A novel mechanism of regulatory T cell-mediated down-regulation of autoimmunity

Hui-Yu Qin¹, Rinee Mukherjee¹, Edwin Lee-Chan¹, Catherine Ewen², R. Chris Bleackley² and Bhagirath Singh¹,³,⁴

¹Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, N6A 5C1, Canada
²Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada
³Robarts Research Institute, London, Ontario, Canada
⁴Institute of Infection and Immunity, Canadian Institutes of Health Research, London, Ontario, Canada

Keywords: autoimmunity, CFA, diabetes, granzyme B/perforin, regulatory T cells

Abstract

We have established a novel CD4 and CD8 double-positive CD25⁺ T regulatory (Treg) clone, MT-5B, from lymph nodes of type 1 diabetes prone non-obese diabetic (NOD) mice immunized with CFA. CFA has previously been shown to prevent the onset of diabetes by inducing Treg cells. In vitro, clone MT-5B was anergic to a panel of antigen stimulations and exerted an immunosuppressive effect in antigen-non-specific and cell contact-independent manners. In vivo, clone MT-5B blocked the adoptive transfer of diabetes. Proteomics and immunoadsorption studies identified the suppressive proteins secreted by clone MT-5B as granzyme B (GrB) and perforin (PFN). GrB-mediated immune suppression was PFN dependent. Removal of GrB or PFN from the culture supernatant (SN) of MT-5B cells or pre-incubation of MT-5B cells with ethyleneglycol-bis(amoethylether)-tetraacetic acid which blocks PFN activity reduced the immunosuppressive effect in vitro. Pre-incubation of diabetogenic splenocytes from NOD mice with MT-5B SN impaired their ability to transfer disease by inducing T cell apoptosis, and removal of GrB from MT-5B SN by immunoadsorption decreased the effector function of MT-5B SN on diabetogenic splenocytes. Immunization of NOD mice with CFA increased the expression of GrB⁺ CD4 T cells, indicating that these cells are present in vivo. In conclusion, we describe a novel mechanism of cell contact-independent immune suppression in which Treg cells maintain immune homeostasis by secreting GrB/PFN.

Introduction

The discovery of CD4⁺ regulatory T (Treg) cells has provided a new understanding of the phenomenon of immunosuppression (1). Naturally arising and induced CD4⁺ Treg cells inhibit both the induction and effector function of autoreactive T cells in autoimmunity (2–5).

CD4⁺ Treg cells can be further differentiated into natural and adaptive Treg subsets based on their distinct ontology, mode of action and suppressive function (3). Each subset of Treg cells expresses CD25. The naturally occurring, thymus-derived CD4⁺CD25⁺ Treg cells have a cell contact-dependent, cytokine-independent mechanism of action (6, 7). Adaptive Treg cells generated in the periphery mediate immune responses by secreting suppressive cytokines and include IL-10-producing Tr1 and transforming growth factor (TGF)-β-producing Th3 cells (8–10). The CD4⁺CD25⁺ Treg cells maintain immune homeostasis by controlling autoimmunity, tumor immunity, infection immunity and transplantation tolerance (11, 12). In type 1 diabetes (T1D), both CD4⁺ T cells (13) and CD4⁺CD25⁺ Treg cells have been widely reported as being involved in the prevention of diabetes in animal model (5, 14–16). The expressions of TGF-β, cytotoxic T lymphocyte-associated antigen (CTLA)-4 and glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) have been shown important to the effector function of CD4⁺CD25⁺ Treg cells (17–20), but they are not necessary for immunosuppression (21–23). It was recently discovered that the transcription factor encoded by the Foxp3 gene is exclusively expressed in CD4⁺CD25⁺ Treg cells and is vital for their development and function (24, 25).

Granzyme B (GrB) is an important member of the granzyme family (26, 27). GrB and perforin (PFN) are the effector molecules that mediate target killing by NK cells and CTLs in

Correspondence to: B. Singh; E-mail: bsingh@uwo.ca
Received 12 September 2005, accepted 3 April 2006
Transmitting editor: C. J. Paige
Advance Access publication 4 May 2006
viral infection and anti-tumor immunity. Dysregulation of this pathway is associated with certain human diseases and genenic abnormalities in mice (28). The expression of GrA and/or GrB has recently been investigated in human and murine CD4+CD25+ Treg cells (29, 30). Similar to NK cells and CTLs, it has been shown that GrB-expressing Treg cells may function in a cell contact-dependent manner. GrB and PFN work synergistically to exert a cytotoxic effect on target cells. The mechanisms underlying the delivery of GrB to the target cells may involve transmembrane pores made by PFN (31), non-specific charge interaction (32) and/or cation-independent mannose 6-P receptor-mediated endocytosis (33). Serglycin, a proteoglycan, has been shown recently to form an apoptosis-inducing multimeric complex with granule proteins GrB and PFN through non-covalent linkage (34, 35). The precise mechanism underlying PFN-dependent delivery, intracellular trafficking and functioning of GrB is still under investigation.

