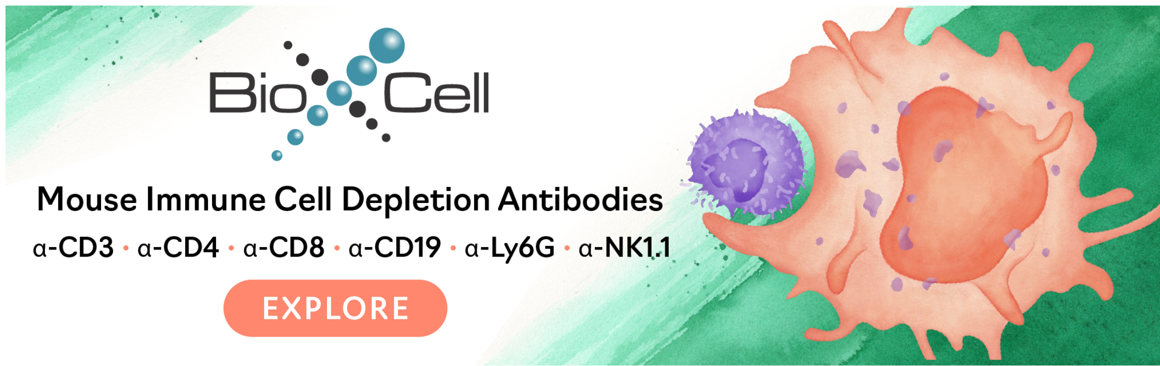

The Journal of Immunology

RESEARCH ARTICLE | OCTOBER 15 2008

B Cells from Patients with Graves' Disease Aberrantly Express the IGF-1 Receptor: Implications for Disease Pathogenesis[1] FREE

Raymond S. Douglas; ... et. al

J Immunol (2008) 181 (8): 5768–5774.
https://doi.org/10.4049/jimmunol.181.8.5768

Related Content

A Novel Murine Model of Graves' Hyperthyroidism with Intramuscular Injection of Adenovirus Expressing the Thyrotropin Receptor

J Immunol (March,2002)

Prevention of Autoantibody-Mediated Graves'-Like Hyperthyroidism in Mice with IL-4, a Th2 Cytokine

J Immunol (April,2003)

Schistosoma mansoni and α-Galactosylceramide: Prophylactic Effect of Th1 Immune Suppression in a Mouse Model of Graves' Hyperthyroidism

J Immunol (August,2004)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

B Cells from Patients with Graves' Disease Aberrantly Express the IGF-1 Receptor: Implications for Disease Pathogenesis[1]

Raymond S. Douglas,*†‡ Vibharavi Naik,† Catherine J. Hwang,† Nikoo F. Afifiyan,* Andrew G. Gianoukakis,*‡ Daniel Sand,* Shweta Kamat,* and Terry J. Smith[2]*†‡

Graves' disease (GD) is an autoimmune process involving the thyroid and connective tissues in the orbit and pretibial skin. Activating anti-thyrotropin receptor Abs are responsible for hyperthyroidism in GD. However, neither these autoAbs nor the receptor they are directed against have been convincingly implicated in the connective tissue manifestations. Insulin-like growth factor-1 receptor (IGF-1R)-bearing fibroblasts overpopulate connective tissues in GD and when ligated with IgGs from these patients, express the T cell chemoattractants, IL-16, and RANTES. Disproportionately large fractions of peripheral blood T cells also express IGF-1R in patients with GD and may account, at least in part, for expansion of IGF-1R$^+$ memory T cells. We now report a similarly skewed B cell population exhibiting the IGF-1R$^+$ phenotype from the blood, orbit, and bone marrow of patients with GD. This expression profile exhibits durability in culture and is maintained or increased with CpG activation. Moreover, IGF-1R$^+$ B cells produce pathogenic Abs against the thyrotropin receptor. In lymphocytes from patients with GD, IGF-1 enhanced IgG production ($p < 0.05$) and increased B cell expansion ($p < 0.02$) in vitro while those from control donors failed to respond. These findings suggest a potentially important role for IGF-1R display by B lymphocytes in patients with GD in supporting their expansion and abnormal Ig production. *The Journal of Immunology,* **2008, 181: 5768–5774.**

B lymphocytes play a critical role in normal immunity and the development of autoimmune disease (1–3). They initiate and support immune function through Ag presentation, expression of costimulatory molecules, and production of Abs (1, 4–6). Moreover, B cells can develop into Ig-producing plasma cells, which not only provoke host defense, but can also recognize host tissue, leading to autoimmunity (2). AutoAb production plays a central role in the pathogenesis of several of these diseases, such as rheumatoid arthritis and Graves' disease (GD)[3] (2, 7). In addition to Ig production and Ag presentation, B cells secrete many cytokines, and thus they modulate immune activation. Among these cytokines are IL-6, TNF-α, and IL-10, all of which can condition T cell responses (5, 8, 9). B cell Ag presentation is robustly up-regulated through the CD40/CD40L (CD154) pathway, which is also essential to T cell activation (10). The concept that B cells play important roles in autoimmunity receives considerable support from the effective interruption of disease progression and activity following therapeutic B cell depletion (11, 12).

GD represents a syndrome comprising thyroid growth, the overproduction of thyroid hormone, and connective tissue manifestations including orbital inflammation and expansion, processes known as thyroid-associated ophthalmopathy (TAO) (3). Aberrant production of IgGs directed at the thyrotropin receptor (TSHR) resides at the heart of the thyroid glandular component of GD (13). A portion of these Abs mimic thyrotropin in their actions, and because they are unencumbered by a normal negative feedback mechanism, result in unregulated TSHR activation and excessive hormone production (14). Patients with fully expressed GD also display infiltration of the orbit by lymphocytes which is characteristic of TAO. Why immunocompetent cells are recruited to the orbit and the circumstances surrounding the generation of Abs directed against self in this disease remain unclear. While anti-TSHR Abs convincingly account for much of the intrathyroidal pathology in GD, very little evidence has been generated to implicate them in the pathogenesis of TAO. Recently, we reported the production of IgGs directed against the insulin-like growth factor-1 receptor (IGF-1R) in patients with GD (GD-IgG) (15, 16). Moreover, IGF-1R has been found to be overexpressed by fibroblasts from these patients, and IGF-1R activation leads to signaling, which is absent in fibroblasts from control donors without autoimmune diseases. Specifically, GD-IgG/fibroblast interactions result in the production of T cell chemoattractants such as IL-16 and RANTES (15, 16). These cytokines may represent the critical signaling that leads to T cell trafficking to the orbit in TAO. Thus, multiple manifestations of GD involve B cells playing important roles in disease pathogenesis through their Ab production, Ag presentation, and participation in T cell function.

IGF-1 and IGF-1R comprise an important signaling pathway that exerts far-reaching influence on growth and development (17–19). In general, IGF-1R signaling is associated with a powerful survival advantage (20, 21). Although ubiquitous, the receptor levels vary among tissues and cell types. Moreover, IGF-1R overexpression has been linked to the malignant phenotype exhibited by certain cancers (19, 22–24). This pathway plays important roles in

*Division of Molecular Medicine, Department of Medicine, Harbor-UCLA Medical Center, Torrance, CA 90502; †Jules Stein Eye Institute, Los Angeles, CA 90095; and ‡David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

Received for publication January 8, 2008. Accepted for publication August 11, 2008.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by National Institutes of Health Grants EY008976, EY011708, DK063121, EY016339, and RR00425, an unrestricted grant from Research to Prevent Blindness, a Research to Prevent Blindness Career Development Award, and the Bell Charitable Foundation.

