

## **Interleukin-10 containing normal human serum inhibits granzyme B release but not perforin release from alloreactive and EBV-specific T cell clones**

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**Interleukin-10 (IL-10), also known as cytokine synthesis inhibitory factor, has pleiotropic effects in immunoregulation and inflammation. It is capable of inhibiting synthesis of pro-inflammatory cytokines like interferon  $\gamma$  (IFN $\gamma$ ), IL-2, IL-3, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and granulocyte macrophage colony stimulating factor (GM-CSF) made by cells such as macrophages and T helper Type 1 cells. We observed that normal human serum, derived from a healthy individual but containing large amounts of IL-10 (arbitrarily designated as “IL-10 serum”), inhibited cytotoxic activity and interfered with granzyme B release from alloreactive cytotoxic T cell (CTL) clones *in vitro*, but did not affect perforin release. The addition of normal human serum containing high levels of anti-IL-10 IgG (arbitrarily designated as “anti-IL-10 IgG serum”) neutralized the inhibitory effects of IL-10 serum. Moreover, we have identified that cytotoxic activity and granzyme B release from an Epstein-Barr virus (EBV)-specific CTL clone was similarly inhibited in the presence of IL-10 serum, while perforin release was unaffected. Anti-IL-10 IgG serum also appeared to neutralize the inhibitory effect of IL-10 serum on an EBV-specific CTL clone.**

Interleukin-10 (IL-10) is a cytokine produced primarily by monocytes and, to a lesser extent, by lymphocytes [1]. This cytokine has an effect on immunoregulation and inflammation [2, 3]. It downregulates the expression of Th1 cytokines, major histocompatibility complex (MHC) class II antigens, and co-stimulatory molecules on macrophages [4, 5]. It also has the ability to inhibit synthesis of pro-inflammatory cytokines such as interferon  $\gamma$  (IFN  $\gamma$ ), IL-2, IL-3, tumor necrosis factor (TNF $\alpha$ ) and granulocyte-macrophage colony stimulating factor (GM-CSF) produced by macrophages and T helper type 1 cells [2, 6]. IL-10 also displays potent ability to suppress antigen presentation by antigen presenting cells [7].

Our aim is to increase the effective utility of donated blood products. Therefore, based on the information from the literature cited above, we analyzed serum containing high levels of IL-10 (arbitrarily designated “IL-10 serum”), derived from normal healthy blood donors, for its ability to inhibit the cytotoxic activity of a number of alloreactive, and one Epstein-Barr virus (EBV)-specific, cytotoxic T cell (CTL) clones. In this study we have specifically focused on the granzyme B [8, 9] and perforin [10, 11] release profiles from these clones. In addition, we have tested whether anti-IL10 IgG containing

serum (arbitrarily designated “anti-IL-10 IgG serum”) derived from normal healthy blood donors can neutralize the inhibitory effects of IL-10 serum.

Our results indicate that, during a 4-hour cytotoxic assay, IL-10 serum inhibited cytotoxic activity against target cells and granzyme B release from both alloreactive CTL clones and an EBV-specific CTL clone. Furthermore, in the presence of anti-IL10 IgG serum, these inhibitory effects were diminished. In contrast, perforin, a serine protease that is released from cytotoxic T cells concomitantly with granzyme B, was not inhibited by the addition of IL-10 serum.

## **RESULTS**

### **An inhibitory effect by IL-10 serum**

In the presence of IL-10 serum, percent specific lysis of target phytohemagglutinin (PHA)-blasted peripheral blood mononuclear cells (PBMCs) was significantly inhibited in two CD4<sup>+</sup> alloreactive T cell clones (arbitrarily designated “clone 1” and “clone 2”) (**Figs. 1a and b**) and in one CD8<sup>+</sup> alloreactive T cell clone (arbitrarily designated “clone 3”) (**Fig. 1c**). In addition, the amount of granzyme B released into the culture supernatant was notably reduced in the presence of IL-10 serum compared to that of control serum for both CD4<sup>+</sup> and CD8<sup>+</sup> clones (**Figs. 1a-c**).