In this study, MT-5B, a double-positive (DP) CD25+ Treg clone established from CFA-protected non-obese diabetic (NOD) mice was analyzed. Although this clone produces immunosuppressive cytokines, we found that these cytokines were not involved in MT-5B-mediated suppression. We identified the suppressive proteins secreted by MT-5B as GrB/PFN by using proteomic and immunoadsorption approaches. Unlike natural CD4+CD25+ Treg cells and GrB-expressing NK cells, CTLs and CD4+ Treg cells, the secretion and effector functions of GrB/PFN by MT-5B cells are cell contact independent. Pre-incubation of diabetogenic splenocytes with GrB/PFN-containing supernatant (SN) down-regulates their ability to transfer disease in a GrB-dependent manner by inducing T cell apoptosis. We report for the first time a novel mechanism involving the modulation of immune responses by DP CD25+ Treg cells through the secretion of GrB/PFN in a cell contact-independent manner.

Methods

Mice

NOD/LtJ and NOD.SCID mice were bred in specific pathogen-free conditions at the Robarts Research Institute animal facilities (London, Ontario, Canada). Animals were used according to the guidelines of the institutional animal care committee at the University of Western Ontario. For adoptive transfer studies, irradiated (800 rads) NOD and NOD.SCID mice were used as recipients. Diabetic NOD mice were kept on daily subcutaneous injection of 1 U human insulin (Humulin, Eli Lilly Co., Indianapolis, USA) until the day of the experiment. C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

Antibodies and reagents

In this study, the following mAbs were used for flow cytometry, western blot, immunoadsorption and ELISA: FITC- or PE-conjugated mAbs to CD3 (145-2C11), CD4 (GK 1.5), CD8α (53-6.7), CD11C (HL3), CD25 (PC61), CD45RB (16A), CD80 (16-10A1), CD86 (GL1), CD90-2 (30-H12), TCRβ (H57-597), CTLA-4 (UC10-4F10-11), Fas (Jo2), FasL (MFL3); PE-conjugated and purified (NA/LETM) mAbs to IFN-γ (XMg1.2), tumor necrosis factor (TNF)-α (TN3-19.12), TGF-β (MAB1835), IL-10 (JESS-2A5), CD3 (145-2C11), isotype controls, avidin-Alkaline Phosphatase and mouse rIL-2 (BD Pharmingen, Ontario, Canada); goat anti-mouse GITR, purified and biotin-conjugated goat anti-mouse GrB antibodies (R&D systems, Ontario, Canada); PE-conjugated mAbs to Foxp3 (FJK-16s) and CD8β (CT-CD8b), purified, PE and biotin-conjugated anti-mouse PFN mAbs (clone JAW 246, eBioscience, San Diego, USA). The following reagents were also used in this study: purified GrB protein, extraluminescence-avidin, ethyleneglycol-bis(aminohexyl ether)-tetraacetic acid (EGTA), propidium iodide (PI) and Concanavalin A (Con A) (Sigma, Ontario, Canada); FITC–TdT-mediated dUTP nick end labeling (TUNEL, Roche, Mannheim, Germany); Mycobacterium bovis BCG (Bacille Calmette–Guérin, Sanofi-Pasteur, Toronto, Canada); mouse recombinant glutamic acid decarboxylase (GAD67) was provided by J. F. Elliott (Edmonton, Canada).

Establishment of CD4+CD8+ CD25+ Treg clones

Four-week-old NOD mice were immunized with CFA in the hind footpad as previously described (36). Ten days later, popliteal lymph nodes were harvested and single-cell suspensions were prepared in complete RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM glutamine, 5 × 10⁻⁵ M 2-mercaptoethanol, 100 U ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin and 10% heat-inactivated FCS. Cells were seeded in 96-well flat-bottom microtitre plates in the presence of 50 μg ml⁻¹ BCG media were changed 5 days later. Ten to 14 days after the initiation of culture, cells well grown in cultures were transferred into 24-well plates in the presence of irradiated syngeneic spleen cells (2500 rads) as antigen-presenting cells (APCs) plus rat natural IL-2 (10 U ml⁻¹, BD Labware, MA, USA). T cell lines were cloned and sub-cloned by limiting dilution in 0.3–0.5 cells per well in the presence of APC and IL-2 as above. MT-5B is one of the clones established and used in this study. Clone MT-5B cells grow in a lightly adherent manner and can be detached by culturing at 4°C for 10 min. Cells were maintained in complete RPMI medium supplemented with 5% FCS and 50 U ml⁻¹ of mouse rIL-2.

Preparation of SN and cell lysates

For preparation of SN, MT-5B cells were washed three times with PBS to remove the residual FCS and incubated for 24–36 h in FCS-free RPMI at a concentration of 10⁵ cells ml⁻¹. Control SN was from nylon wool-enriched splenic T cells of 8-week-old NOD mice (36). SN was also concentrated 40-fold by using Centricon Plus-80 (PL-10 membrane, Millipore Corporation, MA, USA). Cell lysates (LS) were prepared by repeated freeze-thaw cycles in PBS. Equal volumes of concentrated supernatants (SN.C) and LS were prepared from the same number of MT-5B cells. GrB was undetectable in control SN, and was 292, 2602 and 1684 ng ml⁻¹ in MT-5B SN, SN.C and LS, respectively, as determined by ELISA.

