Brief Definitive Report

Human Interleukin 10 Induces Naive Surface Immunoglobulin D$^+$ (sIgD$^+$) B Cells to Secrete IgG1 and IgG3

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Summary

During antigen-induced immune responses, human B cells switch isotype from immunoglobulin M (IgM)-IgD to IgG1-4, IgA1-2, or IgE. In the human, no cytokines have yet been demonstrated to act as switch factors for IgG1, IgG2, and IgG3. In this paper, we report that in response to interleukin 10 (IL-10), anti-CD40 activated tonsillar surface IgD$^+$ (sIgD$^+$) B cells are induced to secrete large amounts of IgM, IgG1, and IgG3 but neither IgG2 nor IgG4. Cord blood purified B cells and lymphocytes from Hyper-IgM patients also produced IgG1 and IgG3 after culture with anti-CD40 and IL-10. In contrast, sIgD$^-$ isotype-committed B cells produce IgG1, IgG2, and IgG3 when activated through CD40 in the presence of IL-10. Thus, in addition to its growth-promoting and differentiating activities on human B cells, IL-10 may represent a switch factor for IgG1 and IgG3.

Human B lymphocytes can produce nine different isotypes of antibody including IgM, IgD, IgG1-4, IgA1-2, and IgE. Each of the four subclasses of human IgG molecules, numbered according to their relative frequency in serum, exhibit a unique profile of effector functions allowing the elimination of pathogenic agents through different mechanisms (1). The IgG1 and IgG3 are predominantly produced in response to protein antigens (2) but chronic stimulation with such antigens results in an increased proportion of IgG4 (1). In contrast, carbohydrate antigens most often induce IgG2 responses (2). A certain commonalty in cytokine regulation of isotype switching between human and murine can be noted. In both species, IL-4 directs switching to IgE secretion (3, 4) and mice with a disrupted IL-4 gene are unable to produce IgE (5). TGF-β is involved in switching towards IgA in both species, although addition of IL-10 is necessary for human B cells (6, 7). IFN-γ is able to induce LPS-activated B cells to secrete IgG2a (8) and dextran-conjugated anti-IgD activated B cells to produce both IgG2a and IgG3 (9). In vivo studies shows that administration of IFN-γ stimulates the IgG2a response. Likewise, mice treated with neutralizing anti-IFN-γ antibodies (10) and mice whose IFN-γ receptor gene has been inactivated (11), show a strong reduction of IgG2a responses. Evolution by duplication of a common ancestral γ chain gene in both species yielded four different IgG subclasses, but this occurred early after speciation for the mouse and later in evolution for humans. Thus, different mechanisms are likely to be used for IgG subclass regulation in mice and in humans. However, it is interesting that IL-4 induces switching towards mouse IgG1 (12) and human IgG4 (13) which are both noncomplement-fixing subclasses. Apart from IL-4 (13) and IL-13 (14) which are involved in the switch towards IgG4, the cytokines that direct the switch towards human IgG subclasses have not been identified. Activation through CD40 allows proliferation but not antibody secretion of naive surface (s)IgD$^+$sIgM$^+$ B cells and further addition of particles of *Staphylococcus aureus* (SAC) enhances their growth and induces them to secrete high levels of IgM. In the presence of IL-10, naive sIgD$^+$sIgM$^+$ B cells activated through CD40 are induced to secrete large amounts of IgM, IgG1, and IgG3, but neither IgG2 nor IgG4. This indicates that IL-10 may represent a switch factor for IgG1 and IgG3.

Materials and Methods

Reagents. The anti-CD40 mAb89 was presented by the CDw32/FCγRII transfected Ltk$^-$ cell line (CDw32 L cells) as described earlier (15). The mAbs used for phenotypic studies were FITC-conjugated anti-CD3, -CD19, -CD14, and -CD20 obtained from Becton Dickinson & Co. (Mountain View, CA).