[2] Address correspondence and reprint requests to Dr. Terry J. Smith, Division of Molecular Medicine, Harbor-UCLA Medical Center, Building C-2, 1124 West Carson Street, Torrance, CA 90502. E-mail address: tjsmith@ucla.edu

[3] Abbreviations used in this paper: GD, Graves' disease; IGF-1R, insulin-like growth factor-1 receptor; MFI, mean fluorescence intensity; TAO, thyroid-associated ophthalmopathy; TSHR, thyrotropin receptor.

Copyright © 2008 by The American Association of Immunologists, Inc. 0022-1767/08/$2.00

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

hematopoietic cell growth and differentiation and normal immune function (19). Peripheral blood T and B cells and monocytes from control human donors express low levels of IGF-1R in vivo (25, 26). Administration of IGF-1 increases the circulating pool of $CD4^+$ T cells and splenic B cells in mice (27, 28), suggesting a role for this growth factor in hematopoietic cell expansion (29). It promotes T cell proliferation during early activation (30) and inhibits apoptosis of both immature and mature T cells through at least three distinct mechanisms (31, 32). With regard to B cell expansion, IGF-1 plays an essential role in the development of bone marrow $CD34^+$ cells into pro-B cells (33). Additionally, IGF-1 increases expression of the IL-4-induced type II IgE receptor (FcεRII/CD23) by both human primary immune cells and established B cell lines. This suggests a role in B cell activation and function (34).

We have recently reported that T lymphocytes from patients with GD represent a cell population skewed toward the $CD3^+$IGF-$1R^+$ phenotype (35). This disproportionate fraction of IGF-1R-displaying T cells can be traced to discrete subsets. In particular, the increased receptor expressing population results from an expanded memory $CD45RO^+$ T cell population. This skew was found in peripheral blood and also in cells harvested from disease-involved orbital connective tissue (35). In contrast, the abundance of $CD45RA^+$ IGF-$1R^+$ naive T cells is similar in peripheral blood from control donors and those with GD. Expression of the receptor by GD-derived T cells has functional consequences in that IGF-1 enhances BrdU incorporation and inhibits Fas-mediated apoptosis (35). Herein we report that the phenotype of B cells in patients with GD is also disproportionately biased toward the $CD19^+$IGF-$1R^+$ phenotype. Moreover, this skewed phenotype affects function, including enhanced B cell expansion and exaggerated Ab production. IGF-1 increases the production of IgG but not IgM in B cells derived from patients with GD compared with those from control donors. Thus, the underlying mechanism for aberrant IgG production that drives multiple aspects of GD may, at least in part, result from the overrepresentation of B cells displaying IGF-1R.

Materials and Methods

Materials

Ficoll-Hypaque was purchased from Sigma-Aldrich. FacLyse buffer, Cytofix, anti-CD19 CyChrome, anti-IGF-1Rα PE (clone 1H7), and isotype mouse IgG1 FITC, PE, allophycocyanin, and CyChrome were purchased from BD Biosciences. FBS was supplied by Invitrogen. IGF-1 and Des1–3 IGF-1 were from Calbiochem and GroPep, respectively.

Patient samples

Subjects, aged 20–65 years, were recruited from the patient population of Jules Stein Eye Institute and Harbor-UCLA Medical Center. Informed consent was obtained as approved by the Institutional Review Boards of the Center for Health Sciences at UCLA and Harbor-UCLA Medical Center. The study population comprised patients evaluated for GD without or with TAO. Control subjects were healthy volunteers without known autoimmune disease who presented for aesthetic or functional eyelid surgery. Individuals excluded from the study included those with nonthyroid autoimmune disease, asthma, granulomatous disease, sinusitis, or HIV infection. Patients with GD comprised a clinically heterogeneous group and included those who were hyperthyroid ($n = 3$) and euthyroid ($n = 27$). Twenty-eight of 30 patients manifested TAO. A minority of patients had active inflammatory disease (clinical activity score ≥ 3; $n = 6$), while most exhibited stable TAO (clinical activity score <3; $n = 22$). No association between IGF-1R display and disease duration or orbital inflammation was noted. Bone marrow samples were derived from a patient with GD, a healthy volunteer, or within 24 h of death. Orbital tissue was obtained from surgical waste during orbital decompression surgery in patients with TAO or from healthy individuals during cosmetic surgery. The tissue was transported on ice, homogenized, and single-cell suspensions were prepared. Tissue was filtered through 70-μm pores and processed for flow cytometry.

Clinical data including age, sex, medications, smoking history, physical exam, and laboratory values were recorded. Careful examination of the skin failed to detect evidence of thyroid-related dermopathy in any of the study participants.

Flow cytometry

Peripheral blood (~5 ml) was obtained and stored in tubes containing EDTA. Staining buffer was prepared in PBS containing 4% FBS with 0.1% sodium azide (Sigma-Aldrich). Staining for flow cytometry was performed within 24 h of blood collection, according to the manufacturer's instructions (BD Biosciences). Briefly, 100 μl whole blood or bone marrow aspirate was placed in 12 × 75 mm polypropylene tubes and fluorochrome-conjugated mAbs were added (1 μg/10^6 cells). These were then incubated in the dark for 20 min at room temperature. FACSlyse solution (2 ml) was added for 10 min at room temperature to lyse RBCs. Cells were washed twice with staining buffer, resuspended in Cytofix (BD Biosciences), and kept in the dark at 4°C until cytometric analysis (within 24 h). Analysis was performed on a FACSCalibur flow cytometer (BD Biosciences). Mean fluorescent intensity (MFI) was calculated as a ratio of mean fluorescence sample/isotype fluorescence. Percentage positive expression was determined as the population of cells with increased fluorescent intensity compared with isotype.

PBMC preparation

PBMCs were prepared using a technique described previously (36). Briefly, whole blood was diluted 1:2 in PBS and layered over Ficoll-Hypaque, centrifuged at 500 × g for 25 min, and washed three times in PBS. Cells (1 × 10^6 cells/ml) were cultured in RPMI 1640 medium supplemented with CpG (2 μg/ml) (Integrated DNA Technologies), IGF-1 (10 nM), and/or Des(1–3) IGF-1 (10 nM).

Quantification of B cell number

PBMCs were cultured as described above. An aliquot of each sample was analyzed by flow cytometry and used to calculate the fractional B cell population after live cell gate. This percentage was then multiplied by the cell number to quantify B cell number. Cell counts were determined according to the manufacturer's protocol (CyQuant; Invitrogen). Briefly, PBMCs were cultured in 96-well plates and incubated for 24–72 h. At the desired time, the 96-well plate was centrifuged at 500 × g for 5 min and the supernatant decanted by inversion. Plates were frozen at −70°C until analysis. Culture solution did not contain phenol red since the dye can interfere with CyQuant GR dye fluorescence. Plates were thawed at room temperature and 200 μl of CyQuant GR dye/cell lysis buffer was added to each well and incubated for 5 min with gentle mixing. Samples were protected from light, and emissions were determined using a Wallac Victor 1420 fluorometer (PerkinElmer) at 480 nm excitation and 520 nm emission wavelengths. They were assayed in quadruplicate and compared with a standard curve.

