Transforming Growth Factor β1 Selectivity Stimulates Immunoglobulin G2b Secretion by Lipopolysaccharide-activated Murine B Cells

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Summary

Bacterial lipopolysaccharide (LPS) has been reported to induce immunoglobulin (Ig)G2b class switching, yet we observed strain differences in IgG2b secretion in response to this mitogen. Specifically, BALB/c B cells, unlike those from DBA/2, synthesized relatively low amounts of IgG2b relative to IgG3, IgG1, or IgM. This report demonstrates that transforming growth factor (TGF)β1, previously shown to induce IgA class switching, selectively stimulates IgG2b secretion by BALB/c resting B cells activated with LPS. This activity was specifically reversed with a neutralizing anti-TGF-β1 antibody. The ability of TGF-β1 to act directly on highly purified membrane (m)IgM+mIgG2b− cells to stimulate IgG2b production, stimulate an increase in IgG2b-secreting cells, and selectively increase the steady-state levels of germline γ2b RNA, suggests that it promotes IgG2b class switching. In this regard, addition of anti-TGF-β antibody to cultures of DBA/2-derived resting B cells activated by LPS, alone, led to selective reduction in IgG2b secretion, indicating that endogenous TGF-β1 accounts for the high IgG2b secretory response observed in that strain. Finally, TGF-β1 failed to stimulate IgG2b secretion by B cells activated with dextran-conjugated anti-IgD antibody. We propose that TGF-β1 is a switch factor for the murine IgG2b subclass for appropriately activated B cells. In combination with other data, this would show that all six non-IgM, non-IgD isotypes in the mouse can be selectively induced by specific cytokines.

T GF-β1 is a pleiotropic cytokine whose primary functions include stimulation of wound healing (1) and suppression of multiple immune cell types (2–4). TGF-β1 can be released by a number of cell types including B cells, T cells, macrophages, and platelets (1–3, 5–8). TGF-β1 was recently shown to stimulate IgA production by LPS-activated murine B cells in vitro by inducing an IgA class switch (9–13). Similar results were recently obtained using human B cells (14–16).

LPS stimulates large amounts of IgM and induces class switching to IgG3 and IgG2b (17, 18). We observed that the ratio of secreted IgG2b to that of IgG3, IgG1, or IgM upon LPS activation varied dramatically depending upon the strain of mouse from which the B cells were derived. Thus, B cells derived from BALB/c mice secreted relatively low

amounts of IgG2b, upon LPS stimulation, relative to cells obtained from DBA/2 mice. Since no cytokine has been described which regulates IgG2b secretion in a positive manner, we used LPS-activated B cells from BALB/c mice to screen for cytokines that selectively stimulate the expression of this Ig isotype. We now show that TGF-β1 selectively induces IgG2b secretion by LPS-, but not anti-IgD-dextran-, activated B cells. This data supports our previous contention that the nature of the B cell activator plays a pivotal role in determining cytokine-directed Ig isotype production (19, 20) and further indicates, in combination with other data, that three cytokines can selectively regulate two of the six non-IgM, non-IgD murine Ig isotypes in a positive manner: IL-4: IgG1 (21) and IgE (22); IFN-γ: IgG2a (23) and IgG3 (20); and TGF-β1: IgG2b and IgA (9, 10).

Materials and Methods

Mice. Female BALB/c and DBA/2 mice were obtained from the National Cancer Institute (Frederick, MD) and were used at 7–9 wk of age.

Medium. RPMI 1640 (Biofluids, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (GIBCO BRL, Gaithersburg, MD), L-glutamine (2 mM), 2-ME (0.05 mM), penicillin (50 μg/ml), and streptomycin (50 μg/ml) were used for culturing cells.

Preparation and Culture of B Cells. Enriched, T-depleted populations of small splenic B cells were obtained as described (24). Functional assays were carried out in 96-well flat-bottomed plates (Costar Corp., Cambridge, MA). Cultured cells were incubated at 37°C in a humidified atmosphere containing 6% CO_2.