Each of the cytokines listed below was tested at various doses and below the optimal concentration point determined in specific bioassays as described previously (15). IL-1α and IL-1β (10^8 U/mg), IL-6 (10^7 U/mg), and TNF-α (2×10^7 U/mg) were purchased from Genzyme Corp. (Cambridge, MA). They were used at 10, 100, 200, and 2.5 ng/ml, respectively. Purified rhIL-2 (Amgen Biologicals, Thousand Oaks, CA, 3×10^6 U/ml), rIL-3 (5×10^6

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U/mg), rhIL-4 (10^7 U/mg), rIL-5 (10^7 U/mg), and rhIL-10 (10^7 U/ml) (Schering-Plough Research Institute) were used at 20 U/ml, 10 ng/ml, 50 U/ml, and 100 ng/ml, respectively. IL-7 (provided by Dr. F. Lee, DNAX Research Institute, Palo Alto, CA) was used at 5% dilution (~10 ng/ml) of a culture supernatant of Cos7 cells transfected with the human IL-7 cDNA clone. TGF-β1 (R&D Systems, Inc., Minneapolis, MN) was tested at 0.5 ng/ml. IFN-γ (10^7 U/mg) was purchased from Amgen Biologicals and was used at 500 U/ml.

Patient. PBMC from a patient (T. G.) with X-linked Hyper-IgM syndrome reported previously (16) were used in this study.

Isolation of $sIgD^+$ and $sIgD^-$ B Lymphocytes. Mononuclear cells from tonsils, peripheral blood, and cord blood were isolated by a standard Ficoll-Hypaque (dose = 1,077 g/ml) gradient method. Tonsillar B cells were first enriched in the E^- fraction and submitted to anti-CD2, -CD3, and -CD14 mAb negative selection with magnetic beads coated with anti-mouse IgG (Dynabeads; Dynal, Oslo, Norway). Purified B lymphocytes were separated using a preparative magnetic cell sorter (MACS®, Becton Dickinson & Co.). The separation based on sIgD expression has been described in details earlier (7). IgD was expressed on >99% of the $sIgD^+$ B cell subpopulation and <1% of $sIgD^-$ B cell subpopulation, as assessed by fluorescence analysis using a FACScan® (Becton Dickinson & Co.).

Culture in the CD40 System. For Ig production, 2.5×10^4 purified B cells or PBMC were cultured in the presence of 2.5×10^3 irradiated (7,000 rad) CDw32 L cells and 0.5 μg/ml of anti-CD40 mAb89 in a final volume of 200 μl (15). Formalinized particles of *S. aureus* strain Cowan I (SAC) were purchased as Pansorbin from Calbiochem-Novabiochem Corp. (La Jolla, CA) and were used at the final concentration of 0.005% (vol/vol). Supernatants were harvested after 10 d (unless otherwise stated) and IgG, IgA, and IgM levels were determined by ELISA.

ELISAs for IgG Subclasses. ELISA specific for IgG subclasses was used as described in detail elsewhere (17). Flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) were directly coated for the titration of human IgG_4 with mouse anti-human IgG4 (HP6023, used at 1 μg/ml) (Calbiochem) or coated with rat anti-mouse IgG1, mAb LO-MG1-13, provided by Dr. Bazin (University of Louvain, Brussels, Belgium) used at 5 μg/ml in carbonate buffer (pH 9.6) for the titrations of IgG1, IgG2, and IgG3. After incubation for 18 h at 4°C, the plates were washed in PBS/0.05% Tween (Merck Sharp & Dohme, West Point, PA) and were incubated 2 h at room temperature with mouse anti-human IgG1 (M15015 used at 0.5 μg/ml) (Oxoid Unipath Ltd., Hampshire, UK) or mouse anti-human IgG2 (HP6002 used at 0.75 μg/ml) (Calbiochem) or mouse anti-human IgG3 (HP6050 used at 0.75 μg/ml) (Calbiochem) in PBS/Tween-1% BSA. Next, the culture supernatants were diluted to the appropriate concentration in PBS/4% nonfat milk, added to the plates, and incubated for 2 h at room temperature. A standard human serum (Janssen, Amsterdam, The Netherlands) was used as reference. The plates were then washed and a goat anti-human IgG Fc coupled to alkaline phosphatase (A-9544) (Sigma Chemical Co., St. Louis, MO) was added at a final dilution of 1:2,000 in PBS/Tween-1% BSA for 2 h at room temperature. After washing, IgG subclasses were measured by incubation with *p*-nitrophenyl phosphate (Sigma) in 1 M diethanolamine/HCl buffer, pH 9.8, and the plates were read with a V-MAX ELISA reader (Molecular Devices, Palo Alto, CA). The limits of sensitivity were 50, 90, 65, and 30 ng/ml for IgG1, IgG2, IgG3 and IgG4, respectively.

