were prepared from 12-wk-old female NOD mice that had been treated with anti-ICAM-1/LFA-1 mAb (black), anti-CD8 mAb (stippled) at 2 wk of age or control NOD mice (white). Purified T cells were cultured in triplicate, either alone, with mitomycin-C-treated NOD islets, or concanavalinA. After 3 days, ^3H -thymidine was added at 16h before harvesting. Data are represented as mean stimulation index (S.I.) $\pm$ SD of three separate experiments.

Transfer of splenocytes from acutely diabetic mice to anti-ICAM-1/LFA-1 mAb and anti-CD8 mAb-treated NOD mice

To evaluate the recipient condition where autoreactive effector T cells might be suppressed by immunoregulatory cells or could not be proliferated, we adoptively transferred splenocytes from acutely diabetic NOD mice into non-irradiated 8-10-wk-old mAb-treated mice. 40% of both mAb-treated recipients became diabetic at 12 to 35 wk after the transfer (Table III). All non-diabetic recipient showed moderate to severe insulinitis. These results suggest that autoreactive T cells that are contained in diabetogenic T cells can proliferate and destroy pancreatic β cells without elimination or inactivation by immunoregulatory cells in mAb-treated NOD mice.

Proliferative ability of T cells from both anti-ICAM-1/LFA-1mAb-treated and anti-CD8 mAb-treated mice

To evaluate the fate of autoreactive T cells in anti-ICAM-1/LFA-1 mAb-treated and anti-CD8 mAb-treated mice, splenic T cells obtained from 12-wk-old mAb-treated NOD

mice were incubated with NOD islets or ConA (Fig.2). Splenic T cells from mAb-treated mice could proliferate well by the mitogen, suggesting absence of general immunosuppression. Moreover, the T cell proliferative ability against islet cells in both anti-ICAM-1/LFA-1 mAb-treated and anti-CD8 mAb-treated mice were quite comparable with the control. Thus, autoreactive T cells against islet cell antigens were not deleted or inactivated in anti-CD8 mAb-treated mice as well as in anti-ICAM-1/LFA-1 mAb-treated mice.

Table III. Transfer of diabetogenic splenocytes in antibody-treated mice.

Recipient	Cell dose ¹	Incidence of Diabetes ² (Age in wk)				
		30	35	40	45	Incidence(%)
Anti-ICAM-1/LFA-1 mAb	6×10^7	0/5	1/5	2/5	2/5	40
Anti-CD8 mAb	6×10^7	1/5	1/5	1/5	2/5	40

¹Donor splenocytes, isolated from acutely diabetic NOD mice, were transferred to 8-10-wk-old mAb-treated NOD mice.

²Recipients were monitored for the development of diabetes until 45 wk of age.

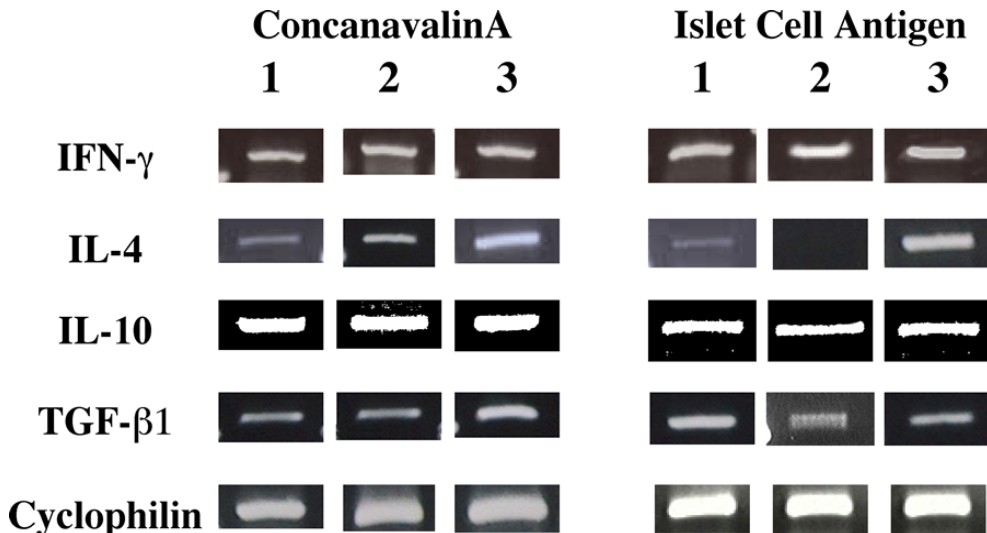


Figure 3. Cytokine expression of tolerarized T cells, from control (Column 1), anti-ICAM-1/LFA-1 mAb-treated (Column 2) or anti-CD8 mAb-treated (Column 3) NOD mice. T cells were cultured with MMC-treated NOD islets and Concanavalin A. At the end of 4 days culture, mRNA was isolated from proliferated T cells and RT-PCR for IFN- γ , IL-4, IL-10, TGF- β 1 and cyclophilin as an internal control, was performed using specific primers, respectively.

Cytokine expressions of autoreactive T cells from both anti-ICAM-1/LFA-1 mAb-treated and anti-CD8 mAb treated mice

To evaluate whether a general shift in the Th1 or Th2/Th3 cytokine profile played an important role in this tolerance mechanism, we evaluated the expression of IFN- γ , IL-4, IL-10 and TGF- β 1 mRNA of islet-reactive T cells by RT-PCR. All control and mAb-treated mice expressed IFN- γ , IL-10 and TGF- β 1. However, IL-4 expression in T cells of anti-ICAM-1/LFA-1 mAb-treated NOD mice was not detected, while both control and anti-CD8 mAb-treated mice expressed IL-4 (Fig.3).