Reagents. LPS W, extracted from *Escherichia coli* 0111:B4, was obtained from Difco Laboratories, Inc. (Detroit, MI). Purified porcine and bovine natural TGF-β_1 were obtained from R & D Systems, Inc. (Minneapolis, MN) and Celtrix Pharmaceuticals (Santa Clara, CA), respectively. Dextran-conjugated monoclonal anti-IgD (Hδ^a/1) ($\alpha\delta$-dex)[1] was prepared as previously described (25). rIL-5 was prepared in the baculovirus system and was a kind gift from Dr. Richard Hodes (National Institutes of Health, Bethesda, MD). PE-labeled polyclonal goat anti–mouse IgM was obtained from Southern Biotechnology Associates (Birmingham, AL). Monoclonal rat IgG1 anti–mouse IgG2b was obtained from Pharmingen (San Diego, CA). Monoclonal mouse IgG1 anti-TGF-β (1D11.16.8) (26) and a control mAb, mouse IgG1 anti–mouse IgM^b-allotype (MB86) (27) were purified from ascites. B cells from BALB/c and DBA/2 mice express IgH of the a-allotype and hence do not bind MB86 (27).

Electronic Cell Sorting. Highly purified (>99%) membrane (m)IgM$^+$mIgG2b$^-$ B cells were obtained by electronic cell sorting of B cells stained with PE-anti-IgM plus FITC-anti-IgG2b using a FACStar Plus® (Becton Dickinson & Co., Mountain View, CA) as described (28).

Quantitation of Secreted Ig Isotypes. Ig isotype concentrations were measured by an ELISA assay as described (24). Briefly, Immulon 2, 96-well flat-bottomed ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA) were used, and a fluorescent product was generated by cleavage of 4-methyl umbilliferyl phosphate (Sigma Immunochemicals, St. Louis, MO) with specifically bound alkaline phosphatase–conjugated antibodies. Ig isotype concentrations were determined by extrapolation from standard curves.

Quantitation of Ig Isotype-secreting Cells. Ig isotype-secreting cells were quantitated by an ELISPOT assay (29). Briefly, flat-bottomed Immulon I microtiter plates (Dynatech Laboratories, Inc.), were coated with goat anti–mouse Ig and blocked with 1% BSA in PBS. Serial dilutions of single cell suspensions, starting with 2 × 10^5 cells/well, were incubated on anti-Ig–coated plates for 7 h at 37°C in a 5% CO_2 in air incubator. The cells were washed away with PBS/0.05% Tween 20, and the plates were overlaid for 2 h with phosphatase-conjugated isotype-specific antibodies to mouse Ig (Southern Biotechnology Associates). The antibodies produced by individual B cells which bound to the plate were visualized by addition of a 5-bromo-3-chloroindolyl phosphate solution (Sigma Immunochemicals).

Detection of Steady-state Levels of Germline $C_H\gamma3$ and $C_H\gamma2b$ RNA. Steady-state levels of germline $C_H\gamma3$ RNA were measured by Northern blot analysis. 20 μg of total RNA, extracted from cultured cells, was separated, by electrophoresis, in a formaldehyde-containing 1% agarose gel and blotted onto a nylon membrane

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[1] *Abbreviations used in this paper:* $\alpha\delta$-dex, dextran-conjugated anti-IgD antibodies; m, membrane.

according to standard protocols. The blot was then hybridized with a cDNA specific for I$\gamma3$ (30) that was labeled with ^{32}P-deoxycytidine by the random hexamer method, and was subsequently exposed to X-ray film. In addition, 20 μg of total RNA, extracted from the same cell populations that were utilized to measure germline $\gamma3$ transcripts, was hybridized to a ^{32}P-labeled probe specific for germline $\gamma2b$ transcripts to assay for steady-state levels of germline $C_H\gamma2b$ RNA by the S1 nuclease protection assay as described (31).

Results and Discussion

In initial experiments we observed that the relative amounts of IgG2b, IgG3, IgG1, and IgM secreted in response to LPS stimulation in vitro differed between B cells from BALB/c vs. DBA/2 mice. In four experiments BALB/c-derived B cells secreted 6–10-fold less IgG2b than DBA/2-derived B cells, whereas their secretion of IgG3, IgG1, or IgM was either comparable or somewhat higher (Table 1). Thus, BALB/c-derived B cells were used to search for cytokines which selectively stimulated IgG2b secretion in response to LPS.

