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Depletion of TGF-β from fetal bovine serum

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Abstract

TGF-β is one of the key cytokines controlling immune responses. Measuring TGF-β from culture supernatants in vitro is an important index of immune function. However, fetal bovine serum (FBS) contains a high level of latent TGF-β that often hampers measuring T cell-derived TGF-β in culture using FBS-supplemented medium. In this report, we generated anti-latency associated peptide (LAP) monoclonal antibodies which cross-react with bovine LAP, and developed a protocol to deplete TGF-β from FBS. This provides the ability to reliably quantify TGF-β in vitro without relying on serum-free media which do not support growth of murine T cells.

Keywords

TGF-β; LAP; fetal bovine serum; depletion; monoclonal antibody

1. Introduction

TGF-β is an important cytokine controlling immune responses. TGF-β suppresses Th1 cells, cytotoxic T cells, and other inflammatory reactions. TGF-β also affects naive T cell differentiation either into $Foxp3^+$ Tregs or Th17 cells (Rubtsov and Rudensky, 2007). Thus, measuring production of TGF-β in T cell culture is a crucial method of describing immune status in vitro.

TGF-β is synthesized as a pro-TGF-β from. Pro-TGF-β is intracellularly processed by furin and forms latent TGF-β which is a non-covalently associated complex consisting of latency-associated peptide (LAP) and mature TGF-β (Miyazono, 1993). Latent TGF-β cannot bind TGF-β receptors, and an activation step is required for TGF-β to have biological activity. How T cell-produced TGF-β is activated is as yet unknown.

In general, murine T cell culture supernatants do not contain active TGF-β, which is measured without the acidification step by ELISA. Investigators usually measure “total” TGF-β after the acidification and neutralization steps on the assumption that total TGF-β reflects TGF-β activity, though this may not be true. Fetal bovine serum-supplemented medium is standardly used for murine T cell culture. 10% FBS-supplemented media contain

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1,000 – 2,000 pg/ml of latent TGF-β (Danielpour, 1989, and our observation). T cell-derived TGF-β in culture supernatants is usually much less than FBS-derived TGF-β. Because of this, some investigators only use serum-free medium for TGF-β measurements to avoid contribution of FBS-derived TGF-β in the culture medium. Commercially available serum-free media, however, often do not support murine T cell growth in vitro. Thus, either subtraction of the background TGF-β level of FBS-supplemented medium or using a poorly growth-supporting serum-free medium, may make TGF-β measurements unreliable. If one would be able to deplete TGF-β from FBS, it would obviate this problem. As part of our investigations of TGF-β, we raised anti-human latency-associated peptide (LAP) mAbs, and selected clones which cross-reacted with bovine LAP. We found that by using these anti-LAP antibodies, the latent TGF-β in FBS was successfully reduced by 90%. We found that the FBS depleted of TGF-β by anti-LAP antibody supported the T cell response as well as normal FBS, and thus enabled the reliable measurement of TGF-β from T cells.

2. Materials and Methods

2.1 Preparation of anti-LAP monoclonal antibodies

Anti-LAP mAbs were raised as described (Oida and Weiner, in press). In brief, BALB/c mice were immunized with recombinant human LAP (R&D Systems, Minneapolis, MN) and boosted with *TGFB1*-transduced P3U1 cells. Lymph node cells were fused with P3U1 myeloma cells and anti-LAP hybridoma clones were selected. Among these anti-human LAP mAbs, clones cross-reacting with bovine LAP were selected by FACS staining of bovine *TGFB1*-transduced P3U1 cells. The Selected hybridoma (TW4-5A8, mouse IgG1,κ) cells were grown in CELLine culture flask (Integra Biosciences, Zizers Switzerland) in ADCF-Mab protein-free medium (Hyclone/Thermo, Rockford, IL) and the antibody was purified T-Gel Absorbent (Pierce/Thermo, Rockford, IL).

2.2 Immunological depletion of latent TGF-β from FBS

20 μg anti-LAP mAb TW4-5A8 was coupled to 200 μl of 1 mg/ml goat anti-mouse IgG BioMag Plus beads (Polysciences, Warrington, PA) in a 1.5 ml microcentrifuge tube according the manufacturer's protocol and the beads were washed with PBS for three times. 1 ml of heat-inactivated FBS (Hyclone) was added to the bead pellet and incubated with rotation at 4°C for 8 hrs. The beads were removed by magnetic separation (DynaMag-Spin, Invitrogen, Carlsbad, CA) and the depletion was repeated for two more times. The depletion volume can be scale-up to 10 times by using a 15 ml tube and a corresponding magnet separator. The final TGF-β-depleted FBS was filtered through 0.22 μm to completely remove the magnetic particles.

2.3 TGF-β ELISA

FBS and culture supernatants were used for TGF-β ELISA with or without acidification. FBS was first diluted tenfold with PBS. Acidification was done by adding 1/10 volume of 1N HCl, incubating at room temperature for 10 min, and neutralizing with 1/10 volume of 1N NaOH/0.1M Tris. The samples were then diluted twofold by adding 25 mM Tris buffered saline. TGF-β ELISA was performed using anti-TGF-β mAb 1D11 (ATCC, Manassas, VA) as a coating antibody and biotinylated chicken anti-TGF-β IgY (BAF240, R&D Systems) as a detection antibody. Recombinant human TGF-β (R&D Systems) was used as a standard (0 – 2,000 pg/ml).

