

Regulatory function of CD4+CD25+ T cells from Class II MHC-deficient mice in contact hypersensitivity responses

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Abstract: Contact hypersensitivity (CHS) is a CD8+ T cell-mediated, inflammatory response to hapten sensitization and challenge of the skin. During sensitization, the magnitude and duration of hapten-specific CD8+ T cell expansion in the skin-draining lymph nodes (LN) are restricted by CD4+CD25+ T regulatory cells (Treg). The regulation of hapten-specific CD8+ T cell priming in Class II MHC-deficient (MHC-/-) mice was investigated. Although hapten-specific CD8+ T cell priming and CHS responses were elevated in Class II MHC-/- versus wild-type mice, presensitization depletion of CD4+ or CD25+ cells in Class II MHC-/- mice further increased CD8+ T cell priming and the elicited CHS response. Flow cytometry analyses of LN cells from Class II MHC-/- mice revealed a population of CD4+ T cells with a majority expressing CD25. Forkhead box p3 mRNA was expressed in LN cells from Class II MHC-/- and was reduced to background levels by depletion of CD4+ or CD25+ cells. Isolated CD4+CD25+ T cells from wild-type and Class II MHC-/- mice limited in vitro proliferation of alloantigen- and hapten-specific T cells to antigen-presenting stimulator cells. These results identify functional CD4+CD25+ Treg in Class II MHC-/- mice, which restrict hapten-specific CD8+ T cell priming and the magnitude of CHS responses. *J. Leukoc. Biol.* 82: 000-000; 2007.

Key Words: suppression · Treg · MHC-/-

INTRODUCTION

Contact hypersensitivity (CHS) is a T cell-mediated, inflammatory response of the epidermis to cutaneous sensitization and subsequent challenge with hapten. Application or sensitization of hapten stimulates the migration of hapten-presenting dendritic cells from the epidermis [i.e., Langerhans cells (LC)] and dermis to the skin-draining lymph nodes (LN), where priming of hapten-specific T cells occurs [1-3]. Hapten challenge induces these primed T cells to traffic to the site of challenge, where they are activated to produce cytokines and express cytotoxicity of keratinocytes, which mediate the characteristic edema/spongiosis of the response [4-6]. These ear-swelling responses typically peak at 24-48 h after challenge and then resolve rapidly.

The primary, antigen-specific effector cells mediating CHS responses are hapten-primed CD8+ T cells. Antibody-mediated depletion of CD8+ cells prior to hapten sensitization results in substantial decreases in the magnitude of the response elicited to hapten challenge when compared with control, Ig-treated mice [7, 8]. In contrast, antibody-mediated depletion of CD4+ T cells increases CHS responses. Similarly, in Class I MHC-deficient (MHC-/-) mice, CHS responses are much lower and in Class II MHC-/- mice, are elevated when compared with responses in wild-type mice [9, 10]. Consistent with the role of CD8+ T cells in this response, in vitro stimulation of T cells from the skin-draining LN of hapten-sensitized mice has demonstrated that the hapten-primed CD8+ T cells produce proinflammatory cytokines, including IFN- γ , and the CD4+ T cells produce IL-4, IL-5, and IL-10 [6, 8].

The level of hapten-specific CD8+ T cell priming to IFN- γ -producing cells in the LN of sensitized mice has been observed to reflect the magnitude and course of the CHS response elicited to hapten challenge. In wild-type mice, hapten-specific CD8+ T cells producing IFN- γ are detected easily in the LN on Days 4 and 5 after sensitization, and the numbers of these cells decline quickly to background by Day 8 or 9 [11]. Depletion of CD4+ T cells during sensitization results in an increased number of hapten-specific CD8+ T cells producing IFN- γ , and these higher numbers are maintained through Day 8. Studies from this and other laboratories have indicated the activity of CD4+CD25+ T regulatory cells (Treg) in limiting the expansion of hapten-primed, effector CD8+ T cell populations and the duration of the CHS response [11, 12]. Treatment of wild-type mice with anti-CD25 mAb during hapten sensitization also increases the numbers of hapten-specific CD8+ T cells producing IFN- γ in the LN and the response elicited to hapten challenge [13]. The immune-enhancing effects of the anti-CD25 mAb treatment are not observed, however, when the wild-type mice are also depleted of CD4+ T cells. These results suggested that the anti-CD25, mAb-mediated enhancement of the response was mediated through CD4+CD25+ Treg. These results predicted that anti-CD25

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mAb treatment of Class II MHC^{-/-} mice during sensitization would provide no further enhancing effect of the treatment on hapten-specific, effector CD8⁺ T cell development or the magnitude of the immune response elicited. In the current study, we have tested this prediction directly and have observed a population of CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice. Although the numbers of these CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice are small when compared with wild-type mice, they express regulatory function during in vivo and in vitro responses. These studies demonstrate for the first time the role of CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice as negative regulators of antigen-specific CD8⁺ T cell development and the immune response.

MATERIALS AND METHODS

Mice

C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were obtained through Dr. Clarence Reeder (National Cancer Institute, Frederick, MD, USA). Class II MHC^{-/-} mice on the C57/BL6 background were purchased from Taconic Farms (Germantown, NY, USA).

Hapten sensitization and elicitation of CHS

Mice were sensitized to 2,4-dinitrofluorobenzene (DNFB) by painting the shaved abdomen and paws with 0.25% DNFB (25 μ l/abdomen; 10 μ l/paw) on Days 0 and +1. On Day +5, hapten-sensitized and control, nonsensitized mice were challenged on each side of each ear with 10 μ l DNFB or oxazolone to elicit the CHS response. Ear thickness was measured using an engineer's micrometer (Mitutoyo, Elk Grove Village, IL, USA) and expressed in units of 10⁻⁴ in. The magnitude of ear swelling is given as the mean increase of each group of three to four individual animals $\pm$ SEM.