### **Depletion of IL-10 from IL-10 serum**

When the IL-10 present in IL-10 serum was depleted using anti-IL-10 monoclonal antibody (mAb)-conjugated protein G-sepharose 4B beads, the inhibitory effect on cytotoxic activity and granzyme B release from alloreactive CTL clones was diminished (**Fig. 2**).

### **Neutralizing activity of anti-IL-10 IgG serum**

Furthermore, by mixing anti-IL-10 IgG serum in the culture, both the cytotoxic activity and granzyme B release from alloreactive CTL clones was recovered (**Figs. 3a and b**). Anti-IL-10 IgG serum did not effect either alloreactive or an EBV-specific CTL clone (data not shown).

### **Exposure to IL-10 serum has a continued effect on alloreactive CTL clones**

To determine the recovery of CTL clones from suppression by IL-10 serum, the clones were mixed with a 10% volume of control or IL-10 serum followed by incubation at 37°C for 4 hours. After washing three times with RPMI 1640 medium, clones were

cultured in fresh medium [RPMI 1640 + 10% fetal calf serum (FCS) + 50 u/ml recombinant IL-2 (rIL-2)] for 24 hours at 37°C, and then subjected to a cytotoxic assay. Cytotoxic activity (**Figs. 4a and b**) and granzyme B release (**Figs. 4c and d**) was still inhibited compared to the control serum.

#### **Activity of an EBV-specific CTL clone**

An EBV-specific CTL clone (arbitrarily designated as “clone 125”) showed cytotoxic activity against an autologous B lymphoblastoid cell line (B-LCL), three allogeneic B-LCLs (arbitrarily designated as “Allo-1 B-LCL”, “Allo-2 B-LCL” and “Allo-3” B-LCL) and Raji cells [12], all of which express abundant levels of EBV protein on the cell surface (**Fig. 5**). The clone showed no activity against K562 cells that are used as a target for natural killer cells (**Fig. 5**). These results suggest that clone 125 is EBV-specific but is not alloreactive because Allo-1, Allo-2 and Allo-3 B-LCLs do not share the same MHC antigens. Clone 125 is thought to recognize EBV antigens expressed on the cell surface, and therefore is not restricted by MHC antigens. Moreover, clone 125 is considered to have no natural killer activity, since it has almost no cytotoxic activity against K562 cells.

#### **The effect of IL-10 serum on an EBV-specific cytotoxic T cell clone**

Cytotoxic activity and granzyme B release from clone 125 against autologous (**Fig. 6a**) and allogeneic B-LCLs (**Fig. 6b**) was also inhibited in the presence of IL-10 serum. However, IL-10 depletion from IL-10 serum was effective in recovering this activity (**Fig. 7a**). It was also recovered by the addition of anti-IL-10 IgG serum with IL-10 serum (**Fig. 7b**).

#### **Perforin release**

In the same experiment, in contrast to granzyme B, perforin release was not inhibited by IL-10 serum in all three alloreactive T cell clones or in the EBV-specific cytotoxic T cell clones (**Figs. 8a and b**).

## **DISCUSSION**

It has been reported that IL-10 can block NF-kappa B activity, and is involved in the regulation of the JAK-STAT signaling pathway. Knockout studies in mice have suggested that IL-10 functions as an essential immunoregulator in the intestinal tract [13, 14]. IL-10 is also known to be released by cytotoxic T cells to inhibit the actions of

natural killer cells during the immune response to viral infection [15].

Our present study indicates that treatment of both alloreactive, and an EBV-specific, CTL clones with IL-10 serum significantly inhibits their cytotoxic activity and granzyme B release. The role of IL-10 in serum appears to be to interfere with granzyme B release from cytotoxic T cell clones at an early time point during their encounter with target cells, resulting in a down-regulation of cytotoxic activity against target cells. As the effect of IL-10 serum is prolonged to least 24 hours, IL-10 appears to have a significant and continued inhibitory effect.