2.4 T cell stimulation

Mouse CD4 T cells were separated from BALB/c spleen and lymph nodes using MACS CD4 Isolation Kit (Miltenyi, Bergisch Gladbach, Germany). CD4 T cells were stimulated

with plate-bound anti-CD3 (145-2C11, BD Biosciences, San Diego, CA, 4 μg/ml) and soluble anti-CD28 (37.51, Biolegend, San Diego, CA, 2 μg/ml) in 10% normal FBS-IMDM, 10% TGF-β-depleted FBS-IMDM, or X-vivo15 serum-free medium (Lonza, Basel, Switzerland) at 5×10^5 cells/ml, and culture supernatants were collected at 48 hrs and 72 hrs.

3. Results and Discussion

3.1 Selection of anti-LAP monoclonal antibodies cross-reacting to bovine LAP

We have recently reported that *TGFB1*-transduced P3U1 cells express LAP on the cell surface (Oida and Weiner, 2010). We made anti-human LAP mAbs by immunizing mice with recombinant human LAP. To select clones cross-reacting bovine LAP, bovine *TGFB1*-transduced P3U1 containing IRES-GFP cells were made, and stained with our in-house anti-LAP mAbs. TW4-5A8 and TW4-3H6 stained bovine *TGFB1*-transduced P3U1 cells (Fig.1, GFP^+ cells) as well as human *TGFB1*-transduced P3U1 cells (Fig1, GFP^- cells), while other clones, such as TW4-2H2, stained only human *TGFB1*-transduced cells. Thus, we found anti-LAP mAbs which cross-react with bovine LAP. TW4-5A8 or TW4-3H6 do not stain mouse *Tgfb1*-transduced P3U1 cells (data not shown), indicating that these anti-LAP mAbs do not cross-react with mouse LAP.

3.2 Immunological depletion of latent TGF-beta from FBS

Fetal bovine serum contains approximately 10 – 20 ng/ml of total TGF-β by ELISA (Danielpour, 1989, and our observation) although it may vary depending on lots. Thus, a 10% FBS-medium usually contains 1,000 – 2,000 pg/ml of total TGF-β. Active TGF-β, which is measured without sample acidification, is virtually not detectable in FBS. Thus, TGF-β exists only as the latent form in FBS, and hence does not have biological activity. However, the amount of FBS-derived TGF-β is often high enough to interfere with measuring T cell-derived TGF-β.

To deplete latent TGF-β from FBS, anti-LAP mAb TW4-5A8 was coupled to goat anti-mouse IgG BioMag beads, and the beads were mixed with FBS. After 8 hr incubation at 4°C, the beads were magnetically removed, and TGF-β depleted FBS was recovered. The depletion process was repeated for a total of three times. We found that the initial total TGF-β amount in our FBS lot was 14.9 ng/ml, and it became 3.2 ng/ml after the first depletion, 2.1 ng/ml after the second depletion, and 1.5 ng/ml after the third depletion (Table 1). As shown in Table 1 the variability of TGF-β measurements was between 5–8%. Clone TW4-3H6 also depleted TGF-β from FBS as well as TW4-5A8 (data not shown). Remaining magnetic particles, if any, were completely removed by filtration through a 0.22 μm filter unit. Free anti-LAP mAb detached from the magnetic beads is expected to be minimal, and it should be noted that the anti-LAP mAb does not cross-react with mouse LAP and hence it does not interfere murine T cell culture even if it is released from the beads.

3.3 Measuring T cell-derived TGF-β

Mouse $CD4^+$ T cells were purified and stimulated with plate-bound anti-CD3 and soluble anti-CD28 at 5×10^5 cells/ml for 48 and 72 hrs in the following media: 1) 10% normal FBS-IMDM, 2) 10% TGF-β-depleted FBS-IMDM, and 3) X-vivo15 serum-free medium. The culture supernatants were measured for TGF-β with/without acidification. IFN-γ was also measured as an indicator of cell activity. T cells cultured in 10% TGF-β-depleted FBS-IMDM showed a similar amount of IFN-γ production as in 10% normal FBS IMDM, indicating that the TGF-β depletion process does not affect overall T cell-supporting ability of FBS. T cells cultured in X-vivo15 produced much less IFN-γ than cultured in 10% FBS medium, suggesting that the serum-free medium does not support T cell growth well.

Active TGF-β was not detected (below 15 pg/ml) without acidification in any T cell culture supernatants or in 10% FBS-IMDM, and hence total TGF-β was measured after acidification.