B cell production of IgM and IgG

PBMCs were stimulated in vitro as previously described (37). Briefly, 2.5 × 10^6 PBMC/200 μl in 96-well plates or 5 × 10^6 PBMC/ml in 24-well plates were cultured in 10% heat inactivated FBS-supplemented RPMI 1640 for 12 days. Cells were stimulated with nothing, CpG 2006 (2 μg/ml) (Integrated DNA Technologies), *Staphylococcus aureus* Cowen strain I (20 μg/ml) (Sigma-Aldrich), and pokeweed mitogen (1/100,000) in the presence or absence of IGF-1 (10 nM). The chosen CpG concentration yielded submaximal mitogen activity. IgM and IgG concentrations were determined after 12 days in culture. Supernatants were harvested and Ig concentrations were assayed using a commercially available ELISA, according to the manufacturer's specifications (Bethyl Laboratories). Sample concentrations were compared with a standard curve.

B cell production of anti-TSHR Abs was assayed in both $CD19^+$IGF-$1R^+$ and $CD19^+$IGF-$1R^-$ populations after sorting by flow cytometry (38). These cells were cultured with monocytes in a 1:2 ratio and incubated with EBV-containing supernatant generated by the B95.8 marmoset cell line for 1 h at 37°C, at a concentration of 2 × 10^6 cells/ml. After EBV exposure, cells were washed and cultured at 1 × 10^6 cells/ml (RPMI 1640, 1% L-glutamine, 1% penicillin-streptomycin, and 10% FBS) in the presence of the monocytes. After 2 wk, conditioned media were analyzed for Ab according to the manufacturer's specifications (TRAK LIA kit, BRAHMS Diagnostica).

Statistics

Values are reported as the mean ± SE. Statistical analysis was performed using a two-tailed Student's *t* test with a confidence level of >95%.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

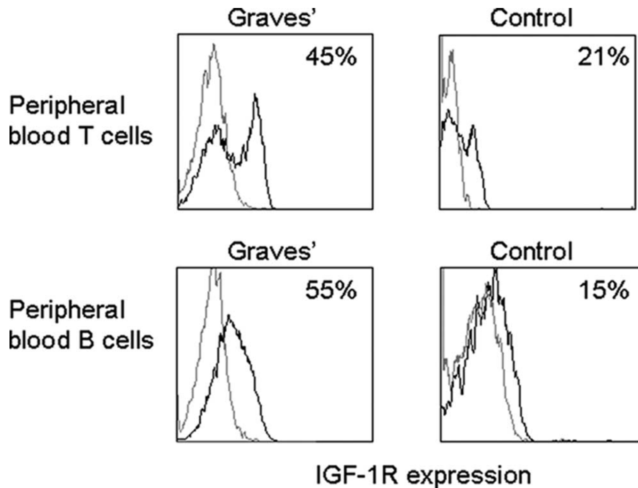

FIGURE 1. Increased fraction of peripheral blood T and B cells from a patient with GD display IGF-1R compared with those from a control donor. PBMCs were stained with anti-CD3, anti-CD19, and anti-IGF-1R Abs as described in *Materials and Methods* and subjected to multiparameter flow cytometry. The gray open histograms represent staining with isotype control Abs.

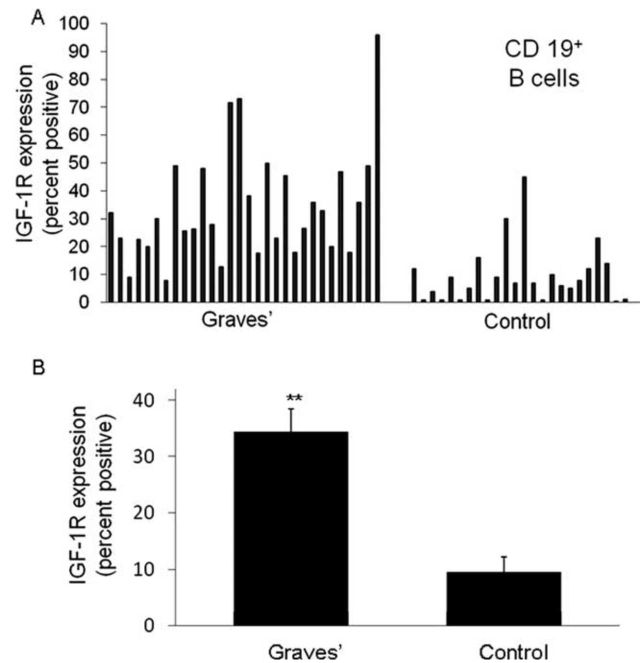

FIGURE 2. A disproportionate fraction of peripheral blood B cells from 30 patients with GD express IGF-1R compared with that found in 24 control donors. *A*, Individual data sets demonstrating fractional IGF-$1R^+$ B cells. *B*, Analysis of IGF-1R display in B cells as an aggregate of multiple patients with GD vs control donors (34 ± 4% IGF-$1R^+$ B cells (mean ± SE, $n = 30$) vs 9 ± 3% IGF-$1R^+$ control B cells ($n = 24$)). Data are expressed as means ± SE (**, $p < 1 \times 10^{-6}$).

Results

A greater proportion of B cells derived from patients with GD express IGF-1R compared with those from control donors

We have previously demonstrated that a disproportionate fraction of peripheral blood T cells from patients with GD express IGF-1R (35). $CD4^+CD45RO^+$ and $CD8^+CD45RO^+$ memory T cells from these patients displayed the greatest divergence from controls with regard to the fractional expression of IGF-1R. Given the prominent role for B cell function in the pathogenesis of GD, we have now investigated IGF-1R display by these lymphocytes in the peripheral blood and a cohort of control donors using multiparameter flow cytometry. Analogous to their T cells, B cells ($CD19^+$, IGF-$1R^+$) from donors with GD exhibit increased fractional expression of the receptor, as demonstrated in a representative study shown in Fig. 1. The disparity between control lymphocytes and those from the patients was similar to that found in their T cells. As shown in Fig. 2, cumulative data from 30 different patients with GD demonstrate that 34 ± 4% (mean ± SE) of B cells express IGF-1R, while the receptor was detected in 9 ± 3% cells from control donors ($n = 24$; $p < 10^{-6}$, GD vs controls). The range of IGF-1R expression was considerably broader among patient-derived B cells (7–98% $CD19^+$IGF-$1R^+$) compared with controls (1–45%). The fraction of IGF-$1R^+$ B cells appears durable since longitudinal examination of lymphocytes obtained serially from four different patients revealed that their abundance was invariant over a 1-year period (data not shown). Moreover, the frequency of IGF-$1R^+$ B cells remained stable following treatment with oral prednisone in a single patient and during clinical remission in two others over 6 mo of follow-up. The fraction of IGF-$1R^+$ B cells from patients with GD does not decline as a function of time in culture (data not shown), exhibiting similar phenotypic stability to that found previously in T cells (35).