These results suggest a therapeutic utility for IL-10 serum. When alloreactive cytotoxic T cells are undesirable, such as in graft-versus-host disease (GVHD) after bone marrow transplantation [16-19], or in the rare but lethal GVHD after blood transfusion [20, 21], infusion of IL-10 serum might be beneficial.

Post transplantation lymphoproliferative disease (PTLD) is a common complication of solid organ transplantation that carries a high morbidity and mortality rate. The majorities of tumors are of B-cell origin and are associated with EBV [12, 22]. Our study suggests that IL-10 interferes not only with alloreactive cytotoxic T cells, but also with EBV-specific cytotoxic T cells, via an inhibition of granzyme B release. Based on these results, it is hypothesized that IL-10 serum may be an effective therapy for PTLD.

It has also been reported that IL-10 levels determine viral persistence *in vivo* because it impairs anti-viral T cell responses [23, 24]. Therapeutic treatment of anti-IL-10 IgG serum may be effective in restoring T cell function, and hence the elimination, of viral infection.

Granzyme B is a cysteine protease found in the cytoplasmic granules of CTLs and natural killer cells. Granzyme B is required for the induction of target cell lysis that occurs as part of cell mediated immune responses, and it can activate apoptosis in target cells [8, 9, 25-27]. In this study, we found that IL-10 serum treatment inhibited granzyme B release at an early stage and effectively eliminated the cytotoxic activity of cytotoxic T cell clones. The mechanism of how this occurs is now under investigation.

Perforin is a serine protease that is released from CTLs, and is one of the main mediators of cell killing [10, 11, 28]. However, in contrast to granzyme B, we found that its release from CTL clones was not inhibited by IL-10. One possible explanation for this is that IL-10 has different effects on the release mechanisms of perforin and granzyme B. The precise mechanism of IL-10 action on granule release is now under consideration.

## **METHODS**

**Serum preparation.** IL-10 concentration in the serum was determined using an enzyme-linked immunosorbent assay (ELISA) kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The IL-10 concentrations in sera obtained from 280 normal healthy blood donors were tested. One serum, in which IL-10 concentration was found to be over four standard deviations from the mean (IL-10 concentration = 29.5 ng/ml), was used in this study and designated “IL-10 serum”. Anti-IL-10 IgG levels in the sera were also measured using ELISA. In brief, 100 µg/ml recombinant IL-10 (rIL-10: BioVission Research Products, Mountain View, CA, USA) was coated onto a 96-well ELISA plate (Corning Incorporated, Corning, NY, USA) and incubated with 10-fold diluted sera in TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) followed by incubation with horseradish peroxidase-conjugated anti-human IgG rabbit F(ab')<sub>2</sub> (DakoCytomation A/S, Glostrup, Denmark). This was followed by incubation with the substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB: DakoCytomation A/S). The reaction was stopped by the addition of H<sub>2</sub>SO<sub>4</sub> and absorbance at 450 nm was measured. One serum in which absorbance levels were over five standard deviations from the mean was used in this study and designated “anti-IL-10 IgG serum”. Serum derived from a normal healthy individual that was free of both IL-10 and anti-IL-10 IgG was used as a control.

**Establishment of alloreactive CTL clones.** Alloreactive cytotoxic T cell clones were established by the co-culture of PBMCs obtained from two unrelated healthy individuals followed by the limiting dilution method as described previously [29]. In brief, PBMCs derived from one healthy individual were prepared by Ficoll density gradient centrifugation [30]. The PBMCs were passed through a nylon-wool column [31] and non-adherent semi-purified T cells were collected. PBMCs derived from the other individual were 25 Gy X-ray irradiated and used as stimulator cells. Semi-purified T cells and irradiated stimulator PBMCs were co-cultured for 3 days at 37 °C in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT, USA). Expanded T cells were then cloned using the limited dilution method. Two CD4<sup>+</sup> T cell clones (arbitrarily designated as “clone 1” and “clone 2”) and one CD8<sup>+</sup> T cell clone (arbitrarily designated as “clone 3”) were obtained. T cell clones were maintained in RPMI 1640 medium supplemented with 10% FCS (HyClone) and 50 u/ml of rIL-2 (Genzyme Diagnostics, Cambridge, MA, USA).