We observed that purified bovine TGF-β_1 stimulated a dose-dependent increase in IgG2b and IgA secretion by LPS-activated B cells in vitro (Fig. 1). Maximal IgG2b and IgA secretion typically occurred between 0.3–1.0 ng/ml, a dose range similar to that reported by others for maximal IgA enhancement (9, 10). Optimal stimulation of IgG2b was observed when TGF-β_1 was added 24 h after addition of LPS and when cells were cultured at relatively high cell densities (2.5–10 × 10^5/ml). The TGF-β_1–induced enhancement in IgG2b secretion was observed in six separate experiments. In contrast, similar concentrations of TGF-β_1 failed to stimulate, and variably inhibited, LPS-induced IgM, IgG3, and IgG1 production (Fig. 1). Addition of TGF-β_1 >1.0 ng/ml typically led to a decrease in all Ig isotypes, and this was associated with a marked reduction in viable cell yields relative to that observed with LPS activation alone. This was consistent with the antiproliferative effect of TGF-β_1 described by others (2, 3). Similar results were obtained using purified porcine TGF-β_1 (data not shown). Although it has been reported that the addition of either IL-5 or IL-2 can selectively enhance TGF-β_1–mediated induction of IgA secretion by LPS-activated B cells (10), we observed no selective induction of IgG2b secretion by either IL-2 or IL-5 on LPS plus TGF-β_1–activated cells (data not shown).

Addition of increasing amounts of a neutralizing monoclonal anti-TGF-β_1 antibody to B cell cultures stimulated with LPS and 1.0 ng/ml of TGF-β_1 resulted in a selective reduction in IgG2b secretion, whereas an isotype-matched control mAb had no significant effect (Table 2). This indicated that the IgG2b-enhancing activity was due to TGF-β_1 and not some contaminant in the TGF-β_1 preparation.

To determine whether the TGF-β_1–mediated increase in IgG2b secretion was due to an increase in the number of IgG2b-secreting cells, or the amount of IgG2b secreted per cell, the number of Ig isotype-secreting cells generated in response to LPS and TGF-β_1 was determined using an

Table 1. *LPS-activated BALB/c-derived B Cells Synthesize Relatively Low Levels of IgG2b*

Strain	Ig secretion				Ratio		
	IgG2b	IgG3	IgG1	IgM	$\gamma3/\gamma2b$	$\gamma1/\gamma2b$	$\mu/\gamma2b$
			ng/ml				
BALB/c	85	5,000	310	52,500	59	3.6	620
DBA/2	850	1,750	24	125,000	2.1	0.03	147

B cells from each strain were cultured at 10^5/ml in the presence of LPS for 4 d. Cells were then washed three times in medium to remove mAbs (murine IgG1), which would otherwise be measured in the IgG1-specific ELISA, and resuspended in medium for an additional 2 d to allow for Ig secretion to occur. Culture supernatants were then removed for measurement of Ig isotype concentrations by ELISA. This data is representative of four similar experiments.

ELISPOT assay (Fig. 2). Cells were analyzed 4.5 d after initiation of culture. When expressed as the percentage of total Ig-secreting cells at a given concentration of TGF-β_1, a dose-dependent increase (maximal: 30-fold at 1.0 ng/ml of TGF-β_1) was observed for IgG2b-secreting cells. By contrast

the percentage of cells secreting IgM, IgG3, or IgG1 either remained stable or decreased. 1.0 ng/ml of TGF-β_1 induced a 5.4-fold increase in the absolute number of IgG2b-secreting cells relative to that observed with LPS alone. By contrast, the absolute numbers of cells secreting IgM, IgG3, and IgG1
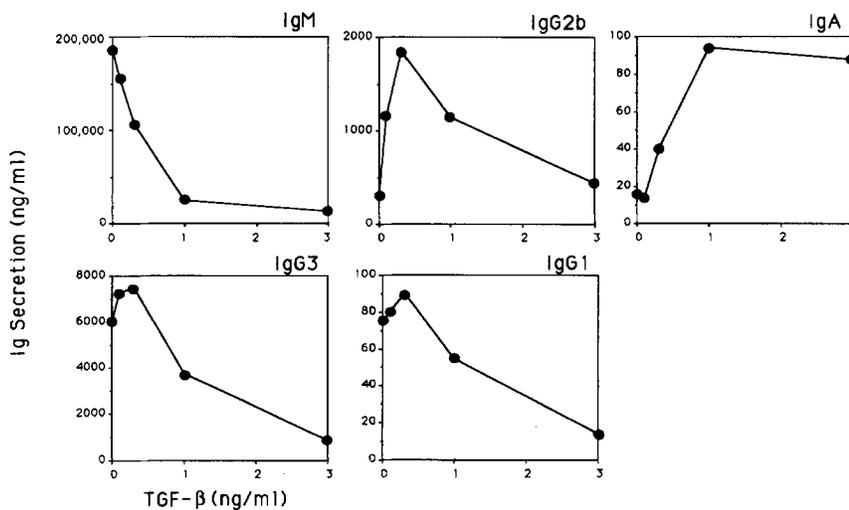
Figure 1. TGF-β_1 selectively stimulates IgG2b and IgA secretion by LPS-activated B cells. BALB/c-derived B cells were cultured for 24 h with LPS (40 μg/ml) at 5 × 10^5 cells/ml in a total of 100 μl of medium upon which varying concentrations of TGF-β_1 were added in 100 μl of medium to achieve the final concentrations illustrated in the figure. Culture supernatants were removed 6 d after initiation of culture and Ig isotype concentrations were measured by an ELISA. All groups were established in triplicate.