Results and Discussion

Total tonsillar B cells cultured in the CD40 system, composed of a fibroblast cell line expressing a CDw32/Fcγ receptor and a mAb specific for the CD40 antigen, proliferate strongly while producing only small amounts of Igs (15, 18, 19). Unseparated tonsillar B cells cultured in the CD40 system alone produced very little IgM and none of the four IgG subclasses (Table 1). Addition of 100 ng/ml IL-10 to these cultures induced B cells to produce considerable amounts of IgM (20,500 ng/ml) and IgG (~80,000 ng/ml) after 10 d of culture (7, 19) (Table 1). Furthermore, in response to IL-10, anti-CD40 activated B cells produced high levels of IgG1, IgG2, and IgG3, representing 80–85%, 4–17%, and 2–5% of the secreted IgG, respectively.

Tonsillar B cells consist of naive B cells, expressing surface IgM and IgD, and isotype-committed B cells that have undergone isotype switch and express surface IgG, IgA, or IgE but have lost expression of IgD and IgM. Naive B cells, separated according to sIgD expression, did not produce detectable amounts of IgM and IgG when triggered through the CD40 antigen (7) (Fig. 1). Addition of IL-10 enhanced IgM production from a mean value of 120–19,200 ng/ml (mean of eight separate experiments, Fig. 1 *A*), in agreement with the notion that IL-10 is a potent B cell differentiation factor (19). Most importantly, in response to IL-10, naive B lymphocytes were induced to secrete IgG from levels <50 ng/ml to a mean value of 3,700 ng/ml (mean of eight separate experiments, Fig. 1 *B*). As shown in Fig. 1 *C*, IL-10 was the only one among 12 recombinant cytokines with recognized B cell tropism (19) to enhance the production of IgG by anti-CD40 activated naive B cells.

Table 1. *Unseparated B Cells Cultured in the CD40 System Secrete IgG1, IgG2, and IgG3 in Response to IL-10*

IL-10	IgG1	IgG2	IgG3	IgG4	IgM
			ng/ml		
–	<50	<90	<65	<30	50 ± 5
+	68,520 ± 1,050	14,235 ± 500	1,830 ± 280	<30	20,500 ± 2,300

Purified tonsillar B cells (5×10^4 cells/well; >99% $CD20^+$) were cultured in flat-bottom microwells for 10 d on 5×10^3 irradiated CDw32 L cells with 0.5μg/ml mAb89 without or with 100 ng/ml IL-10. Supernatants were harvested after 10 d and Ig levels were determined by ELISA. Results are means ± SD of quadruplicates culture. Representative of one out of three experiments.