We next investigated whether the skewed $CD19^+$IGF-$1R^+$ B cell phenotype found in peripheral blood from patients with GD was also present in their bone marrow and orbit. As demonstrated in Fig. 3 (*upper panels*), 49% of marrow-derived $CD19^+CD3^-$ $CD45^+CD15^-CD14^-$ B cells from a single patient with GD express IGF-1R compared with 13% of cells from a control donor, representative of two control subjects (13% and 21%). Fig. 3 (*lower panels*) shows data demonstrating that orbital and peripheral blood B lymphocytes, isolated from matched common donors with GD, exhibit similarly skewed IGF-1R display (peripheral B cells, 34 ± 6%; orbital B cells, 31 ± 5%, $n = 3$). Thus, the increased relative abundance of IGF-$1R^+$ B cells from patients

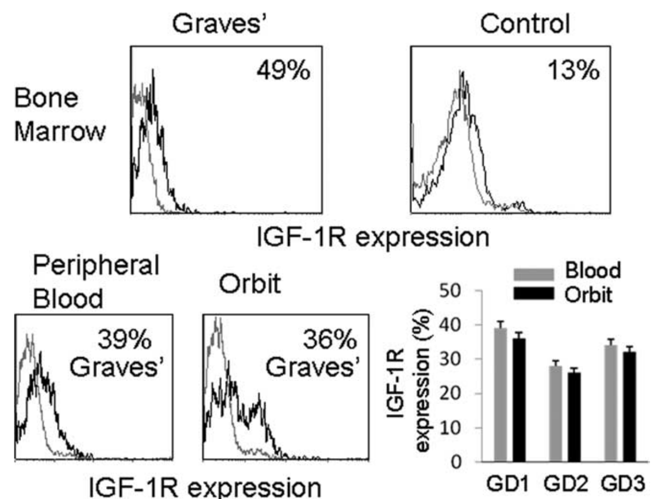

FIGURE 3. Increased fraction of bone marrow- and orbit-derived B cells from patients with GD express IGF-1R. Bone marrow aspirate (*upper panels*) from a patient with GD demonstrates increased fraction of IGF-$1R^+$ B cells compared with a control aspirate. B cells isolated from the orbit and peripheral blood of a patient with GD (*lower panels*) display increased fraction of IGF-1^+ B cells. Isotype Ab staining is shown as an open gray histogram. *Lower right*, IGF-1R expression by B cells from the peripheral blood and orbit; each sample pair was obtained from one of three separate patients.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

Table I. *Exogenous IGF-1 does not alter surface expression of CD80, CD86, or CD23 by GD or control B cells*[a]

	Hours in Culture	GD (n = 5)			Control (n = 5)		
		Total (%)	CD221$^+$ (%)	CD221$^-$ (%)	Total (%)	CD221$^+$ (%)	CD221$^-$ (%)
CD80$^+$	0	6 ± 3	1 ± 1	5 ± 2	6 ± 3	1 ±	6 ± 3
Control	48	8 ± 4	3 ± 2	5 ± 3	14 ± 7	4 ± 2	10 ± 5
CpG	48	16 ± 5	4 ± 2	11 ± 4	21 ± 12	8 ± 4	20 ± 12
IGF-1	48	8 ± 3	3 ± 2	8 ± 3	8 ± 4	2 ± 2	6 ± 4
CpG + IGF-1	48	19 ± 7	4 ± 2	15 ± 6	15 ± 6	3 ± 2	12 ± 5
CD86	0	2 ± 2	na	na	3 ± 1	na	na
Control	48	31 ± 7	9 ± 8	25 ± 8	21 ± 7	5 ± 1	17 ± 6
CpG	48	65 ± 13	15 ± 6	50 ± 14	48 ± 13	8 ± 3	40 ± 9
IGF-1	48	36 ± 16	7 ± 4	29 ± 16	19 ± 8	4 ± 1	15 ± 7
CpG + IGF-1	48	63 ± 19	7 ± 4	55 ± 17	50 ± 14	5 ± 2	45 ± 9
CD23$^+$	0	17 ± 6	4 ± 3	14 ± 7	23 ± 11	6 ± 3	18 ± 8
Control	48	19 ± 9	5 ± 2	17 ± 9	11 ± 5	3 ± 2	9 ± 6
CpG	48	52 ± 14	12 ± 5	37 ± 10	54 ± 19	8 ± 2	45 ± 18
IGF-1	48	21 ± 11	4 ± 2	19 ± 9	13 ± 7	3 ± 2	11 ± 5
CpG + IGF-1	48	52 ± 18	5 ± 2	47 ± 14	65 ± 21	8 ± 3	57 ± 19

[a] PBMC were isolated and cultured with or without CpG (2 μg/ml) and IGF-1 (10 nM) for 48 h and assayed for expression of CD80, CD86, and CD23. Each datum point represents percentage positive-staining CD19$^+$ B cells ± SEM. All treatments compared to their respective controls were not significant. na, not applicable.

with GD is similar in lymphocytes harvested from peripheral blood and orbit.

Impact of IGF-1 on B cell phenotype before and following stimulation with CpG

Since IGF-1 can influence metabolic activity and proliferation of B cells (27, 39), we investigated whether it altered the expression of costimulatory molecules or cell-surface signatures associated with lymphocyte activation. As shown in Table I, basal expression of CD23, CD80, and CD86 is similar on B cells from donors with GD and their controls. As expected, CpG stimulation increased B cell expression of all three markers. In contrast, IGF-1 failed to influence levels of any of these surface molecules after 24, 48, and 72 h (48 h shown in Table I). The vast majority of B cells expressing CD23, CD80, or CD86 fail to display IGF-1R. These cells remain IGF-1R$^-$, regardless of whether they remain untreated or are exposed to CpG, either in the absence or presence of IGF-1.

We have previously reported that expression of IGF-1R on T cells from patients with GD is aberrantly up-regulated following cell activation (35). We therefore investigated whether B cell activation was also associated with increased IGF-1R levels. CpG-provoked lymphocyte activation resulted in divergent patterns of IGF-1R display in cells from patients and their controls. This treatment either maintained or increased B cell expression of IGF-1R in most patients with GD (Fig. 4; n = 9; mean increase, 25 ± 21%). In contrast, identical treatment reduced the abundance of IGF-1R-displaying B cells from control donors (n = 5, mean decrease 64 ± 16%, $p < 0.002$ vs GD).