**Measurement of cytotoxic activity.** PHA-blasted target PBMCs (of the same origin as the PBMCs used as stimulator cells in the T cell cloning), EBV-transformed autologous B-LCL (of the same origin as the T cell clones), three unrelated separate individual-derived allogeneic B-LCLs, Raji and K562 cells were labeled with 10  $\mu$ M calcein-AM (Sigma-Aldrich Corporation, St. Louis, MS, USA) [32, 33]. Effector T cell clones were mixed with calcein-AM-labeled target cells at an effector : target (ET) ratio of 20 : 1, and incubated at 37°C for 4 hours in the presence of 10% of either control, IL-10, or anti-IL10 IgG (the total culture volume was 100  $\mu$ l /well of 96-well culture plate in serum free RPMI 1640 media). After incubation, the fluorescent intensity of calcein-AM released into the culture supernatant was measured by fluorometry using a WALLAC 1423 Multilabel Counter (PerkinElmer Japan, Yokohama, Japan). Percent specific lysis was calculated using the following formula: [release from sample – spontaneous release / total release – spontaneous release] x 100, where ‘spontaneous release’ was measured from wells in which the effector T cell clone was absent, and ‘total release’ was measured from wells in which Triton X-100 was added instead of the effector T cell clone. All samples were run in triplicate.

**Measurement of granzyme B and perforin.** Granzyme B and perforin concentration in the culture supernatant were measured after the 4-hour cytotoxic assay using ELISA kits (Pierce Biotechnology, Inc.). All samples were run in triplicate.

**Depletion of IL-10 from IL-10 serum.** The IL-10 in IL-10 serum was depleted using an anti-IL-10 mAb (clone 23738.11: Affinity Bioreagents, Inc, Golden, CO, USA)-conjugated protein G-sepharose 4B column. In brief, 100 $\mu$ g mAb was conjugated to 100  $\mu$ l of protein G-sepharose 4B. One hundred  $\mu$ l of IL-10 serum was mixed with the conjugate and gently rotated at 4°C overnight. Protein G-sepharose 4B was removed by ultra-centrifugation (15,000 rpm x 10 minutes at 4 °C). The resulting serum was designated “IL-10 depleted serum”. Complete IL-10 depletion was verified by ELISA.

**Establishment of an EBV-specific CTL clone.** Semi-purified T cells were prepared from PBMCs derived from a healthy individual, using a nylon-wool column as described above. These cells were used as responder cells. Autologous PBMCs were 25 Gy X-ray irradiated and mixed with EBV. These cells were used as stimulators. Responder and stimulator cells were co-cultured for 3 days at 37°C in RPMI 1640 medium supplemented with 10% FCS (HyClone). Cloning was performed using the limiting dilution method. One CD4<sup>+</sup> T cell clone (arbitrarily designated as “clone 125”) was obtained that displayed cytotoxic activity against autologous EBV-transformed B-LCL but not autologous PHA-blasted PBMCs. This clone was used as the EBV-specific CTL clone in this study. Clone 125 was maintained in RPMI 1640 medium supplemented with 10% FCS (HyClone) and 50 u/ml of rIL-2 (Genzyme Diagnostics).

**Statistical analysis.** Means and standard deviations (SDs) were calculated and data analyzed using a Student’s t-test. Statistical significance was defined as  $p < 0.05$ .

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#### **COMPETING INTERESTS STATEMENT**

The authors declare that they have no competing financial interests.