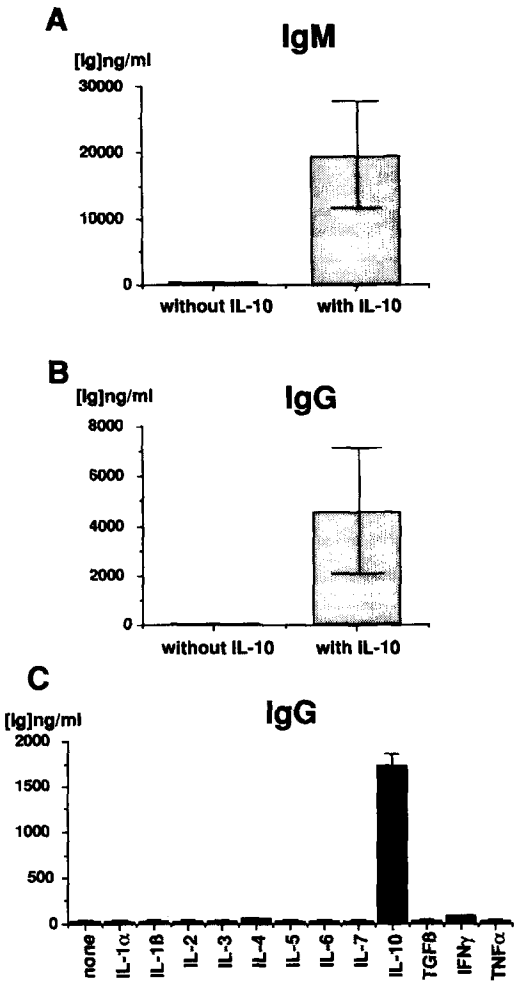

Figure 1. IL-10 induces anti-CD40 activated naive sIgD$^+$ sIgM$^+$ B cells to secrete high levels of IgM and IgG. (*A* and *B*) 5 × 10^4 MACS®-purified sIgD$^+$ B cells were cultured for 10 d with 5 × 10^3 irradiated CDw32 L cells with mAb89 without or with 100 ng/ml IL-10. The results of eight experiments performed over a 2-yr period are shown. (*C*) B cells were cultured with medium alone, 10 ng/ml IL-1α, 100 ng/ml IL-1β, 20 U/ml IL-2, 10 ng/ml IL-3, 50 U/ml IL-4, 10 ng/ml IL-5, 200 ng/ml IL-6, 10 ng/ml IL-7, 100 ng/ml IL-10, 0.5 ng/ml TGF-β1, 500 U/ml IFN-γ, and 2.5 ng/ml TNF-α. Supernatants were harvested after 10 d and IgM and IgG levels were determined by ELISA. Results are means of quadruplicate cultures.

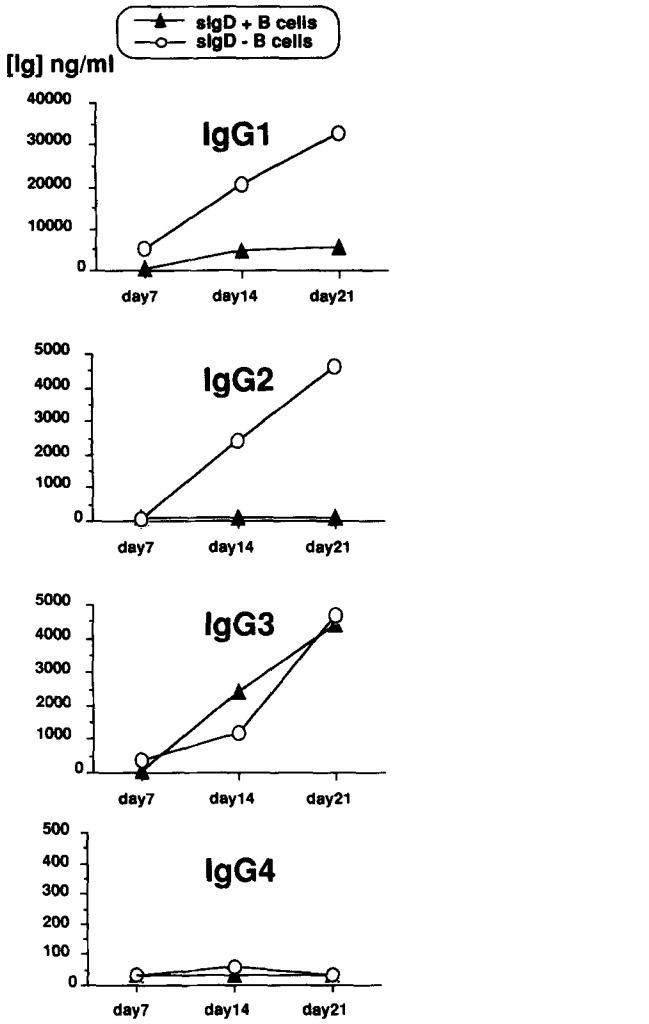

Figure 2. IL-10 induces naive sIgD$^+$ B cells to produce IgG1 and IgG3 and isotype-committed sIgD$^-$ B cells to secrete IgG1, IgG2, and IgG3. 5 × 10^4 MACS®-purified sIgD$^+$ and sIgD$^-$ B cells were cultured for 7, 14, and 21 d with 5 × 10^3 irradiated CDw32 L cells with mAb89 in the presence of 100 ng/ml IL-10. Supernatants were harvested and IgG subclasses levels were determined by ELISA. Ig levels were also measured in day 28 supernatants (data not shown) and found comparable to those of day 21 supernatants. Results are means ± SD of quadruplicate cultures. Representative of three experiments.