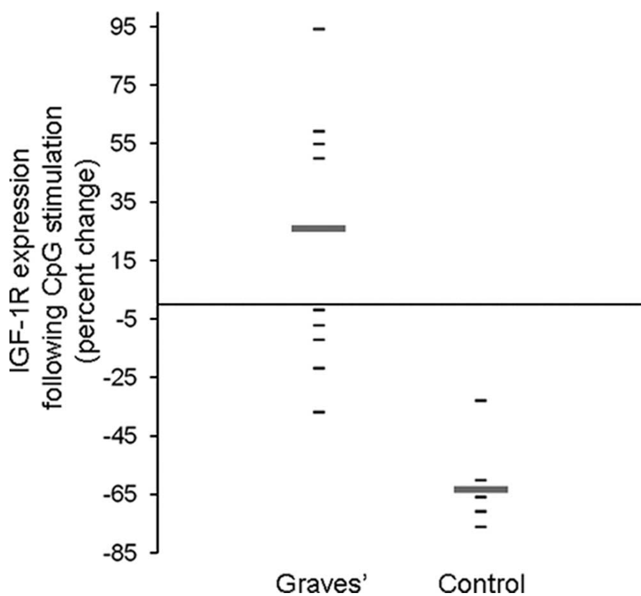

FIGURE 4. Abundance of IGF-1R$^+$ B cells from patients with GD is maintained or increased with CpG activation compared with those from control donors. PBMCs were isolated and cultured in the presence of CpG (2 μg/ml). IGF-1R display was assessed by flow cytometry at 24 h and is expressed as percentage change from unstimulated cultures. Each datum point represents a single patient's sample. Broad horizontal bars indicate mean values for each group (GD vs control, $p < 0.002$).

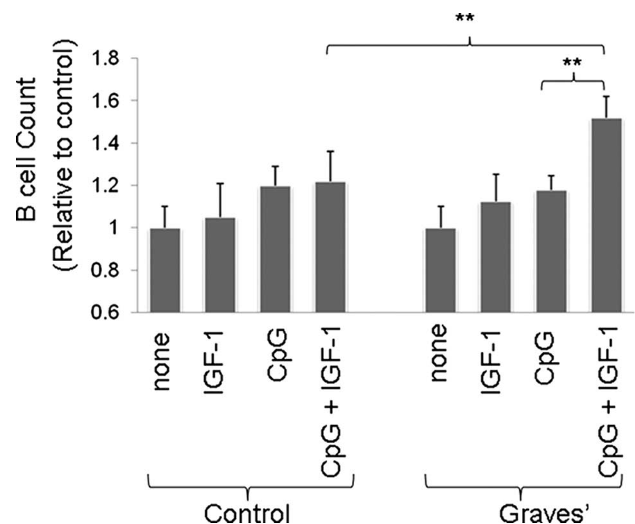

FIGURE 5. IGF-1 potentiates B cell expansion when added together with a concentration of CpG, yielding a submaximal response. B cell number was assessed as described in *Materials and Methods* after 5 days in culture with CpG (2 μg/ml) and IGF-1 (10 nM) as single agents or in combination. Data were derived from five independent experiments (mean ± SE, **, $p < 0.02$).

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

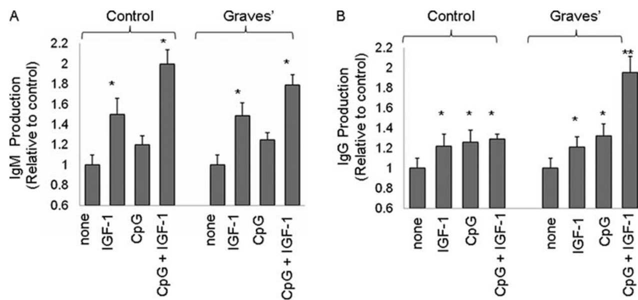

FIGURE 6. IGF-1 potentiates IgM and IgG production both in B cells from patients with GD and in those from control donors. IgM and IgG were assayed after 12 days in culture stimulated with CpG and/or IGF-1. *A*, IGF-1 (10 nM) alone or in combination with CpG (2 μg/ml) significantly increase IgM production by GD and control B cells. *B*, CpG and IGF-1, used as single agents, enhanced IgG production by B cells from both sources (*, $p < 0.05$ vs unstimulated). The combination of CpG and IGF-1 significantly increases IgG production only in B cells from patients with GD compared with treatment with either single agent (**, $p < 0.01$). Results are expressed as the means ± SE of five independent experiments.

The MFI for $CD19^+IGF\text{-}1R^+$ B cells derived from patients and control donors was similar at all time points assayed.

IGF-1 promotes expansion of GD-derived B cells

IGF-1R activation enhances myeloma proliferation and splenic B cell maturation in vivo but these effects in vitro have been found to be variable (40–42). We next investigated whether the increased fraction of circulating IGF-1R$^+$ B lymphocytes found in GD conveys a functionally different cell phenotype. PBMCs or purified B cells (>95% purity) were submaximally stimulated with CpG (2 μg/ml) in the absence or presence of IGF-1 (10 nM). IGF-1 significantly enhances B cell number in cultures derived from patients with GD when added in combination with CpG compared with lymphocytes receiving CpG or IGF-1 alone ($p < 0.02$; Fig. 5). Moreover, Des(1–3) IGF-1 (10 nM), an analog that binds to and selectively activates IGF-1R (43). also enhances the actions of CpG (data not shown; 1.4 ± 0.5-fold increase compared with CpG alone). In contrast, treatment with CpG, IGF-1, or Des(1–3) IGF-1, either as single agents or in combination, fails to expand B cells from control donors.

IGF-1 synergistically enhances the production of IgG by B cells from patients with GD but not those from control donors

Because both autoAb production and action play central roles in the pathogenesis of GD, we next investigated whether the increased fraction of IGF-1R$^+$ B cells in these patients might enhance Ab production. PBMCs from patients with GD and control donors were submaximally stimulated with CpG (2 μg/ml) in the absence or presence of IGF-1 (10 nM) for 12 days, and the conditioned medium was then assayed for IgM and IgG. IGF-1 significantly enhances production of IgM when added alone or in combination with CpG, in both GD-derived and control B cells (Fig. 6*A*; *, $p < 0.05$ treatments vs untreated). The magnitude of effect imposed by IGF-1 was similar in cells from both sources. In contrast, while IgG production is increased with IGF-1 and CpG when added as single agents in both control and disease-derived cells (Fig. 6*B*; *, $p < 0.05$, CpG or IGF-1 compared with unstimulated controls), CpG and IGF-1 appear to act synergistically only in GD-derived B cells, since the combination of these agents enhances IgG production more than either added alone (Fig. 6*B*; **, $p < 0.01$ CpG plus IGF-1 compared with IGF-1 or CpG alone). To assess the potential for IGF-1R$^+$ B cells to produce pathogenic anti-TSHR Abs, B cell populations were sorted by flow cytometry and transformed with EBV (38, 44). The IGF-1R$^+$ B cells consistently produced these Abs, while production by the IGF-1R$^-$ B cell population was variable. Production of anti-TSHR Abs by the respective B cell populations was assessed in three patients. In the first subject, GD1, IGF-1R$^+$ cells produced 20 IU/100 μl, while IGF-1R$^-$ cells produced 10 IU/100 μl. In subject GD2, IGF-1R$^+$ produced 12 IU/100 μl, while production in IGF-1R$^-$ was 0 IU/100 μl; and in subject GD3, IGF-1R$^+$ cells generated 2 IU/100 μl, while in those with the IGF-1R$^-$ phenotype, anti-TSHR Ab production was undetectable (0 IU/100 μl) (IGF-1R$^+$ vs IGF-R$^-$, $p \leq 0.06$). Thus, the expanded IGF-1R$^+$ B cell population found circulating in patients with GD may generate increased levels of pathogenic Abs.

