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Murine Asialo GM1$^+$CD8$^+$ T Cells as Novel Interleukin-12-responsive Killer T Cell Precursors

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Freshly isolated CD8$^+$ T cells, but not CD4$^+$ T cells, contained 20–30% of asialo GM1$^+$ (ASGM1$^+$) T cells which were distinct from ASGM1$^+$NK1.1$^+$ natural killer cells. This novel ASGM1$^+$CD8$^+$ T cell subpopulation showed a strong proliferative response to interleukin-12 (IL-12) in the presence of IL-2. Culture of ASGM1$^+$CD8$^+$ T cells with IL-12 plus IL-2 allowed the generation of anomalous killer T cells concomitantly with the accumulation of cytolytic molecules. Moreover, ASGM1$^+$CD8$^+$ T cells produced high levels of interferon-γ (IFN-γ), but not IL-4, upon stimulation with IL-12 plus IL-2. Such immune responses were not observed in ASGM1$^-$CD8$^+$ T cell subpopulations constituting the majority of CD8$^+$ T cells. These results demonstrated that ASGM1$^+$CD8$^+$ T cells are a novel subpopulation of IL-12-responsive and IFN-γ-producing killer T cell precursors.

Key words: IL-12 — Asialo GM1 — CD8$^+$ T cell — Killer T cell precursor — IFN-γ

Recent work has shown that IL[4]-12 plays a pivotal role in immunoregulation both *in vitro* and *in vivo*.[1,2] One of the important functions of IL-12 is to stimulate Th1-mediated immunity, which facilitates the induction of antitumor immunity *in vivo*.[3] Indeed, *in vivo* administration of IL-12 into tumor-bearing mice caused a marked inhibition of both transplantable and primary tumor cells through the activation of IFN-γ production, NK cell activity and CTL activity.[4–6] To evaluate the mechanisms underlying IL-12-induced antitumor immunity, characterization of the IL-12-responsive cells is important. It has been reported that antigen-stimulated CD4$^+$ Th1 cells, CD8$^+$ T cells or NK cells could respond to IL-12 to produce IFN-γ.[7] However, because of the absence of antibody against IL-12 receptor,[8] the detailed characterization of IL-12-responsive cells has not been conducted yet. Therefore, it is of great interest to find antibodies able to subdivide functionally different mouse CD8$^+$ T or CD4$^+$ T cells in terms of IL-12 responsiveness.

C57BL/6 mice were purchased from Charles River Japan Inc. (Yokohama). All animals were female and used at 5–8 weeks of age. ASGM1$^+$CD8$^+$ T cells and ASGM1$^-$CD8$^+$ T cells were separated from nylon-wool-passaged C57BL/6 mouse spleen T cells using a FACStar (Becton Dickinson, Mountain View, CA) after dual staining with anti-ASGM1 Ab (Wako Chemical, Tokyo) plus FITC-conjugated anti-rabbit Ab (Wako Chemical) and PE-conjugated anti-CD8 mAb. The isolated CD8$^+$ T cells, ASGM1$^+$CD8$^+$ T cells or ASGM1$^-$CD8$^+$ T cells were cultured with IL-2 alone (20 U/ml, kind gift of Shionogi Pharmaceutical Co., Ltd., Osaka), IL-12 alone (20 U/ml, kind gift from Genetic Institute Inc., Cambridge, MA) or IL-12 plus IL-2 for 24–48 h at 37°C using 48-well culture plates at the cell density of 5×10^5/well. The proliferation of the cells was determined by pulsing with ^{3}H-TdR (37 kBq/well) for 4 h. The cytotoxicity was determined by 4-h ^{51}Cr-release assay as described previously.[9] The total cellular content of SE activity was determined using N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester and Ellman's reagent to quantitate the released sulfhydryl product by measuring the absorbance at 405 nm in a plate reader.[9] The IFN-γ activity was determined using The Endogen IFN-γ ELISA kit (Endogen, Cambridge, MA) and IL-4 activity was determined using The Endogen IL-4 ELISA kit (Endogen).

Flow cytometric analyses of ASGM1 antigen expression on freshly isolated C57BL/6 mouse spleen cells demonstrated that the minority (20–30%) of CD8$^+$ T cells expressed ASGM1 antigen on their cell surface (Fig. 1A). Although most NK1.1$^+$ NK cells expressed ASGM1 antigen (Fig. 1D), CD4$^+$ T cells showed no significant ASGM1 antigen expression (Fig. 1B). The unique ASGM1$^+$CD8$^+$ T cell subpopulation appeared to be distinct from ASGM1$^+$NK1.1$^+$ NK cells, because the percentage of NK1.1$^+$CD8$^+$ T cells (0.51%) is negligible (Fig. 1C) and is inconsistent with that of ASGM1$^+$CD8$^+$ T cells (10.38%).