Table 2. *Anti-TGF-β mAb Specifically and Selectively Inhibits IgG2b Secretion by B Cells Activated with LPS Plus TGF-$\beta1$*

	Ig secretion		
	IgG2b	IgM	IgG3
		ng/ml	
LPS	130	47,900	1,250
LPS plus TGF-$\beta1$	1,260	28,900	2,810
LPS plus TGF-$\beta1$ plus anti-TGF-β	265	53,900	2,140
LPS plus TGF-β plus control mAb	1,650	29,400	3,500

BALB/c-derived B cells were stimulated for 24 h at 5 × 10^5/ml in the presence of LPS (40 μg/ml). 24 h later TGF-$\beta1$ (final concentration 1.0 ng/ml) with or without anti-TGF-β (10 μg/ml final concentration) or control mAb (MB86) (10 μg/ml final concentration) were added to culture in an equal volume. Culture supernatants were removed 6 d after initiation of culture for measurement of Ig isotype concentrations by ELISA.

declined at 1.0 ng/ml of TGF-β_1 by 5.5-, 14.8-, and 4.2-fold, respectively. Further addition of 1.0 ng/ml of TGF-β_1 to LPS-activated cultures led to a reduction of 2.5-fold in viable cell yields. Thus, TGF-β_1 increased IgG2b secretion by LPS-activated B cells primarily by increasing the number of cells secreting Ig of that isotype.
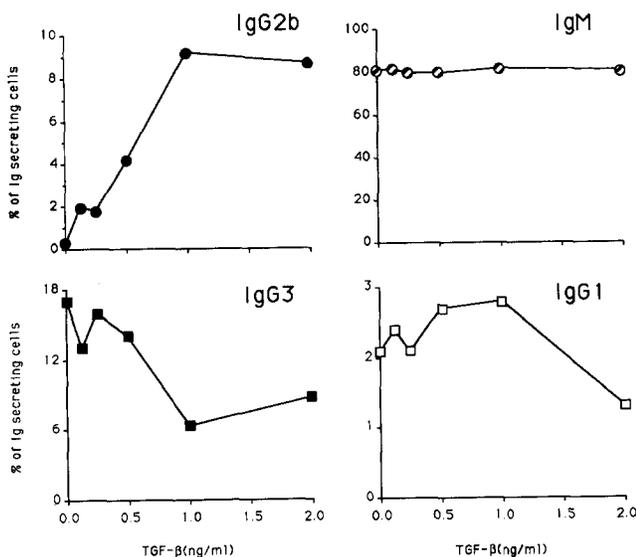
To determine whether TGF-β_1 acted directly on B cells and stimulated class switching, highly purified (>99%) mIgM$^+$mIgG2b$^-$ cells were isolated by electronic cell sorting and stimulated with LPS in the presence or absence of TGF-β_1 (Table 3). TGF-β_1 selectively induced IgG2b secretion by LPS-activated mIgM$^+$mIgG2b$^-$ cells indicating its direct action on the B cell and suggesting that it promoted a class switch to IgG2b.