Antibodies

Purified mAb YTS 191.1.2 and GK1.5 (antimouse CD4) for in vivo treatment were purchased from Ligocyte (Bozeman, MT, USA). Purified mAb PK136 (antimouse NK1.1) for in vivo treatment was purchased from BioExpress (West Lebanon, NH, USA). Culture supernatants of the IgG-producing hybridoma PC 61.5.3 (antimouse CD25) were used to purify mAb by protein G chromatography.

For in vivo depletion of CD4⁺ T cells, mice were treated with 100 μ g each anti-CD4 mAb, YTS 191, and GK1.5 i.p. on 3 consecutive days before hapten sensitization on Days 0 and +1 as described previously [6, 8]. Treatment with anti-CD25 mAb and anti-NK1.1 mAb was performed by injecting mice with 250 μ g PC61.5.3 or PK136, respectively, i.p. on Days 0, +1, +2, and +3 during hapten sensitization. As a control, mice were treated with equivalent amounts of rat IgG (Sigma Chemical Co., St. Louis, MO, USA). In each experiment, treated sentinel mice were used to evaluate the efficiency of CD4⁺ T and NK1.1⁺ cell depletion by antibody staining and flow cytometry analysis of spleen and LN cells and were always >95% when compared with cells from control rat IgG-treated mice.

ELISPOT assays for enumeration of hapten-specific T cells producing IFN- γ

ELISPOT assays were performed as detailed previously [11, 13]. Briefly, ELISPOT plates (Unifilter 350, Polyfiltronics, Rockland, MA, USA) were coated overnight with 4 μ g/ml IFN- γ -specific mAb and blocked with 1% BSA in PBS. LN cells from sensitized or nonsensitized mice were prepared on Day +5 postsensitization and used as responder cells. Syngeneic spleen cells from naïve mice were incubated with 50 μ g/ml mitomycin C for 30 min at 37°C, washed, and labeled with dinitrobenzene sulfonic acid (DNBS) before use as stimulator cells, which were plated at 5 $\times$ 10⁵ cells/well with 5 $\times$ 10⁵ or 2.5 $\times$ 10⁵ responder cells/well in serum-free HL-1 medium (BioWhittaker, Walkersville, MD, USA), supplemented with 1 mM L-glutamine and 1 mM antibiotic. After 24 h of cell culture at 37°C in 5% CO₂, cells were removed from the

plate by extensive washing with PBS/0.05% Tween-20 (PBS-T), and biotinylated anti-IFN- γ mAb (2 μ g/ml) was added. The plate was incubated overnight at 4°C and then washed with PBS-T. Conjugated streptavidin-alkaline phosphatase (Vector Labs, Burlingame, CA, USA) was added to each well for 2 h at room temperature. The plate was washed with PBS-T, and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl substrate (Bio-Rad Laboratories, Hercules, CA, USA) was added for the detection of IFN- γ . The resulting spots were counted with an ImmunoSpot Series I analyzer (Cellular Technology Ltd., Cleveland, OH, USA), which was designed to detect ELISA spots with predetermined criteria for spot size, shape, and colorimetric density.

Flow cytometry analyses

Single cell suspensions were prepared from LN of naïve and hapten-sensitized mice on Day +4 postsensitization. LN cells were washed twice with staining buffer (Dulbecco's PBS with 2% FCS/0.2% NaN₃), and 1 $\times$ 10⁶ cell aliquots were incubated on ice in 150 μ l rat serum (Rockland, Gilbertsville, PA, USA), diluted 1:1000 in the staining buffer. After 30 min, the cells were washed twice and stained with fluorochrome-labeled antimouse mAb to CD4 and CD25 (BD Pharmingen, San Diego, CA, USA). After 30 min on ice, the cells were washed five times, resuspended in staining buffer, and analyzed by two-color using FACScan and CellQuest software (Becton Dickinson, San Jose, CA, USA). Sample data were collected on 20,000 gated cells.

For flow cytometry analyses of forkhead box p3 (Foxp3) expression, a mouse Treg-staining kit (eBioscience, San Diego, CA, USA) was used according to the manufacturer's protocol. Single cell suspensions were prepared from LN and spleens of naïve, wild-type C57BL/6 and Class II MHC^{-/-} mice. Cells were washed with staining buffer, and 1 $\times$ 10⁶ cell aliquots were incubated on ice with FITC anti-CD4 and APC anti-CD25 mAb. After 30 min, the cells were washed and incubated with fixation/permeabilization solution for an additional 30 min on ice. The cells were washed in permeabilization buffer and incubated on ice with Fc block for 15 min before PE anti-Foxp3 antibody was added and incubated for 30 min. Cells were washed in the permeabilization buffer, resuspended in the staining buffer, and analyzed by three-color flow cytometry using FACSCalibur and CellQuest software (Becton Dickinson). Sample data were collected on all gated CD4⁺ cells.

Analysis of Foxp3 gene expression by quantitative RT-PCR

Whole cell RNA was obtained from LN of sensitized mice treated with control IgG, anti-CD4, anti-CD25, or anti-NK1.1 mAb on Day +4 postsensitization by dissolving the cells in Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) with subsequent chloroform extraction. cDNA was synthesized from 2 μ g RNA using the TaqMan RT reagent kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. PCR was performed using custom primers and FAM dye-labeled probes (Applied Biosystems) for mouse Foxp3 and GAPDH (gene assay ID #Mm00475156_m1 and Mm9999915_g1, respectively). The QT of Foxp3 expression for one sample—the RNA sample isolated from LN of anti-CD4, mAb-treated, wild-type mice—was set arbitrarily at 1.0 and used to determine the expression levels of the remaining samples.

