Article

Acquired pMHC I Complexes Greatly Enhance $CD4^+$ Th Cell's Stimulatory Effect on $CD8^+$ T Cell-Mediated Diabetes in Transgenic RIP-mOVA Mice

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$CD4^+$ helper T (Th) cells play pivotal roles in induction of $CD8^+$ CTL immunity. However, the mechanism of $CD4^+$ T cell help delivery to $CD8^+$ T cells *in vivo* is still elusive. In this study, we used ovalbumin (OVA)-pulsed dendritic cells (DC_{OVA}) to activate OT-II mouse $CD4^+$ T cells, and then studied the help effect of these $CD4^+$ T cells on $CD8^+$ cytotoxic T lymphocyte (CTL) responses. We also examined CTL mediated islet β cell destruction which led to diabetes in wild-type C57BL/6 mice and transgenic rat insulin promoter (RIP)-mOVA mice expressing β cell antigen OVA with self OVA-specific tolerance, respectively. In adoptive transfer experiments, we demonstrated that help, in the form of peptide/major histocompatibility complex (pMHC) I acquired from DC_{OVA} by DC_{OVA} activation, was required for induction of OVA-specific CTL responses in C57BL/6 mice. However, in combination with TCR transgenic OT-I mouse $CD8^+$ T cells, the tolerogenic dosage of $CD4^+$ Th cells with acquired pMHC I, but not $CD4^+$ ($K^{b-/-}$) Th cells without acquired pMHC I were able to cause diabetes in 8/10 (80%) RIP-mOVA mice. This study thus expands the current knowledge in T cell-mediated autoimmunity and provides insight into the nature of $CD4^+$ T cell-mediated help in $CD8^+$ CTL induction. *Cellular & Molecular Immunology*. 2008;5(6):407-415.

Key Words: $CD4^+$ Th, pMHC I, dendritic cell, membrane acquisition, diabetes

Introduction

The mechanism of T cell-mediated autoimmunity has been widely investigated in transgenic mice which expressed self antigens (Ags) under the control of tissue-specific promoters. Given the prominent role played by T cells in pathogenicity of diabetes mellitus type I, efforts were made particularly to unravel the nature of T cell tolerance to pancreatic islet β cell Ags. In some of these studies, viral proteins were expressed in pancreatic islets (1, 2). Autoimmune diabetes did not occur in these mice, unless they were infected with the respective virus. These seminal studies demonstrated that $CD8^+$ T cells with specificity for model self antigens usually ignore their specific self antigens under normal circumstances, but could be induced to become auto-aggressive by cross-reactive pathogen-associated Ags (3).

It is now generally been recognized that $CD4^+$ helper T (Th) cells are essential for induction of $CD8^+$ cytotoxic T lymphocyte (CTL) responses against many cell-based Ags, including male-specific Ags, model Ags loaded onto splenocytes, or tumor Ags transfected into cell lines (4-6). Further, it was shown that many cell-based Ags activate $CD8^+$ CTLs through the cross-priming pathway, which involves bone marrow-derived Ag-presenting cells (APCs) that take up exogenous Ags and present them *via* the major histocompatibility complex (MHC) class I pathway (7, 8). Thus, $CD4^+$ T cells are also essential for *in vivo* cross-priming (4-6) and avoiding induction of CTL tolerance (9). Several studies have implicated CD40 signaling (6, 10) and IL-2 secretion (4, 11) from $CD4^+$ T cells as important helper factors in generation of effective CTL responses. Recently, using transgenic mice model (RIP-OVA^{hi}) expressing high amount of ovalbumin (OVA) protein in pancreas under the

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Received Nov 3, 2008. Accepted Dec 18, 2008.

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Abbreviations: DC, dendritic cell; DC_{OVA}, OVA-pulsed DC; FITC, fluorescein isothiocyanate; PE, R-phycoerythrin; TCR, T cell receptor; pMHC I, peptide/major histocompatibility complex; OT-I, OVA-specific class I-restricted TCR transgenic; OT-II, OVA-specific class II-restricted TCR transgenic; RIP, rat insulin promoter; Th, helper T cell; CTL, cytotoxic T lymphocyte.

control of rat insulin promoter (RIP), Behrens and colleagues attempted to define the nature of $CD4^+$ T cell help for induction of islet β cell destruction and diabetes by CTL effectors (10). They demonstrated that the effector phase of CTL responses is influenced by $CD4^+$ T cell help and that CD154 signaling is an important helper factor provided by $CD4^+$ T cells in induction of $CD8^+$ CTL responses. Although considerable knowledge has been acquired in T cell activation, the factors that contributed by the $CD4^+$ T cell help in $CD8^+$ CTL generation are still not well understood (11, 12).

Stimulation of T cells by APCs involves at least two signaling events: one elicited by T cell receptor (TCR) recognition of peptide-MHC (pMHC) complexes and the other by costimulatory molecule signaling (e.g., T cell CD28/ APC CD80) (11, 13). Consequent to Ag-specific T cell-APC interactions, immunological synapse is formed comprising a central cluster of TCR-MHC-peptide complexes and CD28-CD80 interactions surrounded by rings of engaged accessory molecules (e.g., complexed LFA-1-CD54) (14, 15). It has been demonstrated that APC-derived surface molecules are transferred to the Th cells during the course of their TCR internalization (16). Recently, we have shown that $CD4^+$ T cells can acquire not only the synapse-composed MHC class II and costimulatory molecules (CD54 and CD80), but also the bystander pMHC I from APCs, and act as $CD4^+$ Th-APCs in stimulation of CTL responses (11). Although, the efforts to delineate the role played by MHC class II in induction of CTL responses have been investigated in RIP-OVA^{hi} mice (10, 17), the contribution of pMHC I acquisition by $CD4^+$ Th cells in CTL responses resulting in pancreatic islet β cell destruction and diabetes is elusive.