Cell proliferation assays

(1) For proliferation assays, 2 × 10⁵ MT-5B cells were incubated in complete RPMI 1640 medium in the presence of Con A (4 μg ml⁻¹), BCG (100 μg ml⁻¹), GAD67 (10 μg ml⁻¹),...
For co-culture assays, MT-5B cells, MT-5B SN, anti-GrB/perforin-secreting Treg clone (1 μg ml⁻¹) in the presence of 4 × 10⁶ irradiated (2500 rads) syngeneic APC, or C57BL/6 splenocytes (mixed lymphocyte reaction, MLR) for 4–5 days. Nylon wool-enriched splenic T cells of 8-week-old NOD mice were used as controls. [³H]thymidine ([³H]Tdr) (0.5 μCi per well) (PerkinElmer Life Analytical Science, Boston, MA, USA) was added to the plate 16 h before cell harvesting. Uptake of [³H]Tdr was measured by using Liquid Scintillation Counter (MicroBeta, PerkinElmer Wallac, Québec, Canada).

(2) For co-culture assays, MT-5B cells, MT-5B SN, anti-GrB/perforin-secreting splenocytes from acutely diabetic NOD mice were pre-incubated with MT-5B SN, control SN (1:20), GrB+NOD mice. In some experiments, diabetogenic splenocytes cose levels (>300 mg dl⁻¹) with a GlucosCan 2000 blood glucose monitor (Lifescan, Mountain View, CA, USA). Adaptive transfer of diabetes

In adoptive transfer of diabetes experiment, 12 × 10⁶ diabeticogenic splenocytes from acutely diabetic NOD mice were transferred or co-transferred (intravenously) with 6 × 10⁶ MT-5B cells or control NOD T cells into pre-irradiated (800 rads) NOD mice. In some experiments, diabeticogenic splenocytes were pre-incubated with MT-5B SN, control SN (1:20), GrB antibody or control IgG-adsorbed MT-5B SN.C (1:100) at cell concentration of 2 × 10⁶ cells ml⁻¹ for 36 h before transferred into NOD.SCID recipient mouse. Urinary glucose was monitored every other day beginning at 14 days after adoptive transfer with test strips (Miles Canada Inc., Rexdale, Ontario, Canada), and verified for diabetes by examining blood glucose levels (>300 mg dl⁻¹) with a GlucosCan 2000 blood glucose monitor (Lifescan, Mountain View, CA, USA).

Protein extraction, gel filtration chromatography and mass spectral analysis

MT-5B SN.C (1 ml) was run on a 12% polyacrylamide preparation gel under non-reducing conditions. Proteins were extracted from excised slices with 3 ml buffer (0.05 M Tris-HCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1 mg ml⁻¹ BSA and 0.15 M NaCl), and precipitated by adding four volumes of acetone (−20°C) in siliconized Corex tube for 30 min. After centrifugation for 10 min at 10 000 rpm, the protein pellet was rinsed with ice-cold 80% acetone: 20% buffer and reconstituted with 1 ml PBS. For gel filtration, SN.C (0.5 ml) was loaded on 10 × 250 mm Sephacryl S-100 column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). Proteins were eluted with 0.05 M sodium phosphate buffer, pH 7.0 at a flow rate of 0.5 ml per tube min⁻¹ and monitored by spectrophotometry (Spectropec III, LKB Biotechnology AB, Uppsala, Sweden). Each tube of eluates was tested for the ability to suppress a Con A-induced proliferative response. Selected tubes with or without suppressive activity were run on 12% polyacrylamide gels under reducing conditions. Protein bands corresponding to the suppressive activity in SN.C were excised and subjected to mass spectral analysis following in-gel-tryptic digestion at the University of Western Ontario Biological Mass Spectrometry Laboratory using MicroMass ESI-Q-TOF machine.

Analysis of cell-surface markers, intracellular molecules and apoptosis by flow cytometry

For surface staining, cells were directly or indirectly stained with mAbs at 4°C for 30 min. For intracellular staining, cells or surface-stained cells were fixed in 2% formaldehyde–PBS for 15 min at room temperature (RT). After washing with 2% BSA-PBS washing buffer and 0.5% saponin-washing buffer, cells were permeabilized and stained with PE-conjugated antibodies or biotin–avidin system in 0.5% saponin-containing buffer at RT for 30 min. For PI staining, cells were collected from the cultures, washed and stained with 50 μg ml⁻¹ of PI in 0.1% Triton-X 100, 0.1% sodium citrate–PBS. TUNEL staining was performed following manufacturer’s instructions. Briefly, surface-stained cells were fixed and permeabilized with 0.1% sodium citrate, 0.1% Triton-X 100–PBS. Cells were finally incubated with TUNEL–FITC at 37°C for 60 min. In each step, cells were washed twice with washing buffer. Samples were analyzed with a FACScan Flow Cytometer (BD, Sunnyvale, CA, USA) using CELLQuest II software. Ten thousand events were acquired per sample.