Proliferation assays

For allogeneic MLR, LN cells were obtained from C57BL/6 mice pretreated with anti-CD25 mAb by the protocol described above. LN cells were incubated with CD4-specific, antibody-coated magnetic beads (Dynabeads, Dynal A.S., Oslo, Norway) to obtain enriched populations of CD4⁺CD25⁺ T cells, and aliquots of 5 $\times$ 10⁵ cells were delivered to wells of a 96-well plate for use as responder cells. For stimulator cells, single cell suspensions were prepared from spleens of T cell-depleted BALB/c or C57BL/6 mice, treated with 50 μ g/ml mitomycin C, and plated at 5 $\times$ 10⁵ cells/well. To prepare test CD4⁺CD25⁺ cells, the cells were isolated from LN cells of naïve C57BL/6 or B6. Class II MHC^{-/-}, using a commercial isolation kit (Miltenyi Biotec, Auburn, CA, USA), according to the manufacturer's protocol and aliquots of 1 $\times$ 10³ cells, was added to each well of the responder-stimulator cultures.

For the hapten-specific, proliferative assay, LN cell suspensions from unsensitized or DNFB-sensitized C57BL/6 mice on Day +4 following sensitization were used to obtain purified CD8⁺ T cells for use as responder cells (2.5 $\times$ 10⁵

TABLE 1. Increased CHS Responses in Class II MHC^{-/-} Mice Are Enhanced by Treatment with Depleting CD4 mAb

Expt.	Group	Sens.	CD4 depln.	Δ Ear Swelling ($\times 10^{-1}$ in. $\pm$ SEM)		
				24 h	48 h	72 h
1	Wild-type	+	-	63.9 $\pm$ 5.7	46.5 $\pm$ 7.1	ND
	Cl. II MHC ^{-/-}	+	-	101.2 $\pm$ 3.6*	72.5 $\pm$ 3.0*	ND
	Wild-type	-	-	5.67 $\pm$ 0.5	8.53 $\pm$ 0.2	ND
	Cl. II MHC ^{-/-}	-	-	3.57 $\pm$ 0.1	8.64 $\pm$ 0.1	ND
2	Wild-type	+	-	66.3 $\pm$ 3.6	43.1 $\pm$ 6.9	37.5 $\pm$ 4.6
	Wild-type	+	+	98.1 $\pm$ 3.6*	90.5 $\pm$ 5.4*	77.3 $\pm$ 2.0*
	Cl. II MHC ^{-/-}	+	-	105.9 $\pm$ 5.3*	72.5 $\pm$ 3.0*	68.5 $\pm$ 2.7*
	Cl. II MHC ^{-/-}	+	+	102.9 $\pm$ 8.7*	117.3 $\pm$ 12.7**	99.7 $\pm$ 9.4**
	Wild-type	-	-	5.4 $\pm$ 0.1	4.4 $\pm$ 0.5	4.2 $\pm$ 0.4

cells/well). For stimulator cells, hapten-presenting LC (hpLC) from LN cell suspensions of sensitized C57BL/6 mice on Day +2 were isolated using magnetic anti-CD11c mAb-coated beads (Miltenyi Biotec) and were used at 5×10^4 cells/well. CD4+CD25+ T cells were obtained from wild-type and MHC II^{-/-} mice and used in the assay as described above.

In alloantigen- and hapten-specific assays, cultures were pulsed with 1 μ Ci [³H]thymidine 72 h after initiation. Cells were harvested 18 h later onto fiber filter mats, and ³H incorporation was determined by liquid scintillation counting.

RESULTS

Regulation of CHS responses in Class II MHC^{-/-} mice

As previously reported [9, 14], ear-swelling responses to DNFB challenge of sensitized B6 Class II MHC^{-/-} mice were significantly higher than responses in wild-type C57BL/6 mice (**Table 1**, Expts. 1 and 2). To confirm the role of CD4+ T cells in the regulation of the wild-type responses, a group of wild-type mice and as a control, Class II MHC^{-/-} mice were treated with control IgG or depleting, CD4-specific mAb. CHS responses to challenge in wild-type mice depleted of CD4+ T cells before sensitization were higher and of extended duration compared with those induced in control-treated, wild-type mice. At 48 and 96 h postchallenge, the ear-swelling responses in wild-type mice depleted of CD4+ T cells were also higher than responses observed in sensitized and challenged Class II MHC^{-/-} mice. At 48 and 96 h postchallenge, CHS responses in anti-CD4, mAb-treated Class II MHC^{-/-} mice were significantly higher than responses observed in control rat IgG-treated Class II MHC^{-/-} mice (Table 1, Expt. 2).

The magnitude of CHS responses is reflected by the numbers of hapten-specific CD8+ T cells producing IFN- γ in skin-draining LN of sensitized mice [11, 13, 14]. Therefore, the induction of these CD8+ T cells was compared in sensitized, wild-type and Class II MHC^{-/-} mice. CD8+ T cell-enriched cell populations from the skin-draining LN of the mice were isolated on Day +5 after sensitization with DNFB and tested for numbers of hapten-specific CD8+ T cells producing IFN- γ by ELISPOT assay (**Fig. 1**). These CD8+ T cells were clearly present in LN of sensitized, wild-type mice treated with control IgG and were increased two- to threefold in LN of control-sensitized, Class II MHC^{-/-} mice. Anti-CD25 mAb treatment of wild-type mice during sensitization resulted in a threefold increase in the numbers of hapten-specific

CD8+ T cells producing IFN- γ . Treatment of the Class II MHC^{-/-} mice with CD4- or CD25-specific mAb during sensitization resulted in a further twofold increase in the numbers of the hapten-specific CD8+ T cells.