A representative experiment is shown in Fig 2. 10% normal FBS-IMDM contained 1,462 pg/ml TGF-β. The T cell culture supernatant at 48 hrs grown in 10% normal FBS-IMDM showed 1,653 pg/ml TGF-β. Although the subtraction of the background indicates an increase of 191 pg/ml of TGF-β, one cannot rely on this number since the change is only a 13% increase from the base line. On the other hand, by using TGF-β-depleted FBS, The T cell culture supernatant at 48 hr contained 271 pg/ml TGF-β while 10% TGF-β-depleted FBS IMDM only showed 148 pg/ml TGF-β. The subtracted amount (123 pg/ml) is more reliable as the T cell-derived TGF-β amount.

At 72 hrs, The T cell culture supernatant grown in 10% normal FBS-IMDM showed 3,134 pg/ml and the background (1,462 pg/ml) subtraction calculated 1,672 pg/ml. T cell culture sup in 10% TGF-β-depleted FBS IMDM contained 1,286 pg/ml, and the background (148 pg/ml) subtraction became 1,138 pg/ml. Although T cell-derived TGF-β was observed even in 10% normal FBS-IMDM at this time point, the increase in 10% TGF-β-depleted FBS IMDM was more apparent and reliable than the amount in 10% normal FBS.

4. Conclusion

FBS-derived TGF-β makes it difficult to measure in vitro TGF-β production from murine T cells. Here we selected anti-LAP mAbs cross-reacting with bovine LAP from our in-house anti-human LAP mAbs, and we successfully depleted bovine latent TGF-β from FBS by the simple protocol. We did not find that commercially available anti-human LAP antibodies cross-reacted with bovine LAP (data not shown). Our use of anti-LAP antibodies to deplete TGF from FBS enables us to obtain more reliable TGF-β measurements from T cell cultures, especially at low cell number conditions and/or at early time points, without requiring serum-free media which does not support T cell growth well and thus does not provide sensitive biologic assessment of TGF-β.

Acknowledgments

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Abbreviations

mAb	monoclonal antibody
TGF-β	transforming growth factor-β1
LAP	latency-associated peptide

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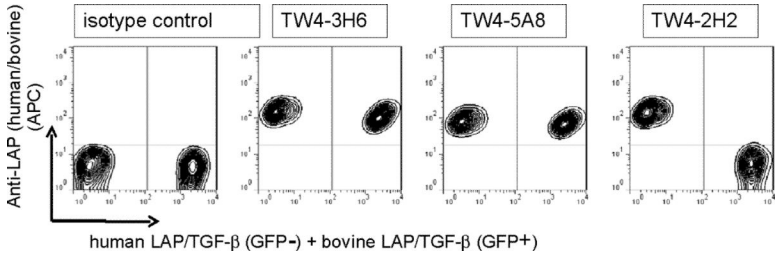

Figure 1. Selection of anti-LAP clones that cross-react with bovine LAP

P3U1-bovine TGF-β #4 cells (with IRES-GFP) mixed with P3U1-human TGF-β #32 cells (lacking IRES-GFP) were surface stained anti-human LAP mAbs. TW4-3H6 and TW4-5A8 stained both GFP^+ cells and GFP^- cells, while TW4-2H2 stained only GFP^- cells.

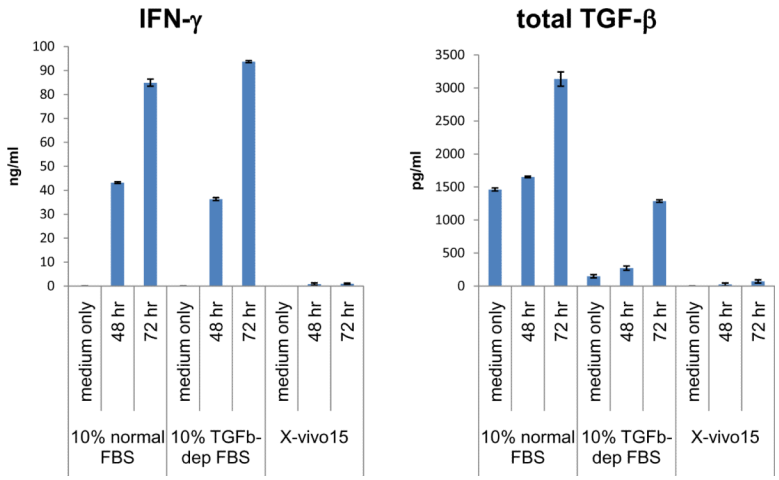

Figure 2. TGF-β measurement from T cell cultures in different media

Murine $CD4^+$ T cells were stimulated with plate-bound anti-CD3 and soluble anti-CD28 for 48 and 72 hrs in 10% normal FBS-IMDM, in 10% TGF-β-depleted FBS-IMDM, or in X-vivo15 serum-free medium. Culture supernatants or the original media were measured for TGF-β and IFN-γ by ELISA.

Table 1

Depletion of TGF-β from FBS

TGF-β was immunologically depleted with anti-LAP TW4-5A8 for three rounds. Remaining TGF-β in FBS was measured by ELISA after acidification. S.D. from three independent depletion procedures is also shown.

	No depletion	1st dep.	2nd dep.	3rd dep.
TGF-β (pg/ml)	14,920 +/− 1,150	3,170 +/− 150	2,100 +/− 80	1,520 +/− 80