An increase in the steady state levels of germline C_H RNA specific for a particular C_H gene typically precedes class switching to the expression of that gene (32, 33). To further assess whether TGF-β_1 acted as a switch factor for IgG2b we determined whether it selectively induced germline $C_H\gamma2b$ RNA in LPS-activated B cells (Fig. 3). TGF-β_1 induced a 2-2.5-fold increase in the steady-state levels of germline $C_H\gamma2b$ RNA in LPS-activated B cells while concomitantly reducing, by 2-2.5-fold, the levels of germline $C_H\gamma3$. The enhancement in steady-state levels of germline $C_H\gamma2b$ RNA by TGF-β_1 was 2-3-fold lower than the associated induction of IgG2b secretion. However, steady-state levels of germline $C_H\gamma2b$ RNA may underestimate the degree of transcriptional activation of the $C_H\gamma2b$ gene, the relationship between germline C_H gene transcription and Ig class switching may not be a linear one, and/or other parameters
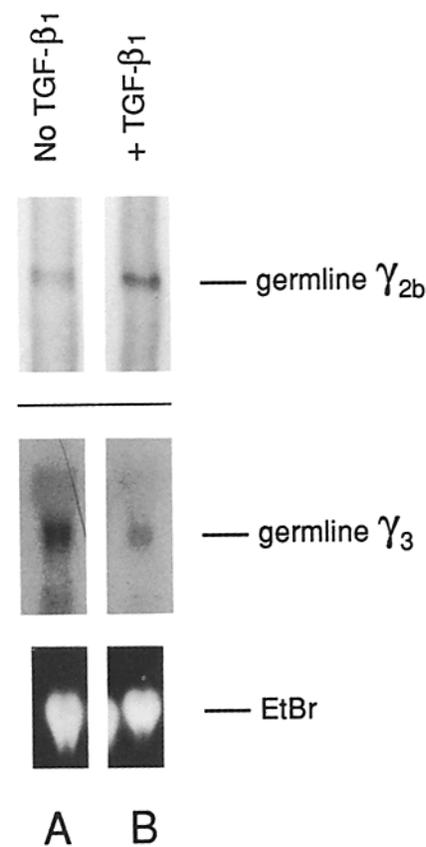
in the response of B cells to TGF-β_1 may further regulate IgG2b synthesis.

Additional experiments demonstrated that a neutralizing anti-TGF-β_1 antibody specifically and selectively reduced IgG2b secretion by LPS-activated DBA/2-derived B cells (Fig. 4). Thus, it appears that endogenous TGF-β_1 accounted for the ability of DBA/2-derived B cells to synthesize relatively high amounts of IgG2b in response to LPS.

We previously determined that cross-linkage of the antigen receptor of small B cells by dextran-linked anti-IgD ($\alpha\delta$-dex) provided a powerful costimulus for cytokine-directed Ig isotype production (34). Yet, $\alpha\delta$-dex was unable to costimulate an IgE response in the presence of even high concentrations of IL-4 (19). We further observed a distinct lack of IgG2b secretion by $\alpha\delta$-dex–activated B cells in the presence of cytokines contained within either activated CD4$^+$ Th1 or Th2 supernatants (19). To determine whether TGF-β_1 could

Figure 3. TGF-β_1 selectively stimulates an increase in steady-state levels of germline $C_H\gamma2b$ RNA by LPS-activated B cells. BALB/c-derived B cells were cultured for 24 h in the presence of LPS (40 μg/ml) and either anti-TGF-β_1 mAb (1D11.16.8) (10 μg/ml) to neutralize any endogenous TGF-β_1, or an isotype-matched control mAb (MB86) (10 μg/ml) at 5 × 10^5/ml in 25 ml of medium upon which medium alone or TGF-β_1 (2.0 ng/ml), respectively, were added in 25 ml of medium. Cells were harvested 3 d after initiation of culture and total RNA was extracted for quantitation of steady-state levels of germline $C_H\gamma2b$ by the S1 nuclease protection assay or $C_H\gamma3$ by Northern blot analysis. Ethidium bromide (EtBr) staining of RNA is included to show essentially equal quantities of RNA used from each group.

Figure 2. TGF-β_1 selectively stimulates an increase in IgG2b-secreting cells in the presence of LPS. BALB/c-derived B cells were cultured for 24 h with LPS (40 μg/ml) at 5 × 10^5 cells/ml in a volume of 5 ml of medium upon which varying concentrations of TGF-β_1 were added in 5 ml of medium to achieve the final concentrations illustrated in the figure. Cells were harvested 4.5 d after initiation of culture for quantitation of Ig isotype-secreting cells by an ELISPOT assay.