A more detailed analysis of IgG production demonstrated a differential effect of IL-10 on the various IgG subclasses. IL-10 induced naive sIgD$^+$ B cells to synthesize exclusively IgG1 and IgG3 (Fig. 2). After 3 wk of culture, IgG1 and IgG3 production by sIgD$^+$ B cells reached a maximum (5,750 and 4,400 ng/ml, respectively) whereas these cells secreted <90 ng/ml IgG2 and <50 ng/ml IgG4. The lack of IgG2 production was not due to inhibition by IL-10 since IL-10 induced high levels of IgG2 from the isotype-committed sIgD$^-$ B cell population (Fig. 2). The difference between IgG2 levels induced in naive and isotype-committed populations also indicates that contamination of the naive B cell population by isotype-committed B cells was minimal. Thus, the high levels of IgG1 and IgG3 produced by the naive B cells in response to IL-10 strongly suggest that they result from isotype switch. The kinetics of IgG1 and IgG3 production were also consistent with switching, since isotype-committed B cells secreted 5,300 ng/ml IgG1 and 540 ng/ml IgG3 as early as 7 d after stimulation whereas these isotypes remained undetectable in the supernatants of naive B cells. The delay in IgG subclasses secretion is consistent with the time required for the genetic recombination within the H chain locus to occur. This IgG1- and IgG3-inducing activity of IL-10 was not related to the organ origin of the B cells as identical results had been obtained with B cells isolated from spleens (data not shown).

Since both IL-10 and anti-CD40 were required for the secretion of IgM as well as IgG1 and IgG3, the results presented above do not allow discrimination between the respective roles of IL-10 and anti-CD40 in inducing the actual isotype switch. To distinguish between these two potential switch signals, we used *S. aureus* Cowan I strain (SAC) as a costimulus in the anti-CD40 system. Addition of SAC resulted in the induction of considerable IgM secretion (30,000 ng/ml) by naive tonsillar B cells, but IgG levels were still undetectable (Fig. 3 *A*). When IL-10 was added to the anti-CD40/SAC system, IgM secretion was moderately enhanced (fourfold), whereas IgG1 and IgG3 were strongly induced (from undetectable values to 2,910 and 3,030 ng/ml, respectively), and IgG2 or IgG4 were still undetectable. By comparison, unseparated B cells activated through CD40 and SAC secreted considerable amounts of IgM but also IgG1, IgG2, and IgG3, without adding exogeneous cytokines. Addition of IL-10 to the anti-CD40/SAC system further enhanced the production of IgM (25-fold), IgG1 (threefold), IgG2 (2.6-fold), and IgG3 (40-fold) from unseparated B cells (Fig. 3 *B*). These results indicate that IL-10 rather than anti-CD40 was responsible for the secretion of IgG1 and IgG3 and that the effect on IgG3 was particularly striking on naive B cells as well as on unseparated B lymphocytes. Furthermore, limiting dilution analysis indicated that IL-10 acted through increasing the frequency of sIgD$^+$ B cells secreting either IgG1 (1:1,800) or IgG3 (1:600) in the anti-CD40/SAC system, whereas in the absence of IL-10, <1:5,000 B cells produced IgG1 or IgG3 (data not shown).