In this study, we chose a well studied transgenic mouse model, RIP-mOVA mice with moderate expression of self OVA Ag and OVA-specific self immune tolerance (17) to retest the stimulatory effect of $CD4^+$ Th cells with or without acquired pMHC I complexes. We then investigated the role of acquired pMHC I complexes on DC_{OVA}-activated $CD4^+$ Th cells in activation of OVA-specific $CD8^+$ CTL responses in wild-type C57BL/6 mice and transgenic RIP-mOVA mice with self OVA-specific tolerance. Our results clearly demonstrated a targeting role of acquired pMHC I complexes on $CD4^+$ Th cells in stimulation of OVA-specific $CD8^+$ CTL responses leading to destruction of islet β cells and diabetes in transgenic RIP-mOVA mice. This study thus expands the current knowledge in T cell-mediated autoimmunity and provides insight into the nature of $CD4^+$ T cell-mediated help in $CD8^+$ CTL induction.

Materials and Methods

Reagents, cell lines and animals

Ovalbumin (OVA) protein was obtained from Sigma (St. Louis, MO). OVA II (ISQAVHAAHAEINEAGR) specific for Ia^b of OVA protein and OVA I (SIINFEKL) peptide (11) and Mut1 (FEQNTAQP) peptide (18) specific for H-$2K^b$ of OVA protein and an irrelevant 3LL lung carcinoma, respectively, were all purchased from Multiple Peptide Systems (San Diego, CA). The biotin-conjugated monoclonal anti-mouse H-$2K^b$, Ia^b, CD4, CD11c, CD69, CD80 antibodies (Abs), and FITC-conjugated anti-CD11c, R-phycoerythrin (PE)-conjugated anti-mouse IL-4 and IFN-γ Abs were all obtained from Pharmingen Inc. (Mississauga, Ontario, Canada). The anti-H-$2K^b$/OVA I peptide (pMHC I) Ab was obtained from Dr. T. Germain, National Institute of Health, Bethesda, MD (19). The recombinant mouse granulocyte/ macrophage-colony stimulation (GM-CSF), IL-2, IL-12 and IL-4 were purchased from R&D Systems (Minneapolis, MN). Female C57BL/6 mice (B6) were obtained from Charles River Laboratories (St. Laurent, Quebec, Canada). The OVA-specific TCR transgenic OT-I and OT-II mice (11), and H-$2K^b$ gene knockout (KO) mice on C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, MA). Homozygous OT-II/H-$2K^{b-/-}$ mice were generated by backcrossing the designated gene KO mice onto the OT-II background for three generations, homozygosity was confirmed by polymerase chain reaction (PCR) according to Jackson Laboratory's protocols. The transgenic rat insulin promoter (RIP)-mOVA mice on C57BL/6 background were obtained from Dr. W. Heath, Walter and Eliza Hall Institute of Medical Research (Melbourne, Australia) (17). These transgenic mice express OVA under the RIP and have, as such, OVA as a neo-self-Ag. They are transgenic for truncated OVA gene that is expressed as a membrane-bound molecule in pancreatic islets, kidney proximal tubules, and testis of male mice. All mice were treated according to animal care committee guidelines of University of Saskatchewan.

Generation of bone marrow-derived dendritic cells

The preparation of bone marrow (BM)-derived dendritic cells (DCs) was previously described (11). Briefly, BM cells prepared from femora and tibiae of normal C57BL/6 mice were depleted of red blood cells with 0.84% ammonium chloride and plated in DC culture medium [DMEM plus 10% FCS, GM-CSF (20 ng/ml) and IL-4 (20 ng/ml)]. On day 3, the nonadherent granulocytes, and T and B cells were gently removed, and fresh media were added. Two days later, the loosely-adherent proliferating DC aggregates were dislodged and replated. On day 6, the nonadherent cells were harvested. These DCs were pulsed with OVA (0.3 mg/ml) for overnight at 37°C, then washed extensively with PBS, and termed DC_{OVA}. DCs generated from the H-$2K^b$ gene KO mice were referred to as ($K^{b-/-}$) DC_{OVA}.

Preparation of OT-II $CD4^+$ and OT-I $CD8^+$ T cells

Naïve OVA-specific $CD4^+$ T and $CD8^+$ T cells were isolated from OT-II and OT-I mouse spleens, respectively, and enriched by passage through nylon wool columns (C&A Scientific Inc, VA). $CD4^+$ and $CD8^+$ T cells were then purified by negative selection using anti-mouse CD8 (Ly2) and CD4 (L3T4) paramagnetic beads (DYNAL Inc, Lake Success, NY) to yield populations that were > 95% $CD4^+/V\alpha 2V\beta 5^+$ and $CD8^+/V\alpha 2V\beta 5^+$ T cells, respectively. To generate DC_{OVA}-activated $CD4^+$ T cells, $CD4^+$ T cells (2 ×

10^5 cells/ml) from OT-II mice were stimulated for three days with irradiated (4,000 rads) DC_{OVA} (1×10^5 cells/ml) in presence of IL-2 (10 U/ml), IL-12 (5 ng/ml) and anti-IL-4 Ab (10 μg/ml) (R&D Systems, Minneapolis, MN) (11). These *in vitro* DC_{OVA}-activated $CD4^+$ T cells were then isolated by Ficoll-Paque (Sigma) density gradient centrifugation and purified using CD4 microbeads (Miltenyi Biotec, Auburn, CA). Activated $CD4^+$ T cells derived from OT-II/H-$2K^{b-/-}$ which was primed by irradiated ($K^{b-/-}$) DC_{OVA} was termed $CD4^+$ ($K^{b-/-}$) Th cells. The phenotype and cytokine profile of the $CD4^+$ ($K^{b-/-}$) Th cells are similar to those of $CD4^+$ Th cells derived from OT-II mice, except for the designated gene deficiency i.e. activated $CD4^+$ ($K^{b-/-}$) Th cells did not express acquired pMHC I .