Table 3. *TGF-β Acts Directly on mIgM⁺mIgG2b⁻ B Cells to Stimulate IgG2b Secretion*

	Ig secretion					
	Nonsorted			mIgM⁺mIgG2b⁻		
	IgM	IgG2b	IgG3	IgM	IgG2b	IgG3
			ng/ml			
LPS	119,000	86	1,800	175,000	290	2,150
LPS + TGF-β1	46,250	500	3,000	106,000	2,600	4,250

Small splenic B cells were stained with FITC-anti-IgG2b plus PE-anti-IgM, mIgM⁺mIgG2b⁻ cells were isolated by electronic cell sorting to >99% purity and stimulated with LPS (40 μg/ml) at 5 × 10⁵ cells/ml. 24 h later, an equal volume of medium with or without 1.0 ng/ml of TGF-β1 was added. Culture supernatant was removed 6 d after initiation of culture with LPS, and IgM, IgG3, and IgG2b concentrations were measured by ELISA.
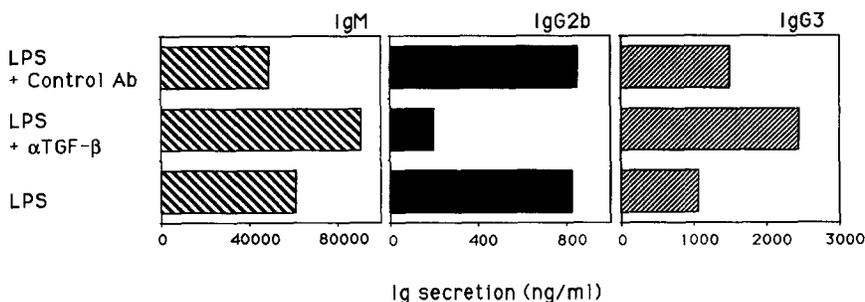
Figure 4. Anti-TGF-β₁ selectively inhibits IgG2b secretion by DBA/2-derived, LPS-activated B cells. DBA/2-derived, B cells were cultured at 1.5 × 10⁵ cells/ml in LPS (20 μg/ml) in the presence or absence of either anti-TGF-β₁ mAb (1D11.16.8) (1 μg/ml) or an isotype-matched control mAb (MB86) (1 μg/ml). Culture supernatants were harvested 6 d after initiation of culture for measurement of Ig isotype concentrations by ELISA. All groups were established in triplicate.
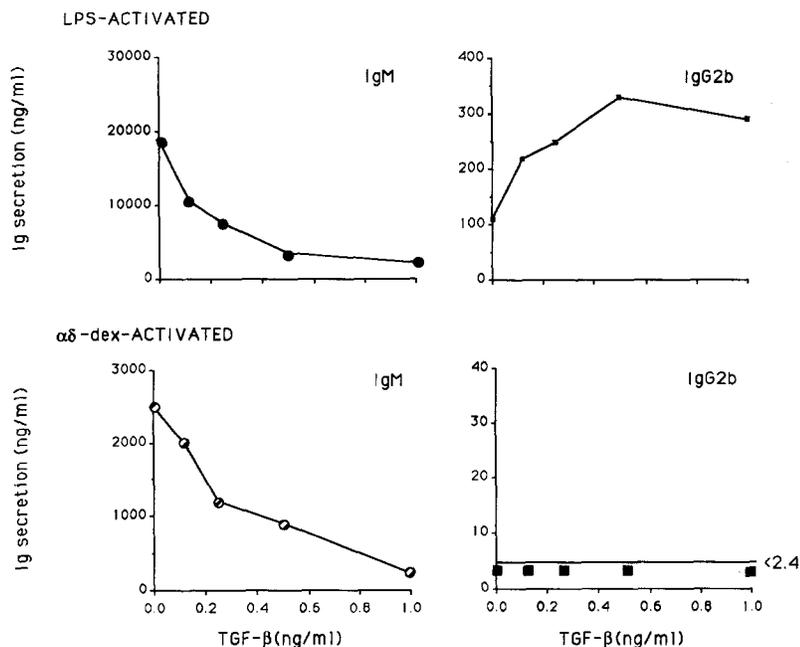
Figure 5. TGF-β₁ stimulates IgG2b secretion by LPS-stimulated, but not αδ-dex plus IL-5-activated B cells. BALB/c-derived B cells were cultured with LPS (40 μg/ml) or αδ-dex (6 ng/ml) plus IL-5 (300 U/ml) for 24 h in 100 μl of medium upon which varying concentrations of TGF-β₁ were added in 100 μl of medium for the final concentration illustrated in the figure. Culture supernatants were removed 6 d after initiation of culture for measurement of IgM and IgG2b concentrations by ELISA. All groups were established in triplicate.