Cells isolated from cord blood were used as another source of B lymphocytes, since this population does not contain detectable numbers of isotype-committed cells. However, neonatal B cells, which are deficient in their ability to produce

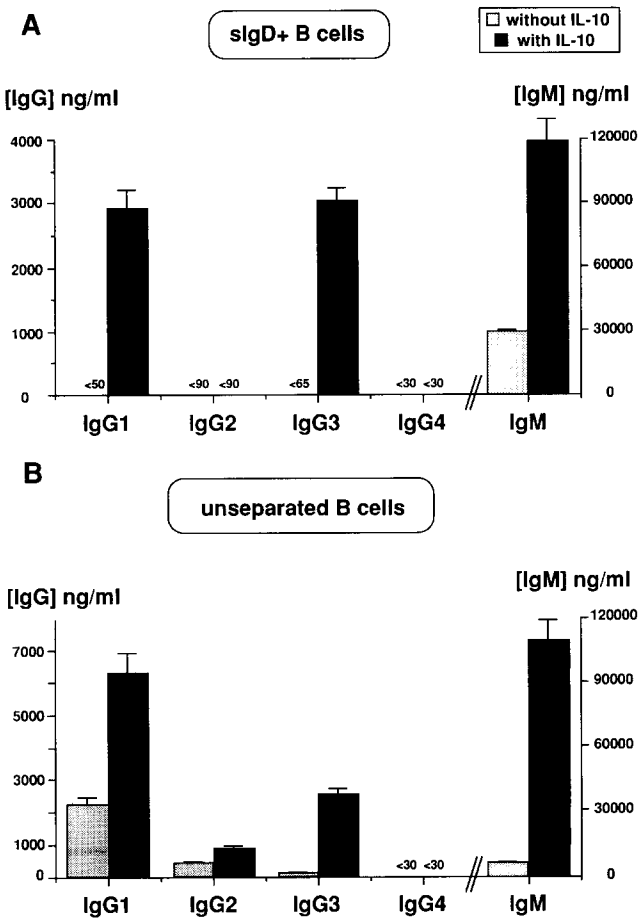

Figure 3. IL-10 further enhances IgM secretion by naive sIgD$^+$ B cells in the CD40/SAC system and induces secretion of IgG1 and IgG3. 5 × 10^4 MACS®-purified sIgD$^+$ B cells (*A*) or unseparated B cells (*B*) were cultured for 14 d with 5 × 10^3 irradiated CDw32 L cells with mAb89 and SAC (0.05% vol/vol) in the presence of 100 ng/ml IL-10. Supernatants were harvested and IgG subclasses levels were determined by ELISA. Results are means ± SD of quadruplicate cultures. Representative of five experiments.

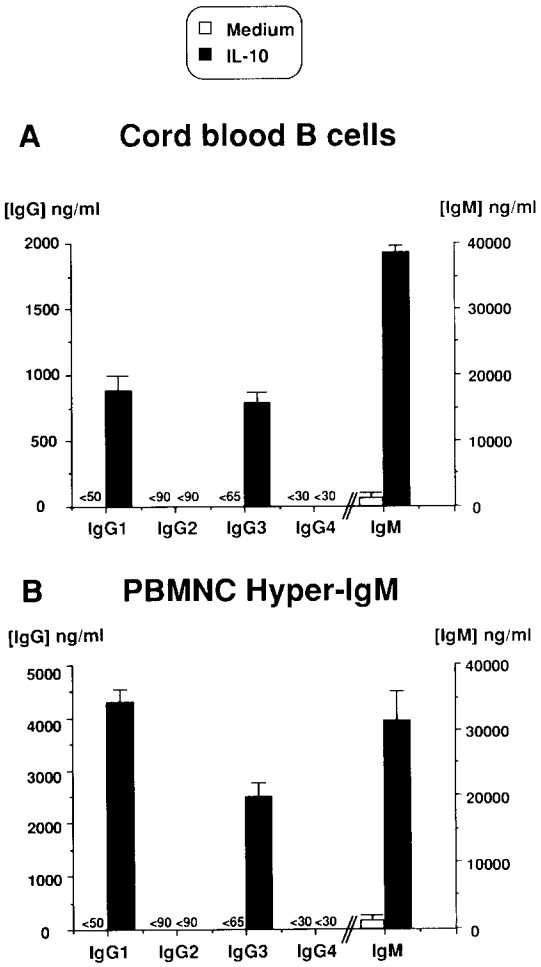

Figure 4. IL-10 induces IgG1 and IgG3 by naive B cells from cord blood or from PBMC from Hyper-IgM patients. 5 × 10^4 B cells purified from cord blood (>98% CD19$^+$ and >98% sIgD$^+$) and total blood mononuclear cells (*PBMNC*) (due to cell number limitation) of a Hyper-IgM patient (G. T.) were cultured with 5 × 10^3 irradiated L cells with mAb89 without or with 100 ng/ml IL-10. Supernatants were harvested after 14 d and IgG subclass levels were determined by ELISA. Results are means ± SD of quadruplicate cultures. Two experiments for (*A*) and one for (*B*).