## FIGURE LEGENDS

**Figure 1.** Alloreactive CTL clones, namely clone 1 (a), clone 2 (b) and clone 3 (c), were mixed with calcein-AM-labeled target PHA-blasted PBMCs at an ET ratio of 20 : 1, and incubated for 4 hours at 37°C in the presence of control or IL-10 serum. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Granzyme B concentration in the supernatant was measured by ELISA. The results shown are representative of four identical, independent experiments. For both cytotoxic activity and granzyme B concentration, IL-10 serum gives significantly different results compared to the control (“\*\*\*” denotes a p value of < 0.001).

**Figure 2.** Alloreactive CTL clones were mixed with calcein-AM-labeled target PHA-blasted PBMCs at an ET ratio of 20 : 1 and incubated for 4 hours at 37°C in the presence of either control, IL-10 or IL-10 depleted IL-10 serum. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Granzyme B concentration in the culture supernatant was measured by ELISA. Experiments on each of the three clones were repeated four times and similar results were obtained. As a representative result, data obtained from clone 1 is shown. For both cytotoxic activity and granzyme B concentration, IL-10 serum gives significantly different results compared to the control and IL-10 depleted IL-10 serum (“\*\*\*” denotes a p value of < 0.001). No significant difference was observed between the control and IL-10 depleted IL-10 serum.

**Figure 3.** Alloreactive CTL clones were mixed with calcein-AM-labeled target PHA-blasted PBMCs at an ET ratio of 20 :1, and incubated for 4 hours at 37°C in the presence of either control, IL-10, or IL-10 + anti-IL-10 IgG serum. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Granzyme B concentration in the culture supernatant was measured by ELISA. Experiments were repeated four times for each clone and similar results were obtained. As representative results, typical data from clone 2 (a) and clone 3 (b) are shown. For both cytotoxic activity and granzyme B concentration, IL-10 serum gives significantly different results compared to the control (“\*\*\*” denotes a p value of < 0.001). No significant difference was observed between the control and IL-10 +

anti-IL-10 IgG serum.

**Figure 4.** Alloreactive CTL clones were mixed with calcein-AM-labeled target PHA-blasted PBMCs at an ET ratio of 20 : 1, and incubated for 4 hours at 37°C in the presence of either control or IL-10 serum. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Granzyme B concentration in the culture supernatant was measured by ELISA. In the same experiment, clones were also treated with a 10% volume of either control or IL-10 serum and incubated for 4 hour at 37°C. After incubation, clones were washed three times with RPMI 1640 medium and incubated in fresh medium (RPMI 1640 + 10% FCS + 50 u/ml rIL-2) for 24 hours at 37°C. Clones were then subjected to a cytotoxic assay using calcein-AM-labeled PHA-blasted PBMCs as targets at an ET ratio of 20 : 1. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Experiments were repeated four times and similar results were obtained. As a representative result, typical data obtained from clone 1 (a) and clone 3 (b) is shown. Using ELISA, granzyme B concentration was also measured in the culture supernatant. Experiments were repeated four times and similar results were obtained. As representative results, date obtained from clone 1 (c) and clone 3 (d) is shown.

**Figure 5.** An EBV-specific CTL clone, clone 125, was mixed with calcein-AM-labeled autologous B-LCL, three allogeneic B-LCLs, Raji or K562 cells at an ET ratio of 20 : 1. Incubation was carried out at 37°C for 4 hours. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Granzyme B concentration in the culture supernatant was measured by ELISA. A representative result from four identical independent experiments is shown. For both cytotoxic activity and granzyme B concentration, K562 cells give significantly different results compared to the other 5 cell lines (“\*\*\*” denotes a p value of < 0.001).