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[4] Abbreviations: IL, interleukin; IFN, interferon; NK, natural killer; CTL, cytotoxic T lymphocyte; ASGM1, asialo GM1; mAb, monoclonal antibody; FITC, fluorescence isothiocyanate; PE, phycoerythrin; SE serine esterase; ELISA, enzyme-linked immunosorbent assay.

To determine the functional differences between ASGM1$^+$CD8$^+$ T cells and ASGM1$^-$CD8$^+$ T cells, each cell population was cultured with IL-2 alone (20 U/ml), IL-12 alone (20 U/ml) or both and then their capability of proliferation, killer cell generation and cytokine production was determined after culture for 48 h. As shown in Fig. 2, only ASGM1$^+$CD8$^+$ T cells, but not ASGM1$^-$CD8$^+$ T cells, showed marked proliferative responses when they were cultured with IL-12 plus IL-2. Such proliferative responses were not seen in other cultures. Consistent with this, anomalous killer cells, which lysed both NK-sensitive YAC-1 and NK-resistant RDM-4 and MBL-2 cells, were also induced only from ASGM1$^+$CD8$^+$ T cells, but not from ASGM1$^-$CD8$^+$ T cells by culture with IL-12 plus IL-2 (Fig. 3). The augmentation of killer cell generation appeared to be due to enhanced induction of cytolytic molecules, because culture of ASGM1$^+$CD8$^+$ T cells with IL-12 plus IL-2

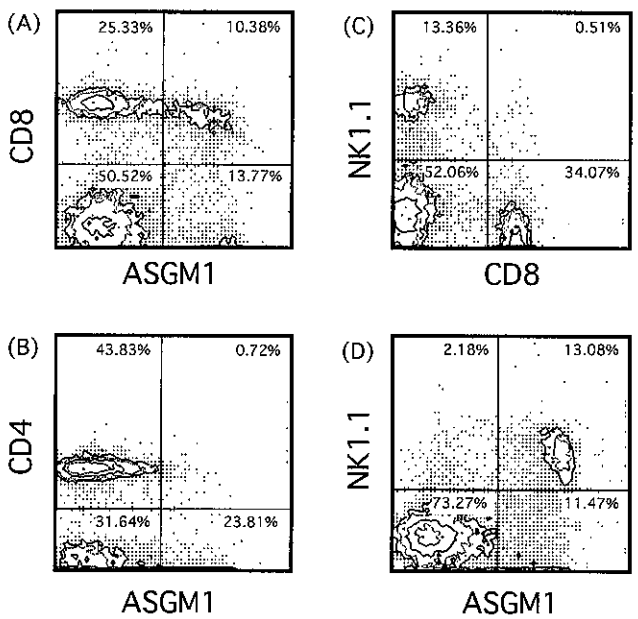

Fig. 1. Existence of ASGM1$^+$CD8$^+$ T cells in freshly isolated mouse T cells. Freshly isolated C57BL/6 mouse spleen cells were passed through a nylon-wool column and then their expression profile of CD8/ASGM1 (A), CD4/ASGM1 (B), NK1.1/CD8 (C) or NK1.1/ASGM1 (D) was determined by using FACScan after dual staining with PE-conjugated anti-CD8 mAb and anti-ASGM1 Ab plus FITC-conjugated anti-rabbit IgG. The method for flow cytometry analyses was described in a previous paper.[10] The percentage of positive cells is indicated in the figures.

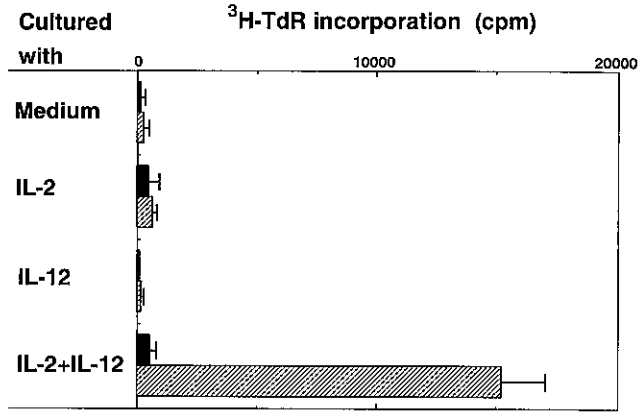

Fig. 2. ASGM1$^+$CD8$^+$ T cells, but not ASGM1$^-$CD8$^+$ T cells, respond to IL-12 plus IL-2 stimulation. ASGM1$^+$CD8$^+$ T cells (hatched bars) and ASGM1$^-$CD8$^+$ T cells (solid bars) were separated by using FACStar. Then, their proliferative response to IL-2 (20 U/ml), IL-12 (20 U/ml) or IL-12 plus IL-2 was determined after culture for 48 h. The proliferation of the cells was determined by pulsing them with [^{3}H]TdR (37 kBq) for 4 h at 48 h after initiation of culture. The data represent mean±SE of triplicate samples.