Phenotypic characterization of DC_{OVA}-activated $CD4^+$ T cells
For the phenotypic analysis, $CD4^+$ T cells were stained with Abs specific for CD4, CD69 and pMHC I, respectively, and analyzed by flow cytometry. To examine the intracellular expression of cytokines, the above cells were processed using a commercial kit (Cytofix/CytoPerm Plus with GolgiPlug; Pharmingen, Inc), and stained with PE-conjugated anti-IL-4 and IFN-γ Abs, according to the manufacturers' protocols (11). Cytokine secretion of $CD4^+$ Th cells in the supernatants was assessed using the commercial enzyme-linked immunosorbent assay (ELISA) kits (Endogen, Woburn, MA). The results were normalized to the recombinant cytokine standard curves.

T cell proliferation assay
C57BL/6 mice and RIP-mOVA mice were *i.v.* immunized with DC_{OVA} (1×10^6 cells per mouse) and $CD4^+$ Th or ($K^{b-/-}$) Th cells (3×10^6 cells per mouse), respectively. Six days after the immunization, 100 μl blood was taken from the tail of each mouse. The blood samples were incubated with 10 μl PE-conjugated H-$2K^b$/$OVA_{257-264}$ tetramer (Beckman coulter, Mississauga, Ontario, Canada) and 1 μl FITC-conjugated anti-CD8 mAb for 30 min at room temperature. The erythrocytes were then lysed using lysis/fixed buffer (Beckman coulter). The cells were washed and analyzed by flow cytometry.

Cytotoxicity assay
C57BL/6 and RIP-mOVA mice were *i.v.* immunized with $DC_{OVA,}$ $CD4^+$ Th or $CD4^+$ ($K^{b-/-}$) T cells (3×10^6 cells per mouse), respectively. Naïve mouse splenocytes were incubated with either high (3.0 μM, $CFSE^{high}$) or low (0.6 μM, $CFSE^{low}$) concentrations of CFSE, to generate differentially labeled target cells. The $CFSE^{high}$ cells were pulsed with OVA I, whereas the $CFSE^{low}$ cells were pulsed with the irrelevant 3LL lung carcinoma H-$2K^b$ peptide Mut1 and served as internal controls. These peptide-pulsed target cells were washed extensively to remove free peptide, and then *i.v.* co-injected at 1:1 ratio into the above immunized mice six days after the immunization. Sixteen hours after target cell delivery, the spleens were removed and residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were sorted and analyzed by flow cytometry.

Animal studies
In RIP-mOVA mice model, the cross-presentation of self OVA Ag was able to delete the autoreactive $CD8^+$ T cells leading to tolerization of the $CD8^+$ T cell compartment to self-Ag OVA (9). We have recently demonstrated that (i) a high frequency of OVA-specific $CD8^+$ T cell precursor can overcome OVA-specific immune tolerance from DC_{OVA} immunization in RIP-mOVA mice and (ii) the OVA-specific $CD8^+$ CTL responses reached a maximal level in presence of 1×10^6 OT-I $CD8^+$ T cell precursors (20). Therefore, we chose the amount of 1×10^6 OT-I $CD8^+$ T cell precursors for transfer to induce maximal $CD8^+$ CTL responses in RIP-mOVA mice immunized with $CD4^+$ Th cells. To assess whether $CD4^+$ Th cells can also overcome immune tolerance and induce mouse diabetes in RIP-mOVA mice in presence of 1×10^6 OT-I $CD8^+$ T cells, the transgenic RIP-mOVA mice (10 mice per group) were *i.v.* transferred with 1×10^6 naïve OT-I $CD8^+$ T cells. One day after the T cell transfer, mice were *i.v.* immunized with variable numbers of $CD4^+$ Th cells with acquired pMHC I (0.1×10^6, 0.5×10^6, 1×10^6 and 2×10^6 cells) or $CD4^+$ ($K^{b-/-}$) Th cells without acquired pMHC I. The above mice were monitored for 2 weeks after immunization for diabetes by urine glucose testing. Animals were considered to be diabetic after 2 consecutive days with readings of more than 55 mmol/L. Pancreatic tissue samples were collected for histopathological examination to ascertain islet β cell destruction.

Results

Activated $CD4^+$ Th cells acquire pMHC I complexes from DC_{OVA} by DC_{OVA} activation
We showed that OVA-pulsed dendritic cells (DC_{OVA}) expressed H-$2K^b$, Ia^b, CD11c, CD80 and pMHC I (Figure 1A), indicating that they are OVA-specific mature DCs. We previously showed that *in vitro* DC_{OVA}-activated OT-II $CD4^+$ T cells acquired DC's MHC I and II and costimulatory molecules (CD54 and CD80) (11). In this study, we more accurately assessed the acquisition of DC's pMHC I complexes by $CD4^+$ Th cells using the specific anti-pMHC I Ab. As shown in Figure 1B, these *in vitro* DC_{OVA}-activated OT-II $CD4^+$ T cells purified by positive selection using CD4-microbeads showed expression of CD4 and CD69 compared to naïve OT-II $CD4^+$ T cells without expression of CD69, indicating that they are activated $CD4^+$ T cells. In addition, these activated $CD4^+$ Th cells also expressed intracellular IFN-γ, but not IL-4, indicating that they are type 1 helper T (Th1) cells. This was further confirmed by ELISA analysis showing that these *in vitro* irradiated OVA-pulsed DC (DC_{OVA})-activated $CD4^+$ T cells secreted IFN-γ (~ 2 ng/ml/ 10^6 cells/24 h), but not IL-4. The naïve OT-II $CD4^+$ T cells did not express pMHC I. However, these DC_{OVA}-activated $CD4^+$ Th cells did show some expression of pMHC I by using the specific anti-pMHC I Ab, indicating that they may acquire pMHC I from DC_{OVA} by DC_{OVA} activation. This was