CD4+CD25+ T cells are present in the LN of Class II MHC^{-/-} mice

As the depletion of CD4+ or CD25+ cells in sensitized Class II MHC^{-/-} mice increased hapten-specific CD8+ T cell priming, the presence of CD4+CD25+ Treg in Class II MHC^{-/-} mice was determined. Cells from LN of DNFB-sensitized and naïve, wild-type and Class II MHC^{-/-} mice were stained and analyzed for the presence of CD4+CD25+ T cells. As expected, a small percent of the CD4+ T cell population from naïve and sensitized, wild-type mice expressed CD25 (**Fig. 2**). It is surprising that a small but distinct population of CD4+ T

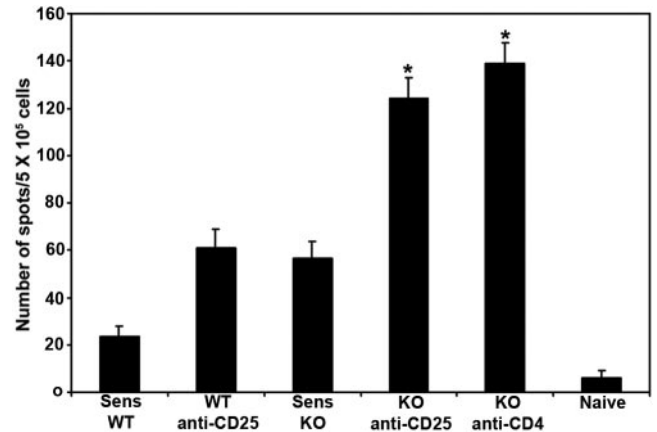


Fig. 1. Regulation of antigen-specific CD8+ T cell development by CD4+ and CD25+ cells in Class II MHC^{-/-} mice. Wild-type (WT) C57BL/6 and B6 Class II MHC^{-/-} mice were treated with 250 μ g control rat IgG, anti-CD25 mAb, or anti-CD4 mAb i.p. and sensitized with 0.25% DNFB on Days 0 and +1. On Day +5, LN cells from the mice were cultured with DNBS-labeled or unlabeled, syngeneic splenocytes on anti-IFN- γ , mAb-coated ELISPOT plates. After 24 h, hapten-specific T cells producing IFN- γ were enumerated. The mean number of IFN- γ -producing T cells in triplicate cultures $\pm$ SEM for two individual mice is shown after subtraction of spots from control wells containing T cells with unlabeled stimulator cells (less than five spots per well). Results are representative of three individual experiments. *, $P < 0.05$, when compared with numbers in control, Ig-treated Class II MHC^{-/-} mice. KO, Knockout.

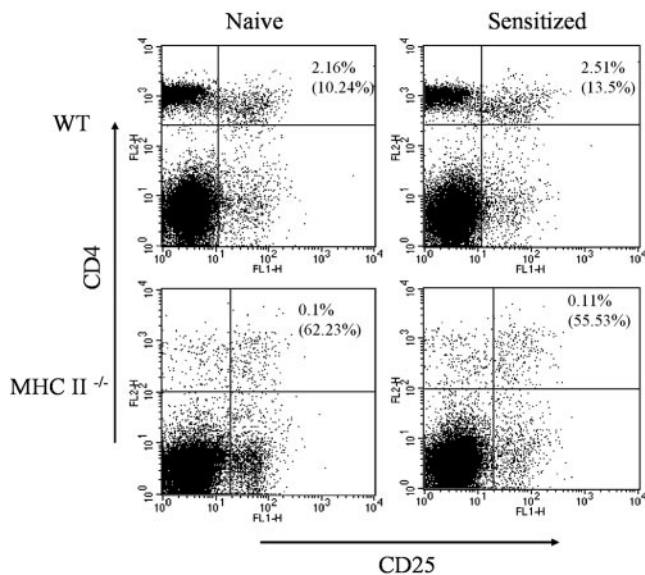


Fig. 2. Presence of CD4+CD25+ T cells in LN of Class II MHC^{-/-} mice. Wild-type C57BL/6 and B6 Class II MHC^{-/-} mice were sensitized with 0.25% DNFB, and on Day +4, LN cell suspensions from the sensitized and naïve mice were prepared and stained with FITC-labeled, anti-CD25 mAb and PE-labeled, anti-CD4 mAb and analyzed by flow cytometry. The percent of CD4+CD25+ cells in the LN cell population is shown in the upper right-hand quadrants. The percent of CD4+ T cells, which express CD25, is shown in parentheses. Results are representative of four individual animals analyzed.

cells was observed in LN from naïve and sensitized Class II MHC^{-/-} mice. More than half of these CD4+ T cells expressed CD25. Hapten sensitization did not increase the percentage of CD4+ T cells expressing CD25 in LN of the wild-type or the Class II MHC^{-/-} mice.