Igs, can be induced to switch after T cell activation (20). As shown in Fig. 4 *A*, neonatal B lymphocytes isolated from cord blood behaved similarly to naive cells purified from tonsils. None of the IgG subclasses were produced when cord blood B cells were cultured in anti-CD40/SAC system alone. Addition of IL-10 resulted in the production of considerable amounts of both IgG1 and IgG3 but not IgG2 and IgG4. The Hyper-IgM syndrome was recently demonstrated to be due to a defective CD40 ligand on T cells, accounting for their inability to drive an isotype switch (16, 21, 22). B cells from these patients can be induced to switch isotype in vitro when cocultured with a malignant human T cell line (23) or after CD40 triggering in the presence of cytokine (16, 21). As shown in Fig. 4 *B*, PBMC from one such patient cultured in the anti-CD40/SAC system behaved similarly to naive B cells from normal individuals. They did not secrete IgG when stimulated with anti-CD40/SAC system alone, but secreted IgG1 and IgG3 but neither IgG2 nor IgG4 after addition of IL-10.

Taken together, the present data support the notion that IL-10 induces naive sIgD$^+$ B cells to switch towards IgG1 and IgG3 because (*a*) naive B cells exclusively synthesize IgG1 and IgG3 in response to IL-10 and do not produce IgG2; (*b*) different sources of naive B cells yield the same isotypes; and (*c*) naive B cells produced these isotypes with a delayed kinetics when compared to isotype-committed B cells.

The CD40 system shares many of the important features occurring within the germinal centers of the lymphoid follicles. Indeed, CD40 activation was sufficient to restore the switching capacity of Hyper-IgM B cells in response to cytokines. Thus, the triggering of CD40 plays a key role in isotype switching, possibly by turning on the isotype switch machinery, which supposedly alters the DNA structure of the H chain locus. Cytokines, on the other hand, are considered to provide isotype specificity. Indeed, IL-10 alone induces switching towards IgG1 and IgG3, and together with TGF-β towards IgA (7), whereas IL-4 and IL-13 induce IgE and IgG4. Yet to be resolved is the cytokine(s) that induces the switch towards IgG2, an isotype most often found in response to carbohydrate antigens (2).

This study brings up a novel activity of IL-10, namely its ability to induce human naive B cells to switch isotype from IgM to IgG1 and IgG3. This function is additional to IL-10's B cell differentiation activity (7, 19) that induces isotype-committed sIgD$^-$ B cells to secrete IgG1, IgG2, and IgG3. Isotype switching was induced in different sources of human naive B cells including highly purified tonsillar or splenic sIgD$^+$ B, neonatal B, and B cells from the Hyper-IgM patient. Further studies at the molecular level are required to determine the respective contribution of IL-10, CD40 triggering, and SAC. It is possible that IL-10 may not account for all IgG3 and particularly IgG1 switch-inducing activity. The ratio IgG1/IgG3 is close to 1 in culture supernatants of naive adult B and in neonatal B cells, whereas it is close to 10 in culture supernatants of unseparated B cells and in serum. The in vivo prevalence of IgG1 could be due to the existence of another factor inducing or enhancing switching to IgG1. In this context, several Ig isotypes are under the control of redundant molecules, such as switching to human IgG4 and IgE in response to IL-4 or IL-13. It is interesting to note that the IgG1 and IgG3 isotypes whose switch is directed by IL-10, preferentially bind to those Fcγ receptors (FcγRI, II, and III) whose expression are modulated on monocytes by IL-10 (24). Finally, this study raises the possibility that patients suffering from IgG3 and IgG1 deficiencies may either display reduced IL-10 secretion, produce IL-10 antagonists, or show an intrinsic B cell impairment resulting in defective IL-10 responses.

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