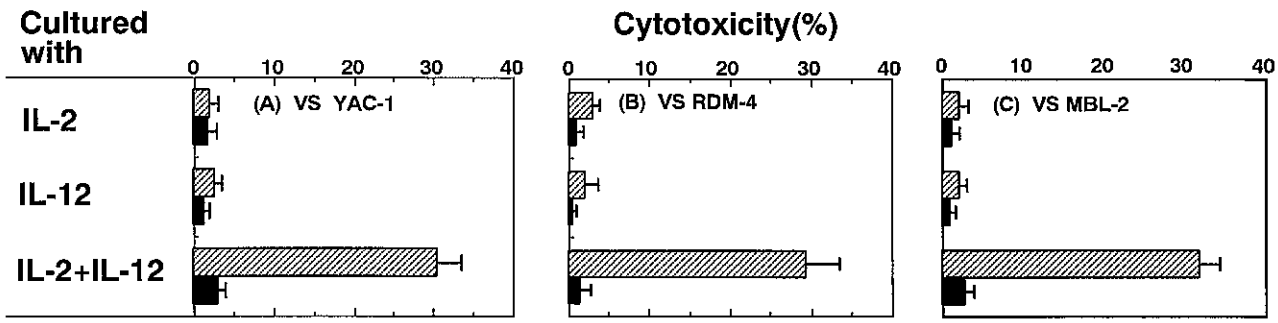

Fig. 3. Generation of anomalous killer T cells from ASGM1$^+$CD8$^+$ T cells by culture with IL-12 plus IL-2. ASGM1$^+$CD8$^+$ T cells (hatched bars) and ASGM1$^-$CD8$^+$ T cells (solid bars) were separated by FACStar. Then, they were cultured with IL-2 (20 U/ml), IL-12 (20 U/ml) or IL-12 plus IL-2 for 48 h. After culture, their cytotoxicity to YAC-1, RDM-4 or MBL-2 was measured by 4-h ^{51}Cr-release assay. The data represent mean±SE of triplicate samples.

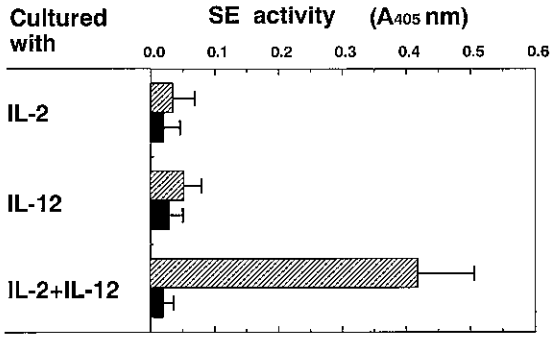

Fig. 4. Induction of serine esterase accumulation in ASGM1$^+$CD8$^+$ T cells by IL-12 plus IL-2. ASGM1$^+$CD8$^+$ T cells (hatched bars) and ASGM1$^-$CD8$^+$ T cells (solid bars) were separated by FACStar. Then, they were cultured with IL-2 (20 U/ml), IL-12 (20 U/ml) or IL-12 plus IL-2 for 48 h. After culture, their total cellular serine esterase activities were measured using N-α-benzyloxycarbonyl-L-lysine thiobenzyl ester and Ellman's reagent. The activity was evaluated in terms of absorbance at 405 nm.