Light and confocal microscopy

MT-5B cells were settled on a glass slide and air dried. Cells were stained with Wright–Giemsa (Sigma) following manufacturer’s instructions. Cells were examined using light microscopy (1000×) (Carl Zeiss Inc., Thornwood, NY, USA). For confocal analysis, cells were stained as previously described for intracellular staining prior to flow cytometric analysis. Subsequently, cells were washed twice in PBS and incubated at RT on polyclonal-lysine-coated glass coverslips (Sigma–Aldrich Canada Ltd., Oakville, Canada) for 10 min. Coverslips were directly mounted on glass slides using Vectorshield (Vector Laboratories Inc., Burlingame, CA, USA) and analyzed using a confocal laser scanning microscope. Confocal microscopy was performed using an Axiovert 100M inverted microscope equipped with a LSM 510 laser scanning unit and a 63X/1.40 NA plan Apochromat objective (Carl Zeiss Inc.). For dual analyses, green fluorescence was detected at >515 nm after
ELISA
A sandwich ELISA was performed to detect GrB in SN and LS (5). Briefly, ELISA plates were coated with capture antibody (0.5 to 1 μg ml⁻¹) in NaHCO₃ buffer, pH 8.6 at 4°C overnight. After saturation with 0.25% Tween–PBS buffer, 50 μl samples were added to each well. The specific binding was detected by a standard antibody–biotin and avidin–AKP system. In each step, the plates were washed with 0.05% Tween–PBS buffer and incubated at 4°C for 3 h. Reactions were developed with phosphatase substrate (p-nitrophenyl phosphate disodium, Sigma). Optical densities were measured at 405 nm by ELISA reader (Bio-Rad, Hercules, CA, USA). The detectable level of GrB was 3–6 ng ml⁻¹.

Western blot
LS, SN.C (10 μl per lane), purified GrB (100 ng per lane), anti-GrB antibody-adsorbed SN.C or GrB adsorbed on beads were run on 12% polyacrylamide gels under reducing conditions. The gel-transferred Immobilon-P Transfer Membrane (Millipore Corporation, MA, USA) was saturated with 0.5% gelatin in Tris buffer–0.05% Tween 20 at RT for 1 h or at 4°C overnight. Membranes were sequentially incubated with biotin–antibodies to GrB or PFN and avidin–AKP (1:1500) for 1 h each at RT. Rinsing and washing in Tris-buffered saline (TBS)–0.05% Tween 20 buffer were performed after each step. Finally, membranes were incubated in nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate chromatin substrate-AKP buffer until the desired levels of color were observed. The reaction was stopped by incubation of membrane with 2 mM EDTA–PBS solution.

Immunoadsorption assay
Two to four micrograms of goat anti-mouse GrB polyclonal antibody, rat anti-mouse PFN mAb or corresponding isotype controls was bound to 30–50 μl of packed GammaBind™ G-Sepharose™ beads (Amersham Biosciences AB, Uppsala, Sweden) in reaction tube with cap (0.2 ml) by incubation in 100 μl of TBS-0.1% BSA buffer, pH 7.5 at 4°C overnight. Beads were washed with buffer and incubated with SN.C, LS (50 to 100 μl) or 0.5 μg of purified GrB and diluted to 100 or 200 μl with buffer. The mixtures were incubated at 4°C for 8 h with rotation. Following centrifugation, antibody-adsorbed SN.C were analyzed by western blotting, tested for suppression of T cell proliferation response or incubated with diabetogenic splenocytes before adoptive transfer of diabetes. The beads were extensively washed with TBS buffer and the immune complexes were eluted from the beads in reducing sample buffer, with ~20 μl of eluate applied to each lane of a 12% polyacrylamide gel. Western blot and/or coomassie blue and silver staining were used for detecting PFN and/or GrB.

Statistical analysis
For statistical analysis, Student’s t-test, two-way analysis of variance (Student–Newman–Keuls) and Kaplan–Meier life survival curves were used. For all analyses, a P-value < 0.05 was considered statistically significant.
suppression is not attributed to the cytokines secreted by these cells. Our results clearly indicate that suppressive effects induced by MT-5B cells are cell contact independent, and mediated by some unidentified proteins.

Identification of GrB in SN from MT-5B cell culture by proteomic analysis

Given the suppressive effects of SN from MT-5B cells, we further investigated the proteins in SN.C by proteomic analysis in two ways. Firstly, proteins in the SN.C were separated by PAGE under non-reducing conditions. Bands designated B1, B2, B3 and a control band B4 were eluted and precipitated by acetone extraction (Fig. 4A) and tested for their suppressive activities (Fig. 4B). We found that band B2 with a molecular weight (MW) of 65 kDa had maximal activity. Secondly, proteins in the SN.C were separated by Sephacryl S-100 gel filtration and various fractions were tested for suppressive activity (Fig. 4C) and run on a 12% polyacrylamide gel under reducing conditions (Fig. 4D). The profiles of bands from fraction 6 (T6) through 13 (T13) of eluates with or without suppressive activity were assessed. Bands (90, 34 and 30 kDa) shown in fractions T8, T9 and T10 that correlated with suppressive activity were further analyzed by mass spectrometric