induce IgG2b secretion by αδ-dex–activated B cells, we added varying amounts of TGF-β₁ to B cells induced to secrete Ig by αδ-dex plus IL-5 (Fig. 5). Although TGF-β₁ clearly acted on αδ-dex plus IL-5–activated cells by virtue of its ability to inhibit IgM secretion in a dose-dependent fashion, it failed to stimulate detectable secretion of IgG2b. This further

supported our model that the nature of the B cell activator plays a pivotal role in cytokine-directed Ig isotype production (19, 20).

Thus, our data strongly suggests that TGF-β₁ stimulates IgG2b class switching by LPS-activated B cells in that TGF-β₁: (*a*) selectively stimulates the secretion of only IgG2b

and IgA; (b) selectively increases the number of IgG2b-secreting cells; (c) acts on mIgM$^+$mIg2b$^-$ cells to induce IgG2b secretion; and (d) increases the steady-state levels of germline C$_H$γ2b RNA.

In light of our findings, it is of interest that an earlier study demonstrating the ability of TGF-β$_1$ to selectively stimulate IgA production found that TGF-β$_1$ inhibited the secretion of IgM, IgG3, IgG1, and IgG2a, but did not inhibit the synthesis of IgG2b (10). The failure to see a significant induction in IgG2b secretion may have reflected, in part, the relatively high levels of IgG2b secretion induced by LPS alone, perhaps due to endogenous TGF-β$_1$. In this regard, we observed that although DBA/2-derived B cells made substantial amounts of IgG2b in response to LPS alone (secondary to endogenous TGF-β$_1$), they secreted only modest amounts of IgA (comparable with that seen utilizing B cells from BALB/c mice). This could in part reflect the need for somewhat higher concentrations of TGF-β$_1$ for optimal IgA vs. IgG2b secretion as is suggested in Fig. 1.

Another group, which earlier reported the ability of joint fluids from rheumatoid arthritis patients to selectively stimulate IgG2b-secreting murine B cells when cultured by LPS in vitro (35), found no affect of TGF-β$_1$ on IgG2b induction (36). Although the reason for this is presently unclear, the failure of human TGF-β$_1$ at 1 and 2 ng/ml, to diminish the number of IgM-, IgG1-, and IgG3-secreting cells over that

seen with LPS alone calls into question the activity of their TGF-β$_1$ preparation. Nevertheless, the IgG2b-inducing activity in joint fluids was ascribed to a 50–60 kD molecule, suggesting that a factor in addition to TGF-β$_1$ may also be capable of enhancing the secretion of IgG2b.

We are currently investigating the basis for the strain-related differences in magnitude of in vitro LPS-induced IgG2b secretory responses. Preliminary work from our laboratory suggests that LPS-activated DBA/2-derived B cells secrete TGF-β$_1$. Whether the differences in IgG2b secretion among B cells derived from other strains of mice reflect differences in the amount of endogenous TGF-β$_1$ produced, or whether these B cells have differing sensitivities to TGF-β$_1$ action, needs to be determined. It is interesting that we recently observed that BALB/c mice immunized with LPS produced lower levels of serum, LPS-specific IgG2b, relative to IgM and IgG3, when compared with similarly immunized DBA/2 mice (Snapper, C. M., unpublished observations). Whether TGF-β$_1$ plays a physiologic role in the in vivo IgG2b response to LPS immunization is being investigated. Finally, it will be interesting to determine whether the constitutive, selective expression of germline C$_H$γ2b transcripts and C$_H$γ2b rearrangement typically seen in murine Abelson virus–transformed pre-B cell lines (33) is also due to endogenous production of TGF-β$_1$.

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