**Figure 6.** Clone 125 was mixed with calcein-AM-labeled autologous B-LCL or three allogeneic B-LCLs at an ET ratio of 20 : 1, and incubated for 4 hours at 37°C in the

presence of either control or IL-10 serum. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant. Granzyme B concentration was measured by ELISA. Experiments were repeated four times and similar results were obtained. As representative results, data obtained from autologous B-LCL (a) and Allo-1 B-LCL (b) is shown. For both cytotoxic activity and granzyme B concentration, IL-10 serum gives significantly different results compared to the control (“\*\*\*” denotes a p value of < 0.001).

**Figure 7.** Clone 125 was mixed with calcein-AM-labeled autologous B-LCL at an ET ratio of 20 : 1, and incubated for 4 hours at 37°C in the presence of either control, IL-10, IL-10 depleted IL-10 or IL-10 + anti-IL-10 IgG serum. After incubation, cytotoxic activity and granzyme B concentration was measured. A representative result from four identical independent experiments is shown (a). For both cytotoxic activity and granzyme B concentration, IL-10 serum gives significantly different results compared to the control and IL-10 depleted IL-10 serum (“\*\*\*” denotes a p value of < 0.001). No significant difference was observed between the control and IL-10 depleted IL-10 serum. Clone 125 was mixed with autologous B-LCL an ET ratio of 20 : 1 and incubated for 4 hours at 37°C in the presence of either control, IL-10, or IL-10 + anti-IL-10 IgG serum. After incubation, cytotoxic activity and granzyme B concentration were measured. A representative result from four identical independent experiments is shown (b). For both cytotoxic activity and granzyme B concentration, IL-10 serum give significantly different results compared to the control or IL-10 + anti-IL-10 IgG serum (“\*\*\*” denotes a p value of < 0.001). No significant difference was observed between the control and IL-10 + anti-IL-10 IgG serum.

**Figure 8.** Three alloreactive CTL clones and an EBV-specific CTL clone were mixed with calcein-AM-labeled target PHA-blasted PBMCs and autologous B-LCL, respectively, at an ET ratio of 20 : 1 and incubated for 4 hours at 37°C in the presence of either control or IL-10 serum. After this, cytotoxic activity was calculated based on the fluorescence intensity of calcein-AM released into the culture supernatant (the same cytotoxicity data is shown in Fig. (a) and (b)). Perforin (a) and granzyme B (b) concentration in the culture supernatant was measured by ELISA. Experiments were repeated four times and similar results were obtained. As a representative result, typical

data obtained from clone 1 is shown. For both cytotoxic activity and granzyme B concentration, IL-10 serum gives significantly different results compared to the control (“\*\*” denotes a p value of  $< 0.001$ ). However, no significant difference was observed between the control and IL-10 serum for perforin concentration ( $p = 0.109$ ).