**Fig. 1.** Phenotypic analysis of clone MT-5B. MT-5B cells were stained directly or indirectly with a panel of labeled antibodies and analyzed by flow cytometry as described in Methods. As shown in overlap histogram plots, cells stained with antibody and isotype control are expressed in solid and dotted lines, respectively. Dot plot shows the double staining of FITC–CD4 and PE–CD8α. Data are representative of at least three independent experiments showing similar results.
Two peptides derived from the MW 34-kDa band were 100% identical to mouse GrB (GeneStream align program). These are peptide 157–170 (YSNTLQEVELTVQK) and peptide 234–242 (VSSFLSWIK). We further confirmed the presence of GrB in the SN of clone MT-5B by using anti-GrB antibody in western blot and ELISA. Western blot analysis showed two bands with MW 32 and 34 kDa in both SN.C and LS that are identical to the mouse rGrB bands (Fig. 4E). The expressions of intracellular and extracellular GrB in LS and SN were also demonstrated in ELISA. Esterase activity of GrB in various preparations was also confirmed by enzymatic assays as previously described (38). Since both GrB and PFN are exported from the granules and work synergistically to kill target cells, we were interested in finding out if they were co-secreted by MT-5B cells. PFN was detected in both SN.C and LS as a 65-kDa band by western blot (Fig. 4E). As shown in Fig. 4(F), the amount of GrB in SN, SN.C and LS from different preparations was quantified by ELISA. The median levels of GrB were 230, 1682 and 865 ng ml\(^{-1}\), respectively.

**Morphological and intracellular GrB/PFN analysis by light and confocal microscopy and flow cytometry**

MT-5B cells were stained with Wright–Giemsa for morphological analysis (Fig. 5A). Clone MT-5B is characterized by its ‘blast like’ larger size, open and clumped nuclear chromatin pattern and abundant and polarized cytoplasmic granules. The cellular membranes appeared more distinct at the site of the polarized granules, which may indicate the exocytosis site of the granules. To determine the intracellular localization of GrB and PFN, MT-5B cells were stained as described in Methods and subjected to confocal microscopy. It was observed that both GrB and PFN are localized in the cells to well-defined sub-cellular vesicles. The overlay images showed coincident staining (yellow in color) specifying co-localization of the green signal from the FITC-labeled GrB and that of the red signal from the PE-labeled PFN antibodies (Fig. 5B). The intracellular co-expressions of GrB and PFN in MT-5B cells were also demonstrated by flow cytometric analysis as shown in Fig. 5(C).

Both GrB and PFN are needed for the suppression induced by MT-5B SN

The roles of GrB and PFN in MT-5B-induced immune suppression were further investigated by depletion assays. GrB or PFN from SN.C and LS was adsorbed by corresponding antibody-bound beads and eluted from these beads. Eluates were denatured and run on a 12% polyacrylamide gel (Fig. 6A). Compared with IgG isotype controls, the specific
adsorptions of GrB and PFN from SN.C or LS were clearly shown in bands with MW 32, 34 and 65 kDa, respectively. The amount of GrB adsorbed on beads was higher in SN.C than in LS. The amount of GrB was largely reduced in SN.C after immunoadsorption with antibody-bound beads compared with that with control goat IgG-bound beads (Fig. 6B), which resulted in the decreased suppressive activity of SN.C correspondingly (Fig. 6C). Removal of both GrB and PFN by immunoadsorption did not have an additive effect (data not shown). Removal of either GrB and/or PFN by immunoadsorption decreased the suppressive activity of SN.C and LS, which indicates that MT-5B-induced suppression is mainly mediated by the secretion of GrB/PFN, and is PFN dependent. This is also indirectly supported by the data in Fig. 4(A) and (B). The maximal activity was found in band B2 (65 kDa) which indicates the composition of both PFN and GrB dimmer under non-reducing condition. Band B3 (34 kDa) which only contained monomeric GrB had no suppressive activity. We also found that purified recombinant mouse GrB alone had no suppressive effect (data not shown).

Fig. 3. Immunosuppression induced by clone MT-5B is cell contact-independent. SN from MT-5B cells suppressed both Con A response (A) and MLR (B) at indicated concentrations. Cell contact-independent suppression was also verified by transwell chamber in which MT-5B cells were separated from the responder cells in cultures at regulator to responder ratio of 1:20 (C). The proteinaceous nature of suppressive component in MT-5B SN was indicated by the loss of activity after pre-incubation of MT-5B SN with trypsin (120 μg ml⁻¹) at 37°C for 30 min (D). The role of cytokines in clone MT-5B-induced suppression was evaluated in Con A response and MLR (two way) by the blocking assays with neutralizing mAbs to IFN-γ, TNF-α, TGF-β or IL-10 (20 μg ml⁻¹) in the absence or presence of MT-5B cells (regulator to responder ratio of 1:30) or MT-5B SN (1:30) (E). The normal levels of Con A response and MLR are shown as solid bars. Isotype controls had no significant effect (data not shown). Results are expressed as mean CPM ± SD of triplicates and are representative of at least three experiments. The dotted line represents the level of Con A response or MLR without modification.
MT-5B cell-induced suppression is sensitive to EGTA pre-treatment