CD4+CD25+ T cells in MHC II^{-/-} mice express Foxp3

To further investigate the possible presence of CD4+CD25+ Treg in Class II MHC^{-/-} mice, the expression of the Treg-associated gene Foxp3 was tested by quantitative RT-PCR. mRNA isolated from LN cells from wild-type and Class II MHC^{-/-} mice had similar levels of Foxp3 expression (**Fig. 3**). The expression of Foxp3 was decreased significantly when wild-type mice were treated with anti-CD4 or anti-CD25 mAb. Likewise, depletion of CD4+ or CD25+ T cells in Class II MHC^{-/-} mice decreased Foxp3 mRNA levels significantly. Treatment with anti-NK1.1 mAb had no significant change in Foxp3 expression in LN cell mRNA isolated from wild-type or Class II MHC^{-/-} mice.

The presence of Foxp3 in CD4+CD25+ T cells in wild-type and Class II MHC^{-/-} mice was tested directly using flow cytometry. In LN and spleen cells (**Fig. 4, a and c**) from wild-type mice, Foxp3-expressing cells constituted ~6% of the CD4 T cells. In Class II MHC^{-/-} mice, Foxp3-expressing cells also constituted ~6% of the CD4+ T cells in the spleen, but this increased to more than 33% of the CD4+ T cells in LN (**Fig. 4, b and d**).

CD4+CD25+ T cells from Class II MHC^{-/-} mice regulate antigen-primed T cell proliferation

Finally, the ability of the CD4+CD25+ T cells from wild-type versus Class II MHC^{-/-} mice to restrict proliferation

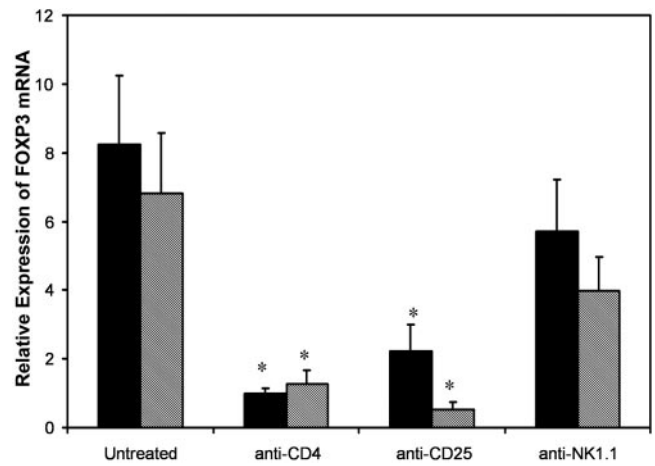


Fig. 3. Foxp3 expression in LN cells of Class II MHC^{-/-} mice. Groups of wild-type C57BL/6 (solid bars) and B6 Class II MHC^{-/-} (hatched bars) mice were sensitized with DNFB on Days 0 and +1 and were treated with 250 µg control rat IgG, anti-CD25, or anti-NK1.1 mAb i.p. on Days 0, +1, +2, and +3 or with 200 µg anti-CD4 mAb i.p. on Days -3, -2, and -1. On Day +4, LN cell suspensions were prepared, and whole cell RNA was extracted. cDNA was synthesized, and real-time PCR, using primers for Foxp3 and GAPDH as the endogenous control, was performed. The results are shown as fold-change compared with the Foxp3 expression observed in the RNA sample isolated from LN of anti-CD4, mAb-treated, wild-type mice. *, $P < 0.02$, when compared with Foxp3 expression in LN cells from control, IgG-treated mice.

of antigen-reactive T cells was tested using two different in vitro approaches. First, in allogeneic MLR, irradiated splenocytes from T cell-depleted BALB/c mice were cultured with CD4+CD25- T cells from C57BL/6 mice,

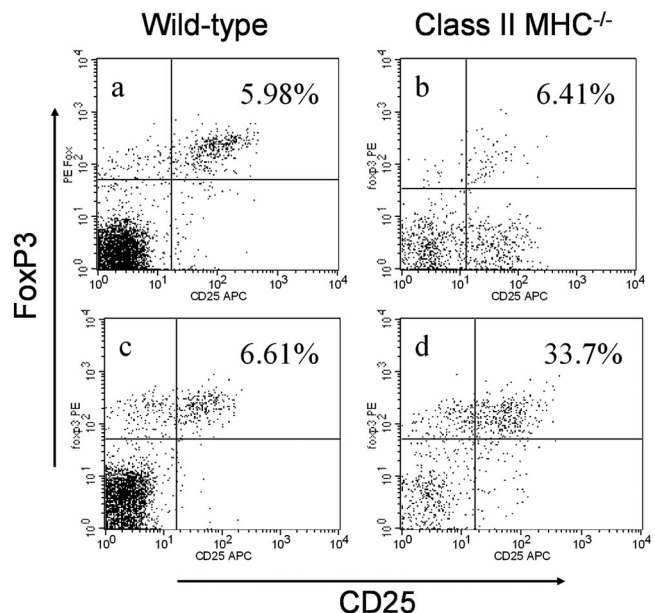


Fig. 4. Presence of CD4+CD25+ Foxp3+ T cells in Class II MHC^{-/-} mice. LN (a and b) and spleen (c and d) cell suspension were prepared from wild-type C57BL/6 and B6 Class II MHC^{-/-} mice and were stained with FITC anti-CD4 mAb, PE anti-Foxp3 mAb, and APC anti-CD25. The CD4+ cells were gated and analyzed for expression of CD25 and Foxp3. The percentage of CD4+ cells expressing CD25 and Foxp3 is shown in the upper right-hand quadrants. Results are representative of each of four individual samples for wild-type and Class II MHC^{-/-} animals.