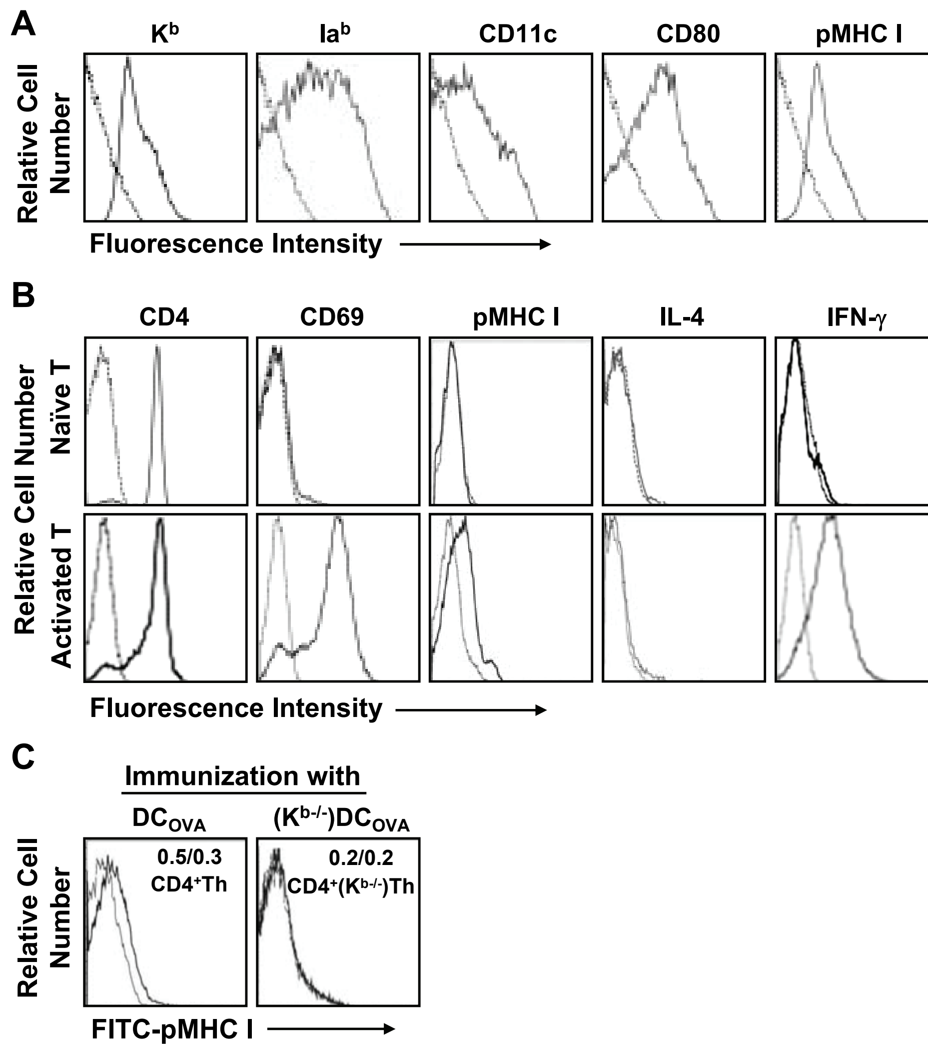

Figure 1. Phenotypic analysis of DCs and CD4$^+$ T cells. (A) OVA-pulsed DCs (DC_{OVA}) were stained with a panel of Abs for analysis of cell surface expression of K^b, Ia^b, CD11c, CD80, pMHCI (thick solid lines). Isotype-matched irrelevant Abs were used as controls (dotted lines). (B) The naïve CD4$^+$ T cells derived from OT-II mice were *in vitro* activated by DC_{OVA}. These activated CD4$^+$ T and the naïve CD4$^+$ T cells were then stained with biotin-conjugated anti-CD4, CD69, pMHCI, IL-4 and IFN-γ Abs and FITC-streptavidin (solid lines), respectively. Biotin-conjugated isotype-matched Abs were used as controls (thin dotted lines). (C) The naïve CD4$^+$ T cells derived from normal OT-II and OTII/K^b KO mice were *in vitro* activated by DC_{OVA} and ($K^{b-/-}$)DC_{OVA}, respectively. These activated CD4$^+$ T cells were then stained with biotin-conjugated anti-pMHC I Ab and FITC-streptavidin (solid lines), respectively. Biotin-conjugated isotype-matched Ab was used as controls (thin dotted lines). The value of mean fluorescence intensity (MFI) of pMHC I expression on CD4$^+$ Th cells with acquired pMHC I or ($Kb^{-/-}$) Th cells without acquired pMHC I using FITC-pMHC I Ab/the control FITC-Ab is presented in each panel. One representative experiment of two in the above different experiments is shown.

further confirmed by the evidence that CD4$^+$ ($K^{b-/-}$) Th-APC did not express any pMHC I complex when stimulated by ($K^{b-/-}$) DC_{OVA} without pMHC I expression (Figure 1C), confirming that these activated CD4$^+$ T cells do acquire pMHC I from DC_{OVA} by DC_{OVA} activation. To rule out the possibility of irradiated-DC_{OVA} contamination in these activated CD4$^+$ Th cell population, we also analyzed them using the anti-CD4 and anti-CD11c Abs specific for CD4$^+$ T cell and DC markers, respectively, by flow cytometry. There was no detectable CD11c$^+$ DC_{OVA} contamination in purified activated CD4$^+$ Th cell population (data not shown) due to not only the powerful positive selection process, but also the killing activity of these activated CD4$^+$ Th cells expressing OVA-specific TCR to pMHC II-expressing DC_{OVA} (21).