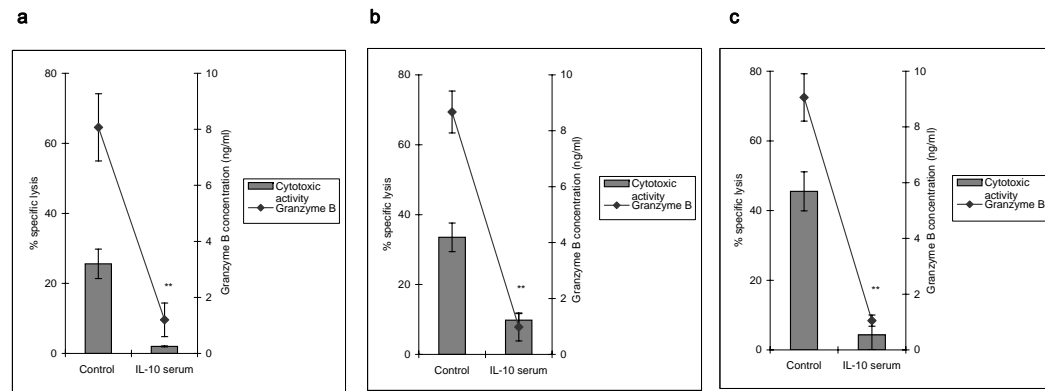


Figure 1



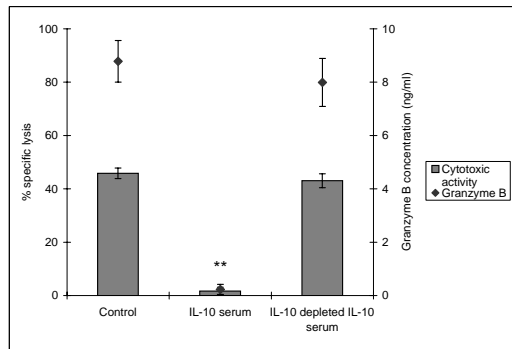


Figure 2

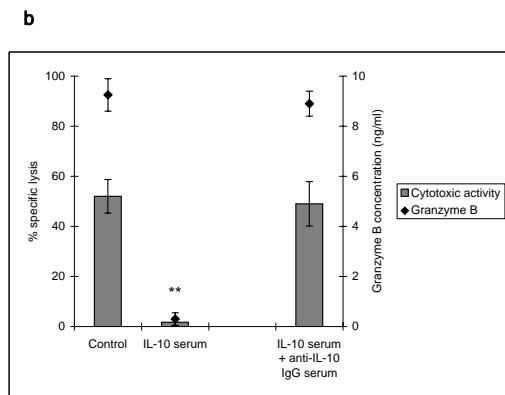
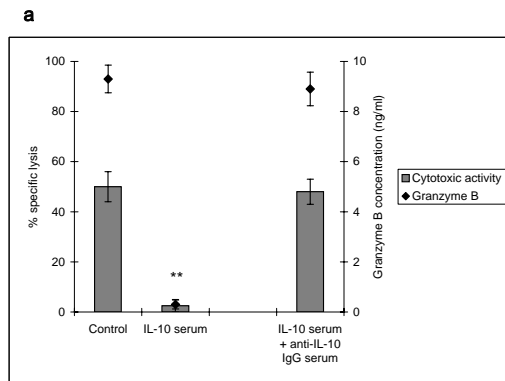
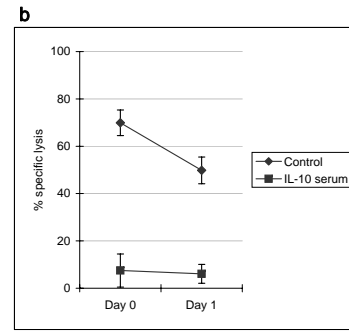
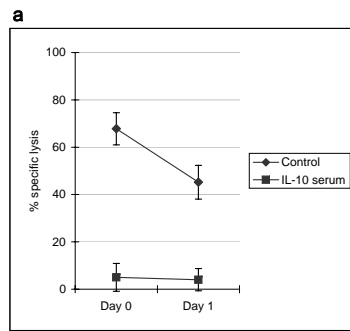


Figure 3

**Cytotoxic activity**



**Granzyme B**

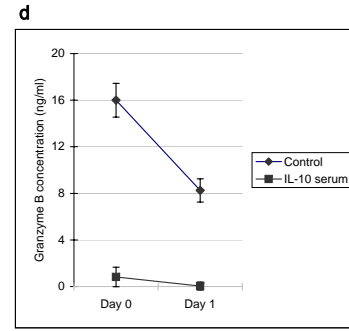
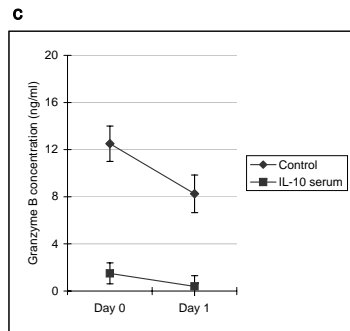