We have demonstrated that MT-5B cell-induced suppression is cell contact-independent and mediated by the secretion of GrB and PFN. EGTA has been shown to inhibit calcium-dependent exocytosis and release of granules and the action of PFN by blocking polymerization of, and pore formation by PFN (39, 40). To further unravel the relationship between GrB and PFN in MT-5B cell or MT-5B SN-induced suppression, MT-5B cells were pre-incubated in the presence of EGTA at different concentrations for different durations. After incubation, MT-5B cells were washed and incubated in medium for another 6 h. MT-5B cells or SN alone and MT-5B cells plus SN were analyzed separately for suppressive activity. As shown in Fig. 7, the suppressive activities of MT-5B cells and SN were significantly blocked by pre-incubation of MT-5B cells with EGTA in a dose-dependent manner after all three durations of incubation. Thus, the suppressive effects induced by

---

MT-5B cell-induced suppression is sensitive to EGTA pre-treatment

We have demonstrated that MT-5B cell-induced suppression is cell contact-independent and mediated by the secretion of GrB and PFN. EGTA has been shown to inhibit calcium-dependent exocytosis and release of granules and the action of PFN by blocking polymerization of, and pore formation by PFN (39, 40). To further unravel the relationship between GrB and PFN in MT-5B cell or MT-5B SN-induced suppression, MT-5B cells were pre-incubated in the presence of EGTA at different concentrations for different durations. After incubation, MT-5B cells were washed and incubated in medium for another 6 h. MT-5B cells or SN alone and MT-5B cells plus SN were analyzed separately for suppressive activity. As shown in Fig. 7, the suppressive activities of MT-5B cells and SN were significantly blocked by pre-incubation of MT-5B cells with EGTA in a dose-dependent manner after all three durations of incubation. Thus, the suppressive effects induced by
MT-5B cells are sensitive to EGTA pre-treatment and cumulative due to the consistent secretion of GrB and PFN in the cultures.

**GrB-dependent down-regulation of diabetogenic effector T cells by MT-5B cells through inducing apoptosis**

The suppressive effect of MT-5B cells was also verified in adoptive transfer of diabetes assay. MT-5B cells significantly blocked the disease transfer when co-transferred with splenocytes from acutely diabetic NOD mice to recipient NOD mice (Fig. 8A). The GrB/PFN pathway is one of the mechanisms underlying the induction of apoptosis (32). Given the presence of both GrB and PFN in SN from MT-5B cells, SN-induced suppression of immune responses was also investigated by pre-incubating diabetogenic splenocytes with MT-5B SN (1:20) or GrB antibody-adsorbed SN.C (1:100) for 36 h before cell transfer. As shown in Fig. 8(A), incubation with MT-5B SN significantly impairs the ability of diabetogenic splenocytes to transfer diabetes. Removal of GrB from SN.C by immunoadsorption damages the ability of SN.C to down-regulate diabetogenic T cells. To assess the mechanism underlying the impairment of diabetogenic T cells, we looked for apoptotic cells after incubation with MT-5B SN using flow cytometry (Fig. 8B). Significantly higher proportions of PI-positive splenocytes and TUNEL-positive CD4⁺ and CD8⁺ T cells were found in cultures incubated with SN from MT-5B than that from NOD T cells, \( P = 0.003 \) for PI-positive cells and \( P = 0.045 \) and 0.011 for TUNEL-positive CD4⁺/CD8⁺ T cells, respectively. Results from both in vitro and in vivo studies confirm that clone MT-5B established from CFA-protected NOD mice down-regulates diabetogenic T cells by inducing apoptosis in a GrB/PFN-dependent fashion.

**CFA immunization up-regulates the expression of GrB in CD4 T cells**

Up-regulation of GrB expression and secretion have been shown in T cells after viral or bacterial infection (41, 42). Since clone MT-5B was established from NOD mice immunized with CFA, a *Mycobacterium tuberculosis*-containing adjuvant, the change of GrB expression in CD4 T cells from freshly prepared or BCG-incubated draining lymph node cells was assessed 12 and 35 days after CFA immunization. As shown in Fig. 9, GrB expression in draining lymph node CD4 T cells was increased by in vivo injection of CFA or and in vitro stimulation of draining lymph node cells with SN of BCG suspension. The significant increases of these cells were found in all groups of cells from

---

**Fig. 5.** Wright–Giemsa staining, confocal and flow cytometric analysis of clone MT-5B. (A) MT-5B cells were stained with Wright–Giemsa on glass slide and examined by a light microscope (1000×). The larger size, abundant cytoplasm, polarized granules and open/clumped chromatin are the features of MT-5B. (B) Intracellular GrB (green), PFN (red) staining and co-localization staining (yellow) were shown by confocal microscopic analysis. (C) Intracellular expressions of GrB/PFN were also shown in histogram plots and dot plots by flow cytometric analysis.
CFA-treated mice except for the fresh cells of 35-day group, and in BCG-incubated cells from all groups except for the incomplete Freund’s adjuvant-treated mice of 12-day group. It seems that immunization with *Mycobacterium* preparation increases the expression of GrB in CD4 T cells both in *vivo* and *in vitro*.