Activated CD4$^+$ Th, but not activated CD4$^+$ ($K^{b-/-}$) Th cells without acquired pMHC I stimulate effective CD8$^+$ T cell proliferation and effector function in wild-type C57BL/6 mice
To dissect the functional effect of pMHC I, we conducted CD8$^+$ T cell proliferation assay. We immunized the wild-type C57BL/6 mice with DC_{OVA}, DC_{OVA}-activated CD4$^+$ Th and CD4$^+$ ($K^{b-/-}$) Th cells. Six days after immunization, we

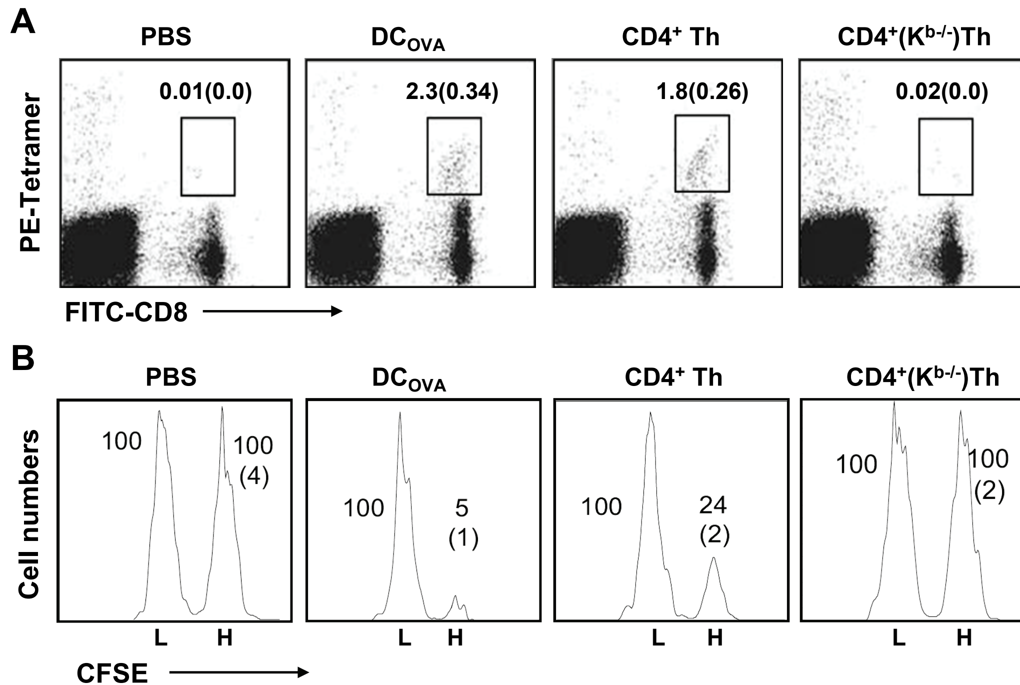

Figure 2. Functional analysis of $CD4^+$ Th cells. (A) Tetramer staining assay. Wild-type C57BL/6 mice were *i.v.* immunized with DC_{OVA} or DC_{OVA}-stimulated $CD4^+$ T or $CD4^+$ ($K^{b-/-}$) T cells. Six days after immunization, the tail blood samples of immunized mice were incubated with PE-H-$2K^b$/OVA I tetramer and FITC-anti-CD8 Ab, and then analyzed by flow cytometry. The value in each panel represents the percentage of tetramer-positive $CD8^+$ T cells versus the total $CD8^+$ T cell population. The value in parenthesis represents the SD. (B) *In vivo* cytotoxicity assay. The above immunized mice were *i.v.* co-injected at 1:1 ratio of splenocytes labeled with high (3.0 μM, $CFSE^{high}$) and low (0.6 μM, $CFSE^{low}$) concentrations of CFSE and pulsed with OVA I and Mut1 peptide, respectively six days after immunization with DC_{OVA} or activated $CD4^+$ T or activated $CD4^+$ ($K^{b-/-}$) T cells. Sixteen hours after target cell delivery, the residual $CFSE^{high}$ and $CFSE^{low}$ target cells remaining in the recipients' spleens were sorted and analyzed by flow cytometry. The value in each panel represents the percentage of $CFSE^{high}$ cells *vs* $CFSE^{low}$ cells remaining in the spleens. The value in parenthesis represents the SD. One representative experiment of three in the above different experiments is shown.

performed tetramer staining assay to detect OVA-specific $CD8^+$ T cells in these immunized mice. As shown in Figure 2A, DC_{OVA} and activated $CD4^+$ Th cells stimulated H-$2K^b$/OVA I tetramer-positive $CD8^+$ CTL responses accounting for 2.3% and 1.8% of the total peripheral blood $CD8^+$ T cells in wild-type mice, respectively, indicating that these activated $CD4^+$ Th cells with acquired pMHC I can efficiently stimulate *in vivo* OVA-specific $CD8^+$ T cell proliferation. However, activated $CD4^+$ ($K^{b-/-}$) Th cells without acquired pMHC I complexes could not stimulate H-$2K^b$/OVA I tetramer-positive $CD8^+$ T cell proliferation (0.02%) in wild-type C57BL/6 mice, indicating the importance of acquired pMHC I in efficient stimulation of *in vivo* OVA-specific $CD8^+$ T cell proliferation. Besides, we adoptively transferred OVA I peptide-pulsed splenocytes that had been strongly labeled with CFSE ($CFSE^{high}$), as well as the control peptide Mut1-pulsed splenocytes that had been weakly labeled with CFSE ($CFSE^{low}$), into the above immunized mice. We found that there were substantial loss of the OVA-specific and $CFSE^{high}$-labeled cells in both positive control DC_{OVA}- (95%) and activated $CD4^+$ Th cell-immunized (76%) mice (Figure 2B), but not in activated $CD4^+$ ($K^{b-/-}$) Th cell-immunized mice (0%), indicating that activated $CD4^+$ ($K^{b-/-}$) Th cells without acquired pMHC I failed in stimulation of $CD8^+$ T cell differentiation into CTL effectors, whereas activated $CD4^+$ Th cells with acquired pMHC I are capable of efficiently eliciting $CD8^+$ CTL responses.