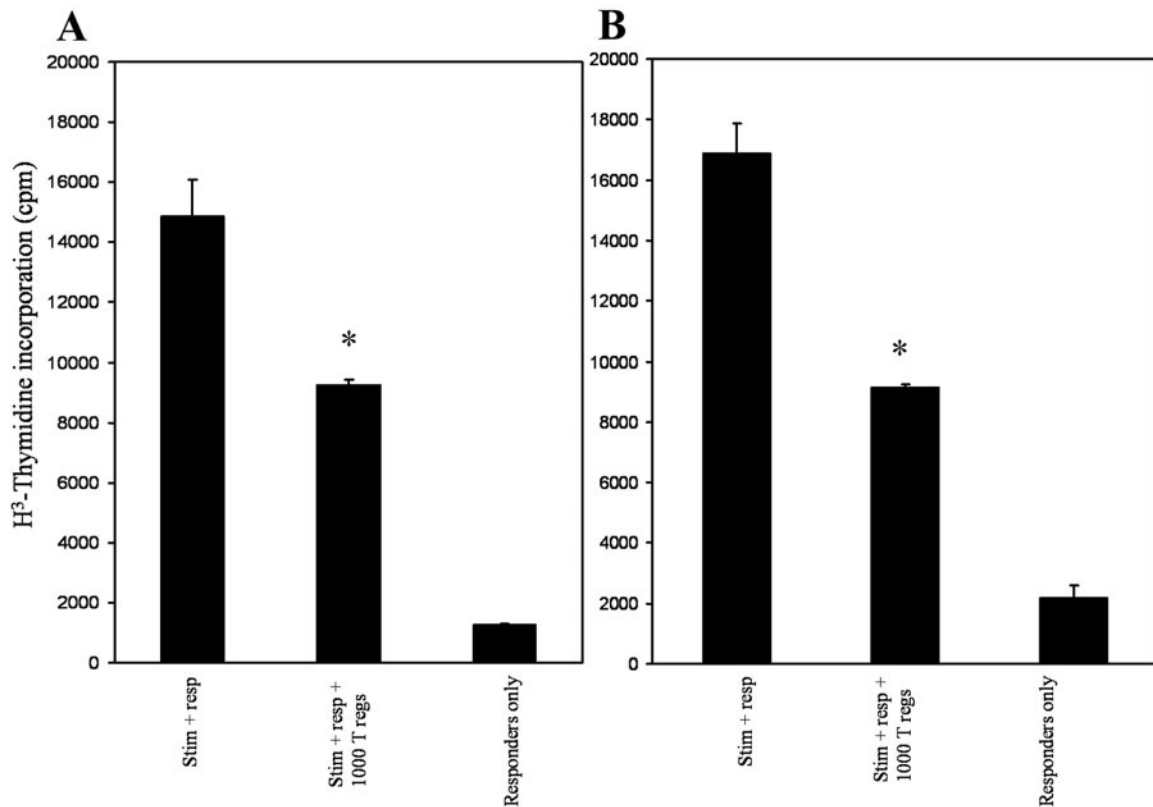


Fig. 5. CD4+CD25+ T cells from Class II MHC^{-/-} mice inhibit the proliferation of alloreactive CD4+ T cells. CD4+CD25+ T cells were isolated from LN of (A) wild-type C57BL/6 and (B) B6 Class II MHC^{-/-} mice. The isolated CD4+CD25+ T cells were added to cultures of syngeneic CD4+CD25- T cells and irradiated, allogeneic splenocytes from T cell-depleted BALB/c mice. After 72 h, cultures were pulsed with [³H]thymidine and harvested 18 h later. The mean ³H incorporation $\pm$ SEM in triplicate cultures is shown. Results are representative of two individual experiments. *, $P < 0.05$, when compared with ³H incorporation in control samples, which contain stimulators (Stim) and responders (resp) without CD4+CD25- T cells.

with or without addition of CD4+CD25+ T cells from C57BL/6 wild-type or Class II MHC^{-/-} mice. Cultures of CD4+CD25- responder T cells alone exhibited low/background levels of [³H]thymidine incorporation. Responder cells cultured with the allogeneic splenocyte stimulator cells exhibited high levels of [³H]thymidine incorporation. Addition of as few as 1×10^3 CD4+CD25- T cells from wild-type mice decreased the proliferation significantly compared with those from cultures of stimulator and responder cells alone (**Fig. 5A**). Similarly, addition of 1×10^3 CD4+CD25- T cells from Class II MHC^{-/-} mice inhibited the proliferation of the CD4+CD25- responder cells in response to the allogeneic stimulator cells (**Fig. 5B**).

Proliferation of CD8+ T cells from sensitized C57BL/6 mice cultured with syngeneic hpLC stimulators was used to test the activity of the CD4+CD25+ T cell populations from wild-type and Class II MHC^{-/-} mice. Again, addition of 1×10^3 CD4+CD25+ T cells from wild-type (**Fig. 6A**) or from Class II MHC^{-/-} (**Fig. 6B**) mice reduced the proliferation of the CD8+ T cells in response to the stimulator hpLC at similar levels. These results suggest that CD4+CD25+ T cells from MHC II^{-/-} mice have regulatory functions in the CHS model similar to those observed in wild-type mice.

DISCUSSION

Many studies have demonstrated the activity of CD4+CD25+ Treg in restricting T cell responses to self- and alloantigens and inhibiting autoimmune disease and allograft rejection [15–20]. Recent studies from this and other laboratories have also reported CD4+CD25+ T cell regulation of CHS responses [11, 12, 21]. These studies were initiated by earlier observations that CHS responses to hapten challenge are elevated when wild-type mice are depleted of CD4+ T cells during hapten sensitization or when the responses are elicited in Class II MHC^{-/-} mice [7–9].