**Discussion**

In this study, we report the morphology, function and mechanism of action of a unique CD4⁺CD8⁺ CD25⁺ Treg cell clone designated as MT-5B. This clone was derived from the lymph node cells of NOD mice immunized with CFA. Immunization of young NOD mice with CFA protects these mice...
also found in CD4+ T cells derived from subjects with chronic (29, 30, 43). The expression of PFN, GrB or granulysin was lymphokine-activated cells exert their cytotoxic effects by mice develop a CD25+ DP T cell population which controls groups and expressed as mean suppression (%) + SEM from three using 24-h splenocyte Con A blasts as responder at 1:20 ratio of SN from the cultures were then tested separately in a 3-day assay complete medium. MT-5B cells or SN (1:3) alone and MT-5B cells plus SN from these cells fail to induce cytotoxicity against target cells (29). We propose that this difference may be due to the maturation state and/or activation state of various Treg cell subsets, otherwise clone MT-5B may represent a new subset of Treg cells.

It has been shown that the activation state of target cells appears to determine their susceptibility to the cytotoxic killing. NK cells kill activated but not resting T cells (56), PFN-expressing T cells kill autoreactive T cells (57), Autologous activated CD4+ and CD8+ T cells are preferentially killed over unactivated T cells (29). CD4+CD25+ Treg cells may control the T cell activation state (58). Based on our observation, GrB/PFN-secreting clone MT-5B exerts similar effect on activated T cells. It seems that the degree of suppression is dependent on the activation state of target cells as shown in Con A response and MLR assays. The mechanism underlying the differential sensitivity of activated and resting T cells to GrB/PFN-mediated suppression has likely evolved to avoid bystander suppressions. In transplantation, CD4+CD25+ Treg cells prevent graft rejection, but this does not compromise anti-virus immunity (59). Therefore, there is no global immunosuppression by Treg cells.

Our perspective is that aberrantly activated cells may cause intrinsic changes such as decrease of serpin protease inhibitor (SPI-6) which makes them sensitive to GrB/PFN-mediated killing and maintains immune homeostasis. Chronic infections, especially intracellular infections, have been shown to increase the proportion of GrA/PFN-expressing T cells which eliminate the pathogen-infected cells (44, 55). The important roles of GrB/PFN in autoimmune disease have been demonstrated in autoimmune encephalomyelitis by using PFN-knockout from T1D (37). MT-5B is characterized by a blast-like morphology with a distinct phenotype. Our results show that MT-5B is capable of suppressing a variety of immune responses by secreting GrB/PFN in a cell contact-independent manner. This clone shares the characteristics of both adaptive and natural CD4+CD25+ Treg cells. It expresses high levels of GrB/PFN but low level of CD3. MT-5B cells showed anergic proliferative response to a panel of immune stimulations. The effector molecule involved in the regulatory function of CD4+CD25+ Treg cells remains unclear, but based on recent studies in human cells and our present study in mouse cells, GrB/PFN pathway appears to be at least one of the suppressive mechanisms underlying the function of CD4+CD25+ Treg cells (29).

It has previously been shown that NK cells, CTLs and lymphokine-activated cells exert their cytotoxic effects by inducing target cell apoptosis through the GrB/PFN pathway (29, 30, 43). The expression of PFN, GrB or granulysin was also found in CD4+ T cells derived from subjects with chronic viral infections, tumors and autoimmune diseases (44–47) or in vitro-activated Tr1 cells (48). GrB and/or PFN-expressing DP T cells were found in human PBL stimulated with human cytomegalovirus (HCMV) and HIV-1 antigens and in peripheral blood lymphocytes (PBL) of normal cynomolgus monkeys (49, 50). DP T cells have also been found in PBL of rodents, swine, monkeys, chickens and humans both in healthy and diseased states (51, 52). CD8α is an activation marker for the subset of peripheral CD4+ T cells (53). MHC class II-deficient mice develop a CD25+ DP T cell population which controls colitogenic CD4+CD25− T cells (54). Their involvement in the adaptive immune responses against infectious pathogens has increased our understanding of the role of T cells in the antiviral immune response (55). Small but increased proportion of DP T cells have been found in peripheral lymphoid tissue of CFA-immunized NOD mice (data not shown). It is uncertain whether the DP phenotype of clone MT-5B is related to the antimycobacterium immunity induced after CFA immunization, but it does prevent the adoptive transfer of T1D to NOD recipients.

Cell contact-dependent killing is a unique feature of natural CD4+CD25+ Treg cells and GrB/PFN-expressing CD4+CD25+ Treg cells (3, 29). In this study, we have clearly demonstrated that clone MT-5B suppresses immune responses in a cell contact-independent manner. The suppression is not primarily mediated by the secretion of suppressive cytokines, as shown by the failure of neutralizing antibodies to block the suppression, but by the secretion of GrB/PFN. The detectable suppressive effects in both SN and LS indicate that GrB/PFN produced by clone MT-5B is stored as well as secreted via granules.