Activated $CD4^+$ Th cells with acquired pMHC I failed in stimulation of $CD8^+$ T cell proliferation and induction of diabetes in RIP-mOVA mice with OVA-specific self immune tolerance

The transgenic RIP-mOVA mice with moderate expression of self OVA Ag have OVA-specific self immune tolerance (10). RIP-mOVA mice were then *i.v.* immunized with DC_{OVA} and activated $CD4^+$ Th cells, respectively. Six days after immunization of DC_{OVA}, mouse tail blood samples were analyzed for $CD8^+$ CTL responses by double (PE-tetramer/FITC-CD8) staining. In contrast to C57BL/6 mice showing significant OVA-specific $CD8^+$ T cell responses (Figure 2B), RIP-mOVA mice did not respond to the above stimulation with OVA-specific T cell responses which accounted for only 0.06% of the total peripheral blood $CD8^+$ T cells (Figure 3A). However, they also resumed their responses to DC_{OVA} stimulation after the treatment of mice with anti-CD25 Ab to

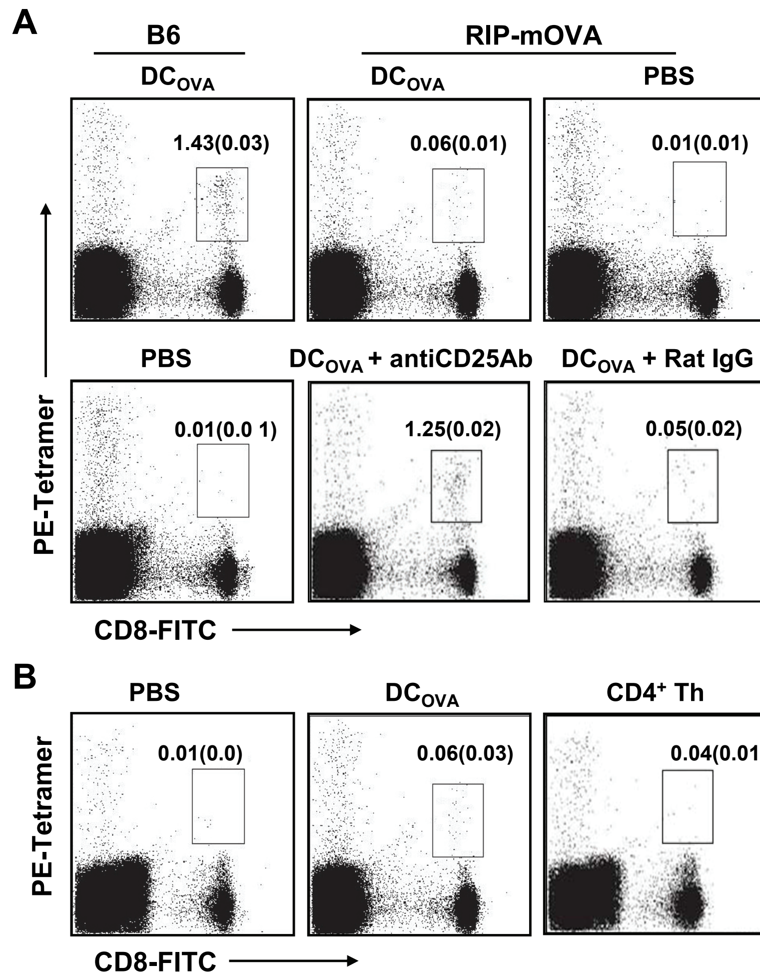

Figure 3. Activated CD4$^+$ T cells failed in stimulating CD8$^+$ T cell proliferation and effector function in RIP-mOVA mice. (A) B6 mice or RIP-mOVA transgenic mice treated with anti-CD25 Ab were immunized with DC_{OVA}. Six days after the immunization, the mouse tail blood samples were stained with PE-tetramer and FITC-CD8 Ab. One representative experiment of two is displayed. (B) RIP-mOVA transgenic mice were *i.v.* immunized with activated CD4$^+$ Th cells. Six days after immunization, CD8$^+$ T cell proliferation was estimated by tetramer staining. The value in each panel represents the percentage of tetramer-positive CD8$^+$ T cells *vs* the total CD8$^+$ T cell population. The value in parenthesis represents the SD. One representative experiment of three in the above different experiments is shown.

deplete CD4$^+$25$^+$ Tr cells, but not with the control rat IgG, confirming the existence of OVA-specific CD4$^+$ Tr cell-mediated immune tolerance in RIP-mOVA mice. Similarly, activated CD4$^+$ Th cells also failed in induction of OVA-specific CD8$^+$ T cell proliferation accounting for only 0.04% of the total peripheral blood CD8$^+$ T cells in RIP-mOVA mice (Figure 3B), indicating that activated CD4$^+$ Th cells alone can not overcome immune tolerance in RIP-mOVA mice.