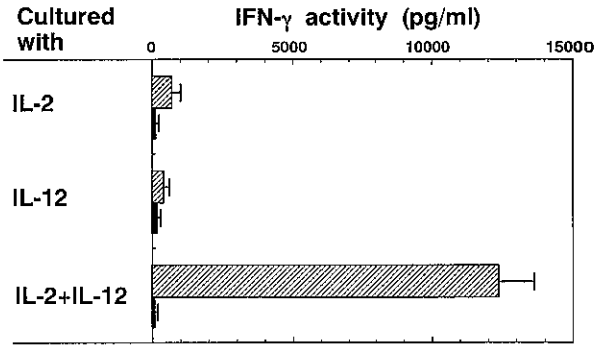

Fig. 5. IFN-γ production of ASGM1$^+$CD8$^+$ T cells upon stimulation with IL-12 plus IL-2. ASGM1$^+$CD8$^+$ T cells (hatched bars) and ASGM1$^-$CD8$^+$ T cells (solid bars) were separated by FACStar. Then, they were cultured with IL-2 (20 U/ml), IL-12 (20 U/ml) or IL-12 plus IL-2 for 48 h. After culture, the IFN-γ activity of their culture supernatants was determined by use of an ELISA kit (Endogen). The data represent mean$\pm$SE of triplicate samples.

resulted in a marked accumulation of serine esterase (Fig. 4), which has been considered to be an important factor for perforin-mediated cytotoxicity.[9]

It has been reported that IL-12 selectively stimulated IFN-γ-producing Th1-type CD4$^+$ T cells.[3,7] To determine whether IL-12 also induced a Th1-like cytokine production pattern in ASGM1$^+$CD8$^+$ T cells, we measured the IFN-γ and IL-4 activity of culture supernatants using ELISA kits. As shown in Fig. 5, culture of ASGM1$^+$CD8$^+$ T cells with IL-12 plus IL-2 caused the production of high levels of IFN-γ. However, IL-2 alone or IL-12 alone was not sufficient to induce IFN-γ production. No significant production of IL-4 was detected in any culture of ASGM1$^+$CD8$^+$T cells (data not shown). ASGM1$^-$CD8$^+$ T cells produced neither IFN-γ nor IL-4 upon culture with IL-12 plus IL-2. The simultaneous existence of both IL-12 and IL-2 is not essential, because ASGM1$^+$CD8$^+$ T cells showed great proliferation, killer cell generation and IFN-γ production when they were incubated with IL-2 alone for 12 h followed by preincubation with IL-12 alone for 12 h (our unpublished data). These data demonstrated that ASGM1$^+$CD8$^+$ T cells are novel IL-12-responsive killer T cell precursor cells which show a Th1-like cytokine production pattern after stimulation with IL-12 plus IL-2.

ASGM1 antigen has been considered as a cell surface marker of NK cells,[11] but it was also demonstrated that ASGM1 antigen is expressed on activated T cells.[12] Therefore, it was speculated that ASGM1$^+$CD8$^+$ T cells might be *in vivo*-activated T cells which were naturally induced in specific-pathogen-free mice. However, this appeared to be unlikely based on the following evidence; (1) IL-2 alone or IL-12 alone, which has sufficient ability to induce the proliferation of activated T cells,[12,13] could not stimulate ASGM1$^+$CD8$^+$ T cells; (2) activation markers such as CD69 and CD25 were not expressed on ASGM1$^+$CD8$^+$ T cells (data not shown).

Recent studies have demonstrated that Th1-Th2 balance is important in tumor immunology, infectious diseases, allergy and autoimmune diseases.[7,14] The role of CD4$^+$ T cells in the regulation of Th1-Th2 balance has been emphasized.[15,16] However, recently, CD8$^+$ T cells showing a Th1-like cytokine production profile have been designated as TC1 cells.[17] Although the precise role of TC1 cells in immunoregulation remains unclear, they might play a pivotal role in the regulation of host surveillance mechanisms against tumor and infection. In this paper, we show that ASGM1$^+$CD8$^+$ T cells are TC1-like CD8$^+$ T cells which are IL-12-responsive and IFN-γ-producing killer-precursor cells distinct from ASGM1$^-$CD8$^+$ T cells. Although it remains unclear whether IFN-γ produced by ASGM1$^+$CD8$^+$ T cells acts in an autocline or a paracline manner, their IFN-γ production appears to be essential for the induction of anomalous killer cells by IL-12 plus IL-2 because addition of anti-IFN-γ mAb to the culture inhibited the maturation of killer cells (data not shown). Therefore, this novel ASGM1$^+$CD8$^+$ T cell population may play an important role in IL-12-induced antitumor activity through the production of IFN-γ, showing cytotoxic activity like CD4$^+$ Th1 cells and NK cells. ASGM1 antigen may be a cell-surface marker which can separate IL-12-responsive TC1-like CD8$^+$ T cells from other CD8$^+$ T cell populations in freshly isolated mouse T

cells. Detailed investigations concerning the functions of ASGM1$^+$CD8$^+$ T cells *in vivo* will be necessary to evaluate their precise role in immunoregulation.

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