Discussion

Herein we report for the first time that B cells from patients with GD are overrepresented by those exhibiting the IGF-1R$^+$ phenotype. This mirrors earlier findings by us that T cells (35) and fibroblasts (15) from these same patients also exhibit a strongly skewed phenotype with regard to cell-surface IGF-1R display. Thus, overexpression of this putative self-Ag appears to be more widely spread in GD than initially thought. The overabundance of IGF-1R$^+$ B cells is not limited to the peripheral circulation but can also be demonstrated in affected orbital tissues from patients with TAO (Fig. 3). This receptor display imparts several functional attributes to B cell behavior in that IGF-1 enhances both survival and Ab production in vitro. Moreover, IGF-1R$^+$ B cells can produce anti-TSHR Abs.

B cells play diverse roles in immune function by virtue of their further differentiation into Ig-secreting plasma cells and their importance in Ag presentation. A critical component of B lymphocyte development concerns how committed precursor cells from the hematopoietic lineage undergo Ig H chain gene rearrangement. This results in L chain production and leads to the release of mature B cells from the bone marrow (45). A number of factors, including IGF-1 emanating from marrow stroma, support early B cell proliferation and drive their differentiation (42, 46, 47). When administered in vivo, IGF-1 expands intrasplenic B cells through increased proliferation of mature cells (28, 48). In marrow, B cells become more abundant following IGF-1 administration in normal adult mice and also in those receiving lethal irradiation followed by reconstitution with syngeneic bone marrow (29). Additionally, IGF-1 enhances IL-7-dependent B cell proliferation in concert with *kit* ligand (39). Studies conducted in vitro disclose that the differentiation of human CD34$^+$ marrow cells is impeded by down-regulating the IGF-1 produced by cocultured MS-5 cells (46). These studies implicate IGF-binding protein-6 (IGFBP-6) as a necessary component of IGF-1-dependent B cell differentiation while IGFBP-3 may act as an inhibitor. Our examination of the bone marrow from a patient with GD discloses the ubiquitous nature of IGF-1R$^+$ B cells within this tissue and suggests the importance of the receptor and the strong survival signals it appears to convey.

IGF-1 binds peripheral human B cells selectively (49), an association that is both saturable and can be displaced with unlabelled IGF-1 and insulin. Administration of IGF-1 to mice results in elevated circulating Ab levels (28, 47). Baudler and colleagues (50) reconstituted Rag2-deficient C57BL/6 mice with fetal liver cells from IGF-1R$^{-/-}$ mice. T cell-independent humoral responses to the type 2 Ag, (4-hydroxy-3-nitrophenyl)acetyl-Ficoll, are substantially diminished while those against the T cell-dependent Ag, (4-hydroxy-3-nitrophenyl)acetyl-chicken globulin, appear normal. B cell development remains normal in IGF-1R-deficient chimeras,

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

as does T cell differentiation. In these animals, IGF-1 enhanced Ig production, an effect that proved independent of B cell proliferation (50). These earlier findings appear congruent with our own observations where incubation of B cells with IGF-1 results in a substantial increase in IgG production without concomitant changes in IgM abundance. Furthermore, Kimata et al. demonstrated that IGF-1 can enhance IgG subtype production in both tonsillar and peripheral B cells (40, 41). This effect was dependent at least in part on CD40 ligation. Our results also demonstrate that IGF-1R-displaying B cells produce activating anti-TSHR Abs and thus may participate specifically in disease pathogenesis.

IGF-1R activation results in the recruitment of docking proteins, including insulin receptor substrate (IRS) (43). This common pathway is shared by several hormone and cytokine receptors, including IL-4R, that regulate B cell Ab production (51). IRS proteins mediate IGF-1- and IL-4-stimulated activation via the PI3K signaling pathway (52). PI3K signaling positively regulates Ig production in vivo (53, 54). Increased expression of the IGF-1R may thus promote Ig production through pathways shared with IL-4R.

IGF-1 enhances DNA synthesis in myeloma cell lines in vitro in the absence of IL-6 (55). Both IGF-1 and IGF-2 up-regulate the effects of IL-6 on plasma cell proliferation. The effects are absent in normal B cells, suggesting that actions of IGF-1 may diverge in normal and malignant cells. Both IGF-1R and insulin receptors are expressed at higher levels in myeloma cell lines than in B lymphoblastoid lines (56). RPMI 8226 cells express high-affinity binding sites for both insulin and IGF-1 (57). The latter increases receptor phosphorylation, PI3K activation, DNA synthesis, and lactate production in these cells. Thus, there exists substantial precedent for IGF-1, its receptor, and the pathways it utilizes in cell signaling for regulating B cells and their plasma cell derivatives.

We have previously reported that circulating GD-IgG is directed against IGF-1R and can be detected in almost all patients with the disease, but in very few control donors (15, 16, 58). When treated with IGF-1 or with GD-IgG, cultured orbital fibroblasts from patients with GD, but not their controls, synthesize high levels of IL-16 and RANTES, two powerful T cell chemoattractants (15, 16). Additionally, orbital fibroblasts from these patients synthesize increased levels of hyaluronan when treated with GD-IgG, an action mediated by IGF-1R (59). We have also reported previously that IGF-1R is expressed by a disproportionately large fraction of fibroblasts from patients with GD (15). We now demonstrate overrepresentation of B cells displaying the IGF-1R$^+$ phenotype in these patients. Activation of these cells through this receptor appears to promote B cell survival and Ab production, including those potentially providing a mechanistic link between orbital and thyroid processes. Collectively, our findings suggest that IGF-1R may play an important role in the pathogenesis of GD. This receptor must now be examined in a broader context of human autoimmunity. It is possible that the relative abundance of IGF-1R$^+$ B cells may represent a spectrum within the general population, and that those individuals with higher levels might be more susceptible to GD and its allied diseases.

Acknowledgments

The authors are grateful to Debbie Hanaya for expert assistance in the preparation of this manuscript and to Dr. Robert Goldberg for patient recruitment. We gratefully acknowledge the invaluable assistance of the Harbor-UCLA General Clinical Research Center staff in efforts to facilitate these studies.

Disclosures

The authors have no financial conflicts of interest.