Tolerogenic dosage of activated CD4$^+$ Th cells has stimulatory effect on CD8$^+$ T cells only in presence of OT-I CD8$^+$ T cell precursors in RIP-mOVA mice leading to islet tissue destruction and diabetes

To break the immune tolerance, RIP-mOVA mice with a previous transfer of 1×10^6 OT-I CD8$^+$ T cells were immunized with different amounts of CD4$^+$ Th cells, and then monitored for 2 weeks for diabetes by urine glucose testing. We found that a previous transfer of a constant amount of OT-I T cells (1×10^6 cells) and subsequent injection of 0.1×10^6, 0.5×10^6, 1×10^6 and 2×10^6 activated CD4$^+$ T cells with acquired pMHC I was able to induce diabetes in 0/10, 3/10, 6/10 and 8/10 RIP-mOVA mice, respectively. Diabetes which occurred 8-14 days after T cell transfer into these RIP-mOVA mice were further confirmed by histopathological examination of pancreas as evidenced by the destruction of pancreatic islet tissues with lymphocyte infiltration (Figure 4A). Our data are consistent with a previous report by Kurts and colleagues demonstrating that when OT-I and OT-II T cells co-injected, OT-II CD4$^+$ T cells stimulate OT-I CD8$^+$ T cells to destroy pancreatic β cells leading to diabetes in RIP-mOVA mice (9). Here, it is interesting to note that 2×10^6 activated CD4$^+$ ($K^{b-/-}$) Th cells without acquired pMHC I were unable to induce diabetes in

RIP-mOVA mice with previous transfer of 1 × 10^6 OT-I $CD8^+$ T cells (Figure 4A), confirming the importance of acquired pMHC I in induction of diabetes in RIP-mOVA mice. To explain the fact that activated $CD4^+$ T cells with acquired pMHC I mediated an enhanced $CD8^+$ CTL response, we and others proposed that activated $CD4^+$ T cells could supply cytokines, such as IL-2 that may enhance the expansion and/or survival of $CD8^+$ T cells (11, 22) and augment the survival of activated $CD8^+$ T cells possibly due to the induction of survival genes like *bcl-xL* or other antiapoptotic mechanism (9, 23) thereby shifting the autoreactive $CD8^+$ T cell response from tolerogenic to immunogenic pathway in RIP-mOVA mice.

Discussion

Generation of effective $CD8^+$ CTL responses to minor histocompatibility or tumor antigens not associated with danger signals often requires help from $CD4^+$ Th cells *via* cross-priming (24). A three-cell interaction model was originally proposed suggesting that antigen-specific $CD4^+$ and $CD8^+$ T cells must interact simultaneously with a common antigen-specific APC and $CD4^+$ T cell activated by APC provides $CD8^+$ T cell help *via* its IL-2 secretion (25). Later, Ridge et al. proposed a dynamic model of two sequential interactions by APC, in which activated $CD4^+$ Th cells license APC *via* CD40/CD40L signalling, which become capable of directly stimulating $CD8^+$ T cell responses (26). Recently, we have proposed a new dynamic model of two sequential interactions by Th-APC (11), in which $CD4^+$ Th cells activated by APC acquire APC's membrane molecules and become $CD4^+$ Th-APC capable of stimulating $CD8^+$ CTL responses. Although there is still no final conclusion regarding these models, we have demonstrated that DCs stimulate effector memory $CD8^+$ CTL responses, whereas $CD4^+$ Th-APC stimulate central memory $CD8^+$ CTL responses (21).

Recently, Hwang et al. (27) reported that the presence of cognate memory $CD4^+$ T cells increases the capacity of memory $CD8^+$ T cells to proliferate after Ag rechallenge, migrate into tumor-associated tissue and differentiate into effector phenotype cells, resulting in substantially enhanced control of tumor outgrowth. Several reports demonstrated improved antitumor (28, 29) and antiviral (30, 31) responses *in vivo* following a combined transfer of transduced $CD8^+$ and $CD4^+$ T cells compared to either T-cell subset alone. Moreover, in a tissue transplantation study, graft rejection was pronounced by simultaneously raising the frequency of $CD4^+$ T cells to ~0.5% and $CD8^+$ T cells to ~5% despite CD28/CD154 blockade. Although the exact mechanism of specific protection of CTL responses by $CD4^+$ Th cells is still unknown, Kennedy and Celis (23) reported that CTL response was tightly controlled by the availability of $CD4^+$ T cell help. By providing direct survival signals to CTL, $CD4^+$ Th cells blocked AICD and increased the functional life span of $CD8^+$ CTL responses. This antiapoptotic effect may explain the need for $CD4^+$ T cell help to maintain CTL responses to viruses such as CMV, HIV, lymphocytic choriomeningitis virus and hepatitis viruses (32-34). Further