DISCUSSION

In this study, we have attempted to reveal the tolerance mechanism induced by the administration of anti-ICAM-1/LFA-1 mAb and anti-CD8 mAb in NOD mice. As previously reported, short-term administration of anti-ICAM-1/LFA-1 mAb and anti-CD8 mAb in young NOD mice completely prevented spontaneous diabetes as well as insulinitis (10,21). However, the previous study of anti-CD8 mAb showed insulinitis of various degrees at later age in some mice (21) and, in this study, insulinitis was also completely prevented even at 30 week of age. It should be noted that we have used different deleting mAb against CD8 molecules. Our deleting anti-CD8 mAb showed rapid and reversible loss of splenic CD8⁺ T cells in treated NOD mice. After complete deletion of CD8⁺ T cells at 4 wk of age, they gradually recovered after cessation of treatment. CD8⁺ T cells began to reappear at 10 wk of age (1.0% of total splenocytes) and reached to nearly 70% of normal level by 18 wk of age (4.7% of total splenocytes in anti-CD8 mAb-treated mice vs. 7.0% in non-treated mice). This suggested that CD8⁺ T cells were required for the induction of autoimmune diabetes especially in the young age.

It is well established that the onset of diabetes in NOD mice is regulated by a fine balance between autoaggressive and suppressive regulatory T cells (15,16). To examine the fate of diabetogenic effector cell in these tolerarized mice, splenocytes from anti-ICAM-1/LFA-1 mAb and anti-CD8 mAb-treated NOD mice were transferred to NOD-SCID mice. All recipients failed to develop diabetes (Fig.1B). In contrast, *in vitro* response of T cells from these protected mice exhibited strong proliferation against syngeneic islet cells (Fig.2), indicating that the induced tolerance was not due to clonal anergy or deletion of islet-reactive T cells. Furthermore, when splenocytes from acutely diabetic NOD mice were adoptively transferred in non-irradiated 8-10-wk-old mAb-treated mice, they could infiltrate the protected islet, leading to insulinitis and diabetes (Table III). We have previously shown that immunoregulatory suppressor T cell is not playing a key role for the long lasting protection induced by anti-ICAM-1/LFA-1 mAb treatment (10). Taken together, these apparently contradictory results suggest that neither enhancement of immunoregulatory cells nor residual APC dysfunction are responsible for the suppression of autoimmune diabetes, implicating that islet-specific T cells in both tolerarized mice changed some phenotypes which rendered them unable to infiltrate the islet *in vivo*.

B. Wang et al. has shown that, in anti-CD8 mAb treated NOD mice, CD4⁺ T cell compartment was unable to transfer insulinitis alone or with CD8⁺ T cells from control or anti-CD8 mAb-treated mice (21), while CD4⁺ T cell compartment of non-manipulated NOD mice are capable of inducing insulinitis (20,23). In a recent study, S.A.Camacho et al. has demonstrated that, during priming, antigen-presenting cell's expression of B7-1 without ICAM-1 led to the generation of effector cells that migrated to the pancreases of RIP-mOVA recipients but did not cause diabetes, while T cells primed with APCs expressing both B7-1 and ICAM-1, could destroy β cells with rapid onset of diabetes. Thus, the ICAM-1/LFA-1 pathway during priming altered in T cell effector function and induced potentially autoreactive response (1). This study revealed that transient blockade of ICAM-1/LFA-1 pathway prevents the effector T cells to obtain the *in vivo* potential of causing insulinitis and diabetes.

An association between progression of diabetes and a Th1 cytokine production of autoreactive T cells has been reported. *In vivo* administration of anti-IFN- γ mAb has been shown to prevent the onset of diabetes in NOD mice (3). Furthermore, it has been shown that transgenic expression of IFN- γ by β cells in normal mice leads to insulinitis, β cell destruction and IDDM (18). *In vivo* administration of Th2 cytokines, IL-4 and IL-10 can prevent the

onset of diabetes in the NOD mice (2,13). Also, Shehadeh et al. has shown in NOD mice protected by CFA injection that Th1 cells are effector in diabetogenesis while Th2 cells play a vital role in the regulation of the disease (19). Adoptive transfer of Th3 cytokine (TGF- β) producing islet-reactive CD4⁺ T cells prevents diabetes in NOD mice (6). Thus, correlation studies between cytokines expressed in islets and autoimmune diabetes development in NOD mice have demonstrated that β cell destructive insulitis is associated with increased expression of Th1 cytokine (IFN- γ), whereas non-destructive insulitis is associated with increased expression of Th2 cytokines (IL-4, IL-10) and Th3 cytokine (TGF- β). In the present study, no Th2 shifting of the cytokine profiles is observed, rather IL-4 expression in T cells of anti ICAM-1/LFA-1 mAb-treated NOD mice was not detected. However, expression of IL-10 was detected in T cells of both tolerarized mice as well as control mice. This phenomenon probably represented a secondary event, which may not be the cause of disease protection.

In conclusion, this study revealed that deletion of CD8⁺ T cells or blockade of anti-ICAM-1/LFA-1 interaction in the young age of an autoimmune diabetes model resulted in alteration of the nature of autoreactive T cells and in the complete prevention of autoimmune diabetes. The detail prevention mechanism is outside of immunoregulatory and Th1/Th2 theory, implicating the loss of capability to infiltrate and to destroy pancreatic β cells. Further studies will reveal these unique tolerance mechanisms, leading to clinical application to prevent type1 diabetes in future.

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