The antigen specificity of CD4+CD25+ Treg cell-mediated suppression is a matter of debate. It has been shown that the activation of CD4+CD25+ Treg cells can be driven by antigen stimulation or via their TCR cross-linking, but the CD4+CD25+ T effector cells are probably not antigen specific (7, 5). It remains unclear why the mechanism of action of Treg cells is cell contact-dependent but their effector function (suppression) is antigen non-specific. The mode of action of clone MT-5B is quite different from other CD4+CD25+ Treg cells in terms of cell–cell contact. Clone MT-5B is probably an activated and fully mature memory T cell population with effector function, and is programmed to produce and secrete GrB/PFN which induces non-specific suppression without cell–cell contact. The other CD4+CD25+ Treg cells or GrB/PFN-expressing CD4+CD25+ Treg cells require target cell contact, and SN from these cells fail to induce cytotoxicity against target cells (29).

The antigen specificity of CD4+CD25+ Treg cell-mediated suppression is a matter of debate. It has been shown that the activation of CD4+CD25+ Treg cells can be driven by antigen stimulation or via their TCR cross-linking, but the CD4+CD25+ T effector cells are probably not antigen specific (7, 5). It remains unclear why the mechanism of action of Treg cells is cell contact-dependent but their effector function (suppression) is antigen non-specific. The mode of action of clone MT-5B is quite different from other CD4+CD25+ Treg cells in terms of cell–cell contact. Clone MT-5B is probably an activated and fully mature memory T cell population with effector function, and is programmed to produce and secrete GrB/PFN which induces non-specific suppression without cell–cell contact. The other CD4+CD25+ Treg cells or GrB/PFN-expressing CD4+CD25+ Treg cells require target cell contact, and SN from these cells fail to induce cytotoxicity against target cells (29). We propose that this difference may be due to the maturation state and/or activation state of various Treg cell subsets, otherwise clone MT-5B may represent a new subset of Treg cells.

It has been shown that the activation state of target cells appears to determine their susceptibility to the cytotoxic killing. NK cells kill activated but not resting T cells (56), PFN-expressing T cells kill autoreactive T cells (57). Autologous activated CD4+ and CD8+ T cells are preferentially killed over unactivated T cells (29). CD4+CD25+ Treg cells may control the T cell activation state (58). Based on our observation, GrB/PFN-secreting clone MT-5B exerts similar effect on activated T cells. It seems that the degree of suppression is dependent on the activation state of target cells as shown in Con A response and MLR assays. The mechanism underlying the differential sensitivity of activated and resting T cells to GrB/PFN-mediated suppression has likely evolved to avoid bystander suppressions. In transplantation, CD4+CD25+ Treg cells prevent graft rejection, but this does not compromise anti-virus immunity (59). Therefore, there is no global immunosuppression by Treg cells.

Our perspective is that aberrantly activated cells may cause intrinsic changes such as decrease of serpin protease inhibitor (SPI-6) which makes them sensitive to GrB/PFN-mediated killing and maintains immune homeostasis. Chronic infections, especially intracellular infections, have been shown to increase the proportion of GrA/PFN-expressing T cells which eliminate the pathogen-infected cells (44, 55). The important roles of GrB/PFN in autoimmune disease have been demonstrated in autoimmune encephalomyelitis by using PFN-knockout
mice (60). Previously, we have shown that BCG down-regulates the activity of diabetogenic T cells by inducing apoptosis through both Fas/FasL and TNF-α pathways (61). However, clone MT-5B may represent another kind of Treg cells under a certain activation stage, and exert its effector function mainly by using GrB/PFN pathway. GrB-induced cytotoxicity against target cells has been shown to be PFN-dependent (29, 43) but the precise role and acting mode of PFN in GrB cell-surface binding, internalization, intracellular trafficking and targeting needs to be further elucidated. To verify the requirement of PFN in GrB secretion and GrB-induced suppression, two experiments were performed in this study: One is removal of GrB or PFN from SN by immunoadsorption. The suppressive activity is significantly decreased but not completely removed after immunoadsorption. Another is the pre-incubation of MT-5B cells with EGTA, a Ca²⁺ chelator. The decreased suppressive effect of EGTA-pre-incubated MT-5B cells or its SN was significant. It suggests that GrB/PFN is constitutively secreted and the suppressive activity is accumulated in SN. It also indicates that decreased suppression after EGTA pre-incubation mainly results from the changes in secretion and function of PFN, and
that cell contact-independent secretion of GrB by clone MT-5B is dependent on PFN for its function. The requirement of both GrB and PFN for triggering in vitro apoptosis has been confirmed by other studies (32, 62), but the opposite conclusion was also reported in PFN-knockout mice (30).

In this study, the establishment of a cell contact-independent DP CD25+ Treg clone raises an interesting issue regarding the mechanism of action of CD4+CD25+ Treg cells and their application for immune interventions. We propose that the cell contact-independent suppression by soluble GrB/PFN is a major mechanism for the highly activated and mature memory CD4+CD25+ Treg cells. GrB has been shown to be exocytosed as a complex with proteoglycans and PFN plays a key role in its effector mechanism (35). The restricted targeting of activated cell populations makes these Treg cells superior in suppress-
References