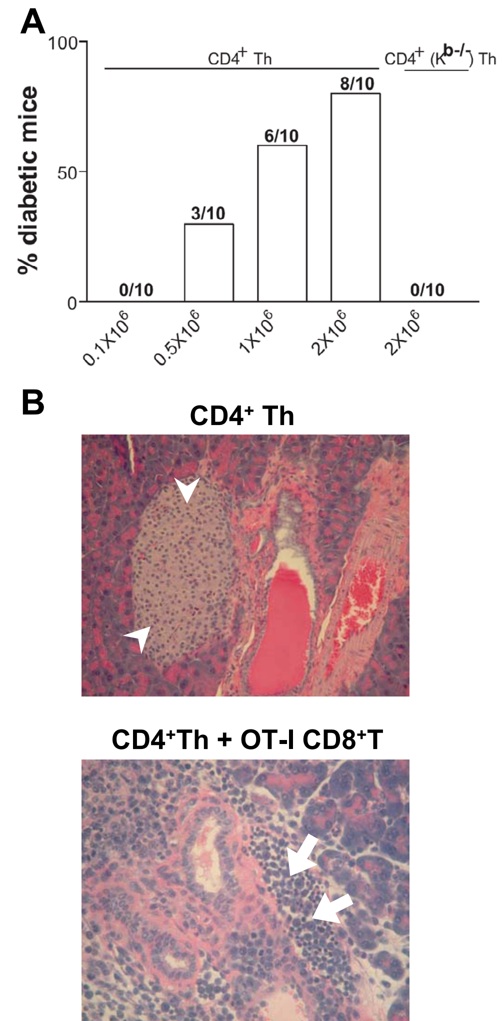

Figure 4. The stimulatory effect of $CD4^+$ Th cell is targeted to $CD8^+$ T cell responses against mouse pancreatic islet β cell antigen leading to diabetes in RIP-mOVA mice *via* acquired pMHC I. (A) RIP-mOVA transgenic mice were *i.v.* immunized with constant number of OT-I $CD8^+$ T cells (1 × 10^6 cells/mouse) alone or in combination with different numbers (0.1 × 10^6, 0.5 × 10^6, 1 × 10^6 and 2 × 10^6) of $CD4^+$ Th cells/mouse or 2 × 10^6 $CD4^+$ ($K^{b-/-}$) Th cells/mouse, respectively. Mice were monitored for diabetes from day 6 post immunization for at least 20 days by urine glucose testing. Animals were considered diabetic after 2 consecutive days with readings ≥ 56 mmol/L urine glucose. (B) Pancreatic tissues of the transgenic RIP-mOVA mice immunized with $CD4^+$ Th cells alone or in combination with OT-I T cells were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were stained with HE and examined by microscopy (× 150). The destruction of pancreatic islet tissues and lymphocyte infiltration (arrows) were only found in OT-I $CD8^+$ T cells (1 × 10^6 cells) + $CD4^+$ Th cells (2 × 10^6 cells) immunized RIP-mOVA mice, whereas RIP-mOVA mice injected with $CD4^+$ Th cells (2 × 10^6 cells) alone showed normal pancreatic tissues (arrow head). One representative experiment of three in the above different experiments is shown.

work is needed to elucidate the exact mechanism(s) by which $CD4^+$ and $CD8^+$ T cells interact for CTL responses. Our unpublished results suggest that it is the acquired pMHC I complexes on $CD4^+$ Th cells, that target the stimulatory effects derived from IL-2 secretion, CD40L and acquired CD80 costimulations to the Ag-specific $CD8^+$ T cells *in vivo*, leading to induction of OVA-specific central memory CTL responses and antitumor response. These studies corroborate the importance of $CD4^+$ T cell's stimulatory effect on effective $CD8^+$ CTL responses.

More recently, Cox et al. have demonstrated that $CD4^+$ Th cells with acquired bystander pMHC I complexes from APCs became susceptible to CTL killing in an Ag-specific manner (35). However, the molecular mechanism for $CD4^+$ Th cell acquisition of the bystander pMHC I from DCs by DC activation is unclear. We have recently demonstrated that $CD4^+$ Th cells acquired the bystander pMHC I because the bystander pMHC I complexes colocalized with pMHC II complexes in the same synapse formed between a DC and a $CD4^+$ T cell, which was internalized by $CD4^+$ T cell and subsequently recycled on the surface of the $CD4^+$ T cell (36). In this study, we have further elucidated the critical role of the acquired pMHC I in regulation of immune responses. We note that it is the acquired pMHC I complexes on $CD4^+$ Th cells, that target its stimulatory effect to OVA-specific $CD8^+$ T cell responses, leading to destruction of pancreatic islet tissues and diabetes. These results are also supported by our another two recent reports showing that the acquired pMHC I complexes on DC_{OVA}-activated $CD4^+$ Th and $CD8^+$ Tc cells can target their helper effects onto adoptive $CD8^+$ Tc cell-immunotherapy of cancer and stimulation of $CD8^+$ CTL responses, respectively (37, 38). The targeting role of the acquired pMHC I complexes on $CD4^+$ Th-APC may also be applied to interpret the generation of Ag-specific regulatory T (Tr) cells *in vivo* after encounter with Ag presented by DCs (39, 40). Zhang et al. demonstrated that double negative Tr cells used their TCR to acquire allo-MHC peptides from APCs, and became Ag-specific Tr cells in suppression of graft rejection (41). Tarbell et al. also showed that $CD4^+25^+$ Tr expanded *in vitro* by Ag-specific DC stimulation became 20-fold more efficient in suppression of autoimmune diabetes caused by diabetogenic T cells in nonobese diabetic mice than polyclonal unexpanded $CD4^+25^+$ Tr cells (42). However, they did not elucidated the molecular mechanism on how the immune suppressive effect of these Tr cells can be specifically or more efficiently delivered to the *in vivo* T-cell-mediated graft rejections or autoimmune diseases. Based upon the above principle elucidated in this study, we assume that these $CD4^+$ or DN Tr cells may become Ag-specific after acquisition of pMHC I or pMHC II complexes by DC stimulation *in vivo*. This assumption has recently been proved. It has been shown that these DN Tr cell-mediated antigen-specific suppression is *via in vivo* trogocytosis of alloantigen (43). We have also demonstrated that antigen specificity of adoptive $CD4^+$ regulatory T cells was obtained *via* acquired pMHC I complexes from IL-10-secreting antigen-specific DCs by DC stimulation (44).

Taken together, our data provide direct evidence that it is the acquired pMHC I complexes on $CD4^+$ Th cells that enhances $CD4^+$ T cell helper effect in induction of $CD8^+$ T cell-mediated pancreatic islet destruction and diabetes. Thus, the present findings may have significant implications in autoimmunity and antitumor immunotherapy.

Acknowledgements

This study was supported by research funds from Canadian Institute of Health Research (MOP 79415, 81228 and 89713).

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