References

1. Dorner, T., and P. E. Lipsky. 2006. Signalling pathways in B cells: implications for autoimmunity. *Curr. Top Microbiol. Immunol.* 305: 213–240.
2. Martinez-Gamboa, L., H. P. Brezinschek, G. R. Burmester, and T. Dorner. 2006. Immunopathologic role of B lymphocytes in rheumatoid arthritis: rationale of B cell-directed therapy. *Autoimmun. Rev.* 5: 437–442.
3. Prabhakar, B. S., R. S. Bahn, and T. J. Smith. 2003. Current perspective on the pathogenesis of Graves' disease and ophthalmopathy. *Endocr. Rev.* 24: 802–835.
4. Tandon, N., R. A. Metcalfe, D. Barnett, and A. P. Weetman. 1994. Expression of the costimulatory molecule B7/BB1 in autoimmune thyroid disease. *Q. J. Med.* 87: 231–236.
5. Sato, S., M. Fujimoto, M. Hasegawa, K. Takehara, and T. F. Tedder. 2004. Altered B lymphocyte function induces systemic autoimmunity in systemic sclerosis. *Mol. Immunol.* 41: 1123–1133.
6. Bour-Jordan, H., B. L. Salomon, H. L. Thompson, R. Santos, A. K. Abbas, and J. A. Bluestone. 2007. Constitutive expression of B7-1 on B cells uncovers autoimmunity toward the B cell compartment in the nonobese diabetic mouse. *J. Immunol.* 179: 1004–1012.
7. El Fassi, D., C. H. Nielsen, H. C. Hasselbalch, and L. Hegedus. 2006. The rationale for B lymphocyte depletion in Graves' disease. Monoclonal anti-CD20 antibody therapy as a novel treatment option. *Eur. J. Endocrinol.* 154: 623–632.
8. Aggarwal, B. B., S. Shishodia, Y. Takada, D. Jackson-Bernitsas, K. S. Ahn, G. Sethi, and H. Ichikawa. 2006. TNF blockade: an inflammatory issue. *Ernst Schering Res. Found. Workshop* 56: 161–186.
9. Moore, K. W., R. de Waal Malefyt, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19: 683–765.
10. Salmaso, C., D. Olive, G. Pesce, and M. Bagnasco. 2002. Costimulatory molecules and autoimmune thyroid diseases. *Autoimmunity* 35: 159–167.
11. Yanaba, K., Y. Hamaguchi, G. M. Venturi, D. A. Steeber, E. W. St. Clair, and T. F. Tedder. 2007. B cell depletion delays collagen-induced arthritis in mice: arthritis induction requires synergy between humoral and cell-mediated immunity. *J. Immunol.* 179: 1369–1380.
12. Nielsen, C. H., D. El Fassi, H. C. Hasselbalch, K. Bendtzen, and L. Hegedus. 2007. B-cell depletion with rituximab in the treatment of autoimmune diseases. Graves' ophthalmopathy the latest addition to an expanding family. *Expert Opin. Biol. Ther.* 7: 1061–1078.
13. Chen, C. R., P. Pichurin, Y. Nagayama, F. Latrofa, B. Rapoport, and S. M. McLachlan. 2003. The thyrotropin receptor autoantigen in Graves disease is the culprit as well as the victim. *J. Clin. Invest.* 111: 1897–1904.
14. Akamizu, T. 2003. Monoclonal antibodies to thyroid specific autoantigens. *Autoimmunity* 36: 361–366.
15. Pritchard, J., R. Han, N. Horst, W. W. Cruikshank, and T. J. Smith. 2003. Immunoglobulin activation of T cell chemoattractant expression in fibroblasts from patients with Graves' disease is mediated through the insulin-like growth factor I receptor pathway. *J. Immunol.* 170: 6348–6354.
16. Pritchard, J., N. Horst, W. Cruikshank, and T. J. Smith. 2002. Igs from patients with Graves' disease induce the expression of T cell chemoattractants in their fibroblasts. *J. Immunol.* 168: 942–950.
17. Bateman, J. M., and H. McNeill. 2006. Insulin/IGF signalling in neurogenesis. *Cell Mol. Life Sci.* 63: 1701–1705.
18. Binz, K., P. Joller, P. Froesch, H. Binz, J. Zapf, and E. R. Froesch. 1990. Repopulation of the atrophied thymus in diabetic rats by insulin-like growth factor I. *Proc. Natl. Acad. Sci. USA* 87: 3690–3694.
19. Zumkeller, W. 2002. The insulin-like growth factor system in hematopoietic cells. *Leuk. Lymphoma* 43: 487–491.
20. Reiss, K., P. Porcu, C. Sell, Z. Pietrzkowski, and R. Baserga. 1992. The insulin-like growth factor 1 receptor is required for the proliferation of hemopoietic cells. *Oncogene* 7: 2243–2248.
21. Roldan, A., E. Charreau, R. Schillaci, E. M. Eugui, and A. C. Allison. 1989. Insulin-like growth factor-1 increases the mitogenic response of human peripheral blood lymphocytes to phytohemagglutinin. *Immunol. Lett.* 20: 5–8.
22. Kurmasheva, R. T., and P. J. Houghton. 2006. IGF-I mediated survival pathways in normal and malignant cells. *Biochim. Biophys. Acta* 1766: 1–22.
23. Ahlen, J., J. Wejde, O. Brosjo, A. von Rosen, W. H. Weng, L. Girnita, O. Larsson, and C. Larsson. 2005. Insulin-like growth factor type 1 receptor expression correlates to good prognosis in highly malignant soft tissue sarcoma. *Clin. Cancer Res.* 11: 206–216.
24. Zadik, Z., Z. Estrov, Y. Karov, T. Hahn, and Y. Barak. 1993. The effect of growth hormone and IGF-I on clonogenic growth of hematopoietic cells in leukemic patients during active disease and during remission: a preliminary report. *J. Pediatr. Endocrinol.* 6: 79–83.
25. Kooijman, R., M. Willems, C. J. De Haas, G. T. Rijkers, A. L. Schuurmans, S. C. Van Buul-Offers, C. J. Heijnen, and B. J. Zegers. 1992. Expression of type I insulin-like growth factor receptors on human peripheral blood mononuclear cells. *Endocrinology* 131: 2244–2250.
26. Kooijman, R. K., L. E. Scholtens, G. T. Rijkers, and B. J. Zegers. 1995. Differential expression of type I insulin-like growth factor receptors in different stages of human T cells. *Eur. J. Immunol.* 25: 931–935.
27. Clark, R., J. Strasser, S. McCabe, K. Robbins, and P. Jardieu. 1993. Insulin-like growth factor-1 stimulation of lymphopoiesis. *J. Clin. Invest.* 92: 540–548.
28. Jardieu, P., R. Clark, D. Mortensen, and K. Dorshkind. 1994. In vivo administration of insulin-like growth factor-I stimulates primary B lymphopoiesis and enhances lymphocyte recovery after bone marrow transplantation. *J. Immunol.* 152: 4320–4327.
29. Alpdogan, O., S. J. Muriglan, B. J. Kappel, E. Doubrovina, C. Schmaltz, R. Schiro, J. M. Eng, A. S. Greenberg, L. M. Willis, J. A. Rotolo, R. J. O'Reilly, and M. R. van den Brink. 2003. Insulin-like growth factor-I enhances lymphoid