The specificity and functional mechanisms these cells express to mediate regulation of CHS remain rather unclear. Studies by Ring and colleagues [21] have indicated the regulatory activity of CD4+CD25+ Treg in inhibiting effector CD8+ T cell adherence to the challenge site vasculature and infiltration into the skin parenchymal tissue through production of IL-10 during elicitation of CHS responses. We have reported that CD4+CD25+ T cells restrict the development of hapten-specific CD8+ T cells, which are the effector T cells mediating the response [11, 13]. The number of hapten-specific CD8+ T cells producing IFN- γ in the skin-draining LN of hapten-sensitized, wild-type BALB/c and C57BL/6 mice peaks on Day +5 after sensitization and then declines rapidly, and the

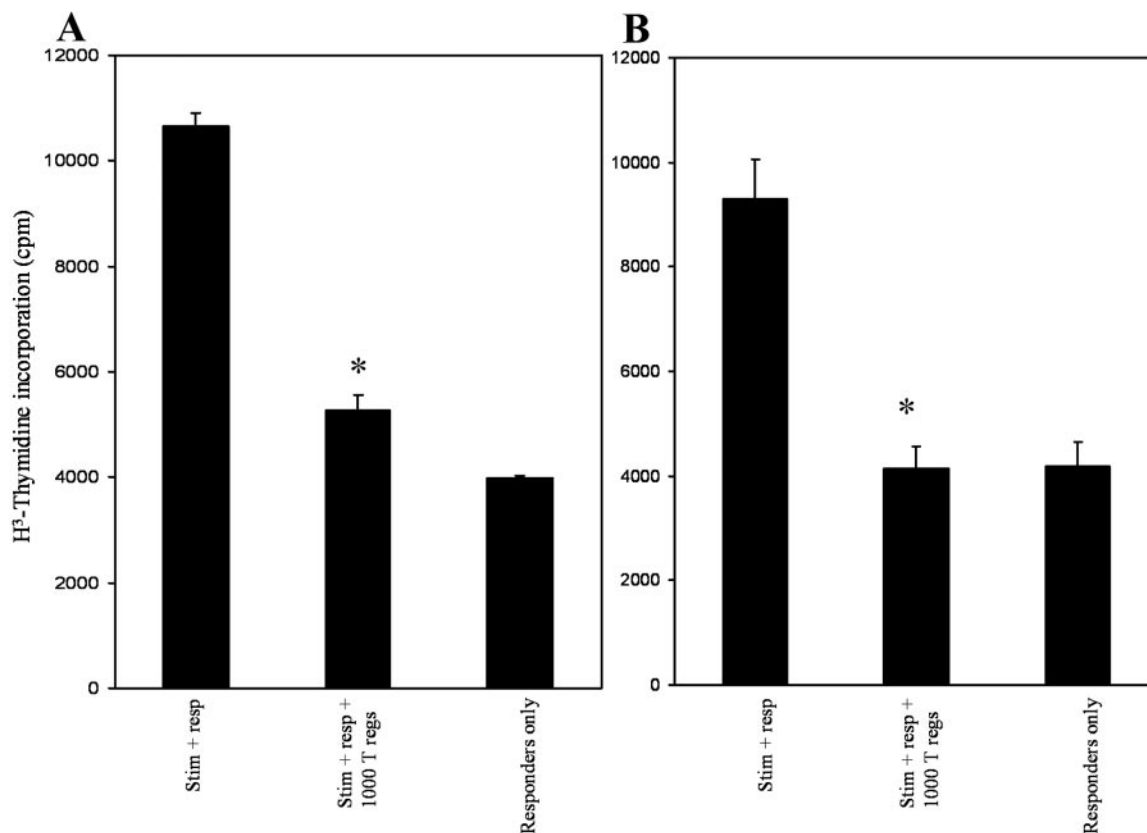


Fig. 6. CD4+CD25+ T cells from Class II MHC^{-/-} mice inhibit the proliferation of hapten-reactive CD8+ T cells. CD4+CD25+ T cells were isolated from LN of (A) wild-type C57BL/6 and (B) B6 Class II MHC^{-/-} mice. The isolated CD4+CD25+ T cells were added to cultures of isolated CD8+ T cells from LN of hapten-sensitized C57BL/6 mice and hpLC, also isolated from sensitized mice. After 72 h, cultures were pulsed with [³H]thymidine and harvested 18 h later. The mean ³H incorporation $\pm$ SEM in triplicate cultures is shown. Results are representative of two individual experiments. *, $P < 0.03$, when compared with ³H incorporation in control samples, which contain stimulators and responders without CD4+CD25+ T cells.

characteristics of this CD8 T cell priming reflect the increase and decline of the ear-swelling response to hapten challenge. In sensitized, wild-type mice depleted of CD4+ T cells, the number of these T cells as well as the CHS response are increased on Day +5, and these levels are extended in duration for several days. We reported recently that antagonism of IL-2 or CD25 results in a similar dysregulation of hapten-specific CD8+ T cell priming and CHS responses in wild-type mice [13]. During experiments extending these latter studies, the CHS response in Class II MHC^{-/-} mice was used as a control model for the presumed absence of this regulation. However, we observed that treatment of Class II MHC^{-/-} mice with anti-CD4 or anti-CD25 mAb during hapten sensitization resulted in further increases in the magnitude and duration of CHS swelling responses to challenge, as documented in this report.