Figure 4

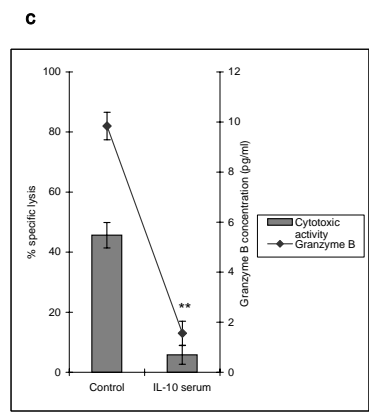
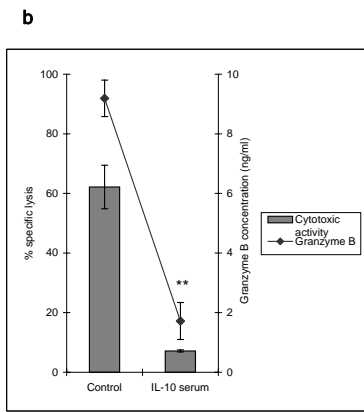
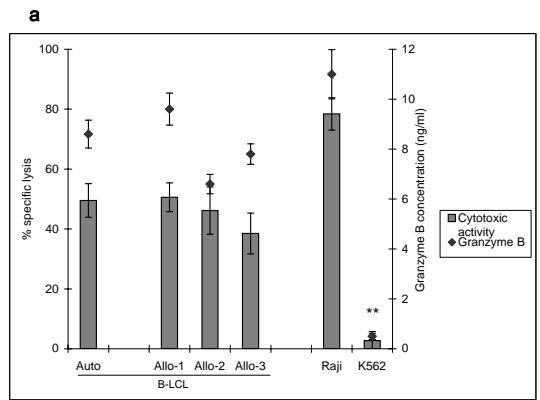
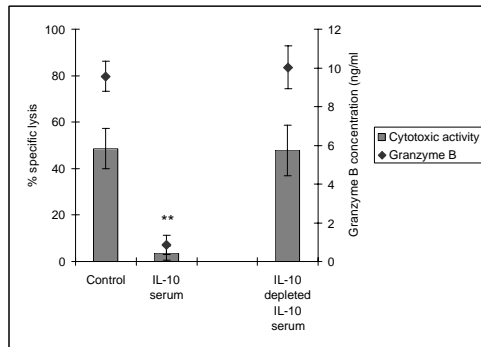


Figure 5

**a**



**b**

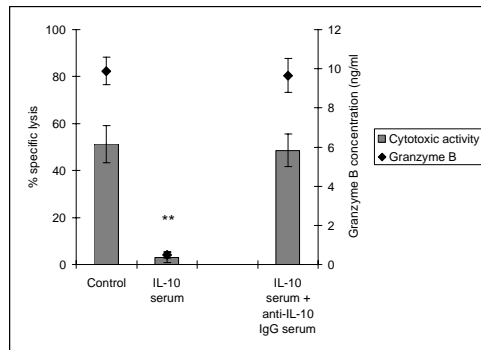
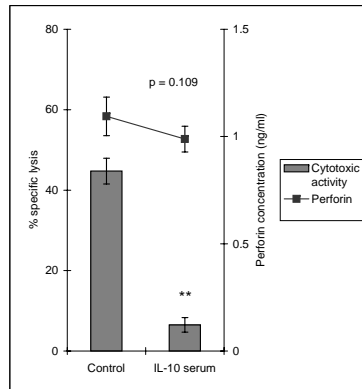


Figure 6

**a**



**b**

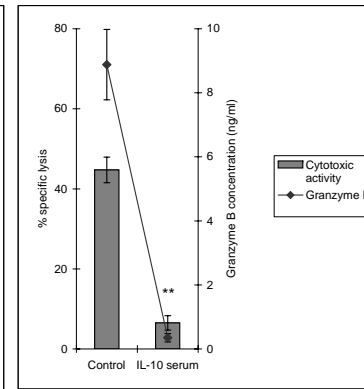


Figure 7