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024

and myeloid reconstitution after allogeneic bone marrow transplantation. *Transplantation* 75: 1977–1983.
30. Hettmer, S., L. Dannecker, J. Foell, M. W. Elmlinger, and G. E. Dannecker. 2005. Effects of insulin-like growth factors and insulin-like growth factor binding protein-2 on the in vitro proliferation of peripheral blood mononuclear cells. *Hum. Immunol.* 66: 95–103.
31. Liu, E., H. K. Law, and Y. L. Lau. 2003. Insulin-like growth factor I promotes maturation and inhibits apoptosis of immature cord blood monocyte-derived dendritic cells through MEK and PI 3-kinase pathways. *Pediatr. Res.* 54: 919–925.
32. Navarro, M., and R. Baserga. 2001. Limited redundancy of survival signals from the type 1 insulin-like growth factor receptor. *Endocrinology* 142: 1073–1081.
33. Landreth, K. S., R. Narayanan, and K. Dorshkind. 1992. Insulin-like growth factor-I regulates pro-B cell differentiation. *Blood* 80: 1207–1212.
34. Kim, J. H., H. H. Park, and C. E. Lee. 2003. IGF-1 potentiation of IL-4-induced CD23/FcεRII expression in human B cells. *Mol. Cells* 15: 307–312.
35. Douglas, R. S., A. G. Gianoukakis, S. Kamat, and T. J. Smith. 2007. Aberrant expression of the insulin-like growth factor-1 receptor by T cells from patients with Graves' disease may carry functional consequences for disease pathogenesis. *J. Immunol.* 178: 3281–3287.
36. Coligan, J., A. Kruisbeek, D. Marguiles, E. Shevach, and W. Strober, eds. 2006. *Current Protocols in Immunology.* John Wiley and Sons, New York.
37. Ryan, E. P., S. J. Pollock, T. I. Murant, S. H. Bernstein, R. E. Felgar, and R. P. Phipps. 2005. Activated human B lymphocytes express cyclooxygenase-2 and cyclooxygenase inhibitors attenuate antibody production. *J. Immunol.* 174: 2619–2626.
38. Fan, J. L., R. K. Desai, J. S. Dallas, N. M. Wagle, G. S. Seetharamaiah, and B. S. Prabhakar. 1994. High frequency of B cells capable of producing anti-thyrotropin receptor antibodies in patients with Graves' disease. *Clin. Immunol. Immunopathol.* 71: 69–74.
39. Gibson, L. F., D. Piktel, and K. S. Landreth. 1993. Insulin-like growth factor-1 potentiates expansion of interleukin-7-dependent pro-B cells. *Blood* 82: 3005–3011.
40. Kimata, H., and M. Fujimoto. 1994. Growth hormone and insulin-like growth factor I induce immunoglobulin (Ig)E and IgG4 production by human B cells. *J. Exp. Med.* 180: 727–732.
41. Kimata, H., and A. Yoshida. 1994. Differential effect of growth hormone and insulin-like growth factor-I, insulin-like growth factor-II, and insulin on Ig production and growth in human plasma cells. *Blood* 83: 1569–1574.
42. Kimata, H., and A. Yoshida. 1994. Effect of growth hormone and insulin-like growth factor-I on immunoglobulin production by and growth of human B cells. *J. Clin. Endocrinol. Metab.* 78: 635–641.
43. De Meyts, P., J. Palsgaard, W. Sajid, A. M. Theede, and H. Aladdin. 2004. Structural biology of insulin and IGF-1 receptors. *Novartis Found. Symp.* 262: 160–171; discussion 171–176, 265–268.
44. Traggiai, E., S. Becker, K. Subbarao, L. Kolesnikova, Y. Uematsu, M. R. Gismondo, B. R. Murphy, R. Rappuoli, and A. Lanzavecchia. 2004. An efficient method to make human monoclonal antibodies from memory B cells: potent neutralization of SARS coronavirus. *Nat. Med.* 10: 871–875.
45. Paul, W., ed. 2003. *Fundamental Immunology,* 5th Ed. Lippincott Williams & Wilkins, Philadelphia, pp. 159–194.
46. Taguchi, T., H. Takenouchi, J. Matsui, W. R. Tang, M. Itagaki, Y. Shiozawa, K. Suzuki, S. Sakaguchi, Y. U. Ktagiri, T. Takahashi, et al. 2006. Involvement of insulin-like growth factor-I and insulin-like growth factor binding proteins in pro-B-cell development. *Exp. Hematol.* 34: 508–518.
47. Robbins, K., S. McCabe, T. Scheiner, J. Strasser, R. Clark, and P. Jardieu. 1994. Immunological effects of insulin-like growth factor-I: enhancement of immunoglobulin synthesis. *Clin. Exp. Immunol.* 95: 337–342.
48. Hinton, P. S., C. A. Peterson, E. M. Dahly, and D. M. Ney. 1998. IGF-I alters lymphocyte survival and regeneration in thymus and spleen after dexamethasone treatment. *Am. J. Physiol.* 274: R912–R920.
49. Stuart, C. A., R. T. Meehan, L. S. Neale, N. M. Cintron, and R. W. Furlanetto. 1991. Insulin-like growth factor-I binds selectively to human peripheral blood monocytes and B-lymphocytes. *J Clin. Endocrinol. Metab.* 72: 1117–1122.
50. Baudler, S., J. Baumgartl, B. Hampel, T. Buch, A. Waisman, C. M. Snapper, W. Krone, and J. C. Bruning. 2005. Insulin-like growth factor-1 controls type 2 T cell-independent B cell response. *J. Immunol.* 174: 5516–5525.
51. Keegan, A. D., K. Nelms, M. White, L. M. Wang, J. H. Pierce, and W. E. Paul. 1994. An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. *Cell* 76: 811–820.
52. Keegan, A. D., K. Nelms, L. M. Wang, J. H. Pierce, and W. E. Paul. 1994. Interleukin 4 receptor: signaling mechanisms. *Immunol. Today* 15: 423–432.
53. Clayton, E., G. Bardi, S. E. Bell, D. Chantry, C. P. Downes, A. Gray, L. A. Humphries, D. Rawlings, H. Reynolds, E. Vigorito, and M. Turner. 2002. A crucial role for the p110δ subunit of phosphatidylinositol 3-kinase in B cell development and activation. *J. Exp. Med.* 196: 753–763.
54. Omori, S. A., and R. C. Rickert. 2007. Phosphatidylinositol 3-kinase (PI3K) signaling and regulation of the antibody response. *Cell Cycle* 6: 397–402.
55. Jelinek, D. F., T. E. Witzig, and B. K. Arendt. 1997. A role for insulin-like growth factor in the regulation of IL-6-responsive human myeloma cell line growth. *J. Immunol.* 159: 487–496.
56. Freund, G. G., D. T. Kulas, B. A. Way, and R. A. Mooney. 1994. Functional insulin and insulin-like growth factor-1 receptors are preferentially expressed in multiple myeloma cell lines as compared to B-lymphoblastoid cell lines. *Cancer Res.* 54: 3179–3185.
57. Freund, G. G., D. T. Kulas, and R. A. Mooney. 1993. Insulin and IGF-1 increase mitogenesis and glucose metabolism in the multiple myeloma cell line, RPMI 8226. *J. Immunol.* 151: 1811–1820.
58. Smith, T. J. 2003. The putative role of fibroblasts in the pathogenesis of Graves' disease: evidence for the involvement of the insulin-like growth factor-1 receptor in fibroblast activation. *Autoimmunity* 36: 409–415.
59. Smith, T. J., and N. Hoa. 2004. Immunoglobulins from patients with Graves' disease induce hyaluronan synthesis in their orbital fibroblasts through the self-antigen, insulin-like growth factor-I receptor. *J. Clin. Endocrinol. Metab.* 89: 5076–5080.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/181/8/5768/1264744/zim02008005768.pdf by guest on 19 April 2024