The goal of the current study was to investigate the potential existence of a CD4+CD25+ T cell population in Class II MHC^{-/-} mice and the potential regulatory role of this population. The presence of a small population of CD4+ T cells was reported originally following generation of the Class II MHC^{-/-} mice, although the potential function of these cells was undetermined [22]. Staining of LN cells from hapten-sensitized and naive Class II MHC^{-/-} mice indicated the presence of a distinct population of CD4+ T cells, a majority expressing

CD25. Hapten-specific CD8+ T cell priming was increased when Class II MHC^{-/-} mice were treated with anti-CD4 or anti-CD25 mAb during hapten sensitization, raising the first suggestion of a regulatory function of CD4+CD25+ T cells in Class II MHC^{-/-} mice. To test this potential function directly, in vitro assays were used to show that the CD4+CD25+ T cells from Class II MHC^{-/-} mice inhibit the proliferation of wild-type CD8+ T cells and CD4+ T cells in hapten- and alloreactive assays.

We considered the possibility that the CD4+CD25+ T cells in Class II MHC^{-/-} mice were CD1-restricted NKT cells. These lymphocytes have been shown to have positive and negative regulatory functions in many immune responses including CHS [23–26]. Several observations argued against negative regulation of CHS by NK T cells in Class II MHC^{-/-} mice. First, NKT cells express CD122, the IL-2 receptor β chain, but not CD25, the α chain [27, 28]. This is consistent with NKT cell dependency on IL-15 rather than IL-2. Second, few of the CD4+ T cells in LN or spleen of Class II MHC^{-/-} mice expressed NK1.1 (D. D. Kish, data not shown). Third, sensitization of CD1d^{-/-} mice, which lack NKT cells, indicated a slight (25–30%) increase in the number of hapten-specific CD8+ T cells producing IFN- γ but not the two- to threefold increases observed when Class II MHC^{-/-} mice were depleted of CD4+ or CD25+ cells before sensitization (A. V.

Gorbachev, data not shown). Fourth, CD4⁺ cells in LN of Class II MHC^{-/-} mice express Foxp3, which is required for the development and function of the CD4⁺CD25⁺ Treg. Depletion of CD4 or CD25, but not NK1.1-expressing cells, eliminated Foxp3 transcripts in the cells.

The level of hapten-specific CD8⁺ T cell priming and CHS responses is higher and extended in duration in Class II MHC^{-/-} mice when compared with wild-type mice, and this has led to the use of the former as a model of CHS responses induced and elicited in the absence of CD4⁺CD25⁺ T cell-mediated regulation. The regulatory activity of the CD4⁺CD25⁺ T cells during CHS responses in Class II MHC^{-/-} mice was uncovered in the current study, when the mice were depleted of CD4⁺ or CD25⁺ cells during sensitization. The number of CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice is considerably lower than is observed in wild-type mice and is likely to account for the increased responses observed to hapten sensitization and challenge, when responses in Class II MHC^{-/-} versus wild-type mice are compared. The lower numbers of CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice make experiments transferring the cells to naïve recipients impractical and have restricted our analysis of these cells to in situ and in vitro studies. It is important, however, that similar levels of regulatory activity were observed on a cell-per-cell basis when equivalent numbers of CD4⁺CD25⁺ T cells from wild-type and Class II MHC^{-/-} mice were tested using in vitro proliferation assays. The current results are consistent with previous studies from this laboratory, indicating that a primary point of regulation in the CHS response is the CD4⁺CD25⁺ T cell-mediated restriction of effector CD8⁺ T cell development to sensitization [11, 13]. This in turn limits the magnitude and duration of the swelling/inflammation elicited by the hapten-primed CD8⁺ T cells in response to hapten challenge.

The identification of CD4⁺CD25⁺ Treg in I-A^b/I-E^b (Class II MHC^{-/-}) mice raises several issues about the specificity and origin of these cells. First, requirements for the selection and/or development of CD4⁺CD25⁺ Treg in the Class II MHC^{-/-} mice are unknown. In wild-type mice, these T cells develop through high-affinity interactions with self-peptide/Class II MHC complexes, although CD4 interaction with the Class II MHC molecule is not a prerequisite for Treg development [29–31]. The expression of I-A^b/I-E^a chimeric molecules has been documented on APC and transfected L cells at low levels [32–34], whereas to our knowledge, the expression of I-A^b/I-E^b molecules has not [3]. It is also possible that the CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice develop independently of Class II MHC expression in the thymus. A second issue arising from these studies is that the CD4⁺CD25⁺ Treg observed in Class II MHC^{-/-} mice may represent a small compartment of the Treg repertoire of wild-type mice or may arise because of the lack of competition for selection ligands in the thymus. CD4⁺CD25⁺ Treg development and function in Class II MHC^{-/-} mice also raise questions about the specificity of the regulatory cells during function to restrict hapten-specific CD8⁺ T cell priming to hapten sensitization, albeit to a lower degree than is observed in wild-type mice. Populations of antigen-specific and nonspecific CD4⁺CD25⁺ Treg have

been shown to function in controlling auto- and alloimmune responses in wild-type mice [35–37].

In summary, the current report defines a population of CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice, which although low in numbers, do exert a regulatory influence on hapten-specific T cell priming to mediate CHS responses. In CHS, this population plays a role in negative regulation of the response. Depletion of this population prior to hapten sensitization results in increases in the magnitude and duration of the CHS response compared with those observed in sensitized, control-treated, Class II MHC^{-/-} mice. Functionally, CD4⁺CD25⁺ T cells from Class II MHC^{-/-} mice are as potent as wild-type CD4⁺CD25⁺ T cells in suppressing antigen-driven T cell proliferation in vitro. These studies demonstrating the presence and function of CD4⁺CD25⁺ T cells in Class II MHC^{-/-} mice indicate that caution must be taken regarding the use of Class II MHC^{-/-} mice as a model, where this regulation is presumed to be absent.

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