The interplay between T helper subset cytokines and IL-12 in directing human B lymphocyte differentiation

Alenka Gagro[1, 2] and John Gordon[1]

[1] The Medical Research Council Centre for Immune Regulation, The University of Birmingham, The Medical School, Birmingham, GB
[2] Institute of Immunology, Zagreb, Croatia

This study asks how T helper (TH) subset cytokines impact upon IL-12-directed change in B cells engaged in signaling via the B cell receptor and CD40, essential components in the initiation of T-dependent B cell responses. For B cells stimulated in this way, IL-12 promoted a distinct phenotype highlighted by the hyper-expression of CD38: the Th1 cytokine IFN-γ reproduced the IL-12 effects while neutralizing antibody to IFN-γ reversed IL-12-dependent change. The divergent pathway of differentiation promoted by the Th2 cytokine IL-4 (characterized by hyper-induction of CD23) was left unchecked by IL-12. IL-10 was found to dampen IL-12 actions by suppressing IL-12-dependent IFN-γ production but failed to perturb the effects of exogenous IFN-γ. Thus, IL-12 – by invoking autocrine IFN-γ production – promotes phenotypic deviation in B cells engaging T-dependent signals. The reversal of such Th1 driving of B cells by IL-10 only when the source of IFN-γ is endogenous and the inability of IL-12 to impact upon IL-4-directed differentiation suggest a progressive and hierarchical commitment of B cells to polarization during a developing T-dependent response dominated at the level of the Th cell rather than that of the dendritic cell.

Received	4/2/99
Revised	19/7/99
Accepted	20/7/99

Key words: Th1/Th2 / IL-12 / B lymphocyte / CD40 / IL-4

1 Introduction

Dendritic cells (DC) are the initiators of T-dependent (TD) antibody responses. Their importance in priming naive, antigen-specific Th cells via captured and processed antigen presented on MHC class II molecules is well established [1]. The APC is a major arbiter of the subsequent pathway taken by the developing Th cell, influencing its bifurcation toward a Th1- or a Th2-type response [2]. DC-derived IL-12 appears to be a key determinant in the initial skewing towards a Th1 pathway [3, 4]. In more developed responses, IFN-γ – a product of Th1 and CD8 T cells – promotes further commitment along this same pathway, characterized by the manifestations of cell-mediated immunity [5, 6]. By contrast, a classical humoral response is generated largely under the guidance of IL-4, a prototype Th2 cytokine [7, 8]. Candidates for initiating IL-4-dependent Th2 responses are CD1-restricted T cells [9]. IL-10 (produced by Th2 cells, among others) has an important dampening role in APC-dependent Th cell activation [10]. While often manifested at the level of Th1 inactivation (particularly by inhibiting the production of Th1-promoting cytokines) there are studies indicating that it can down-regulate Th responses in a Th subset-unrestricted manner [11–13]. It has been suggested that IL-10 turns off "noxious" inflammatory responses in favor of a less aggressive form of immune defense [14].

Recent studies indicate that DC may contribute to the recruitment of B cells in TD responses and influence their subsequent maturation. DC generated *in vitro* from $CD34^+$ progenitors present in human cord blood have found to enhance the growth and differentiation of CD40-activated naive B cells [15]. Recently, IL-12 was shown to be critical in the development of this DC-dependent amplification of a B cell response [16]. Previous reports had highlighted that IL-12 could serve as a growth and differentiation factor for human B cells pre-stimulated with *Staphylococcus aureus* Cowan I (SAC) and IL-2 or anti-μ-coupled beads [17, 18]. Studies in rats have revealed that naive B cells can interact directly with

[I 19342]

Abbreviations: SAC: *Staphylococcus aureus* Cowan I **DC:** Dendritic cell(s) **GC:** Germinal center(s) **TD:** T dependent **BCR:** B cell receptor **CD40L:** CD40 ligand

0014-2980/99/1010-3369$17.50+.50/0

DC to form short-lived clusters [19]. This work has been extended both *in vitro* and *in vivo* to indicate that DC retaining unprocessed antigen can transfer this to naive B cells to initiate a specific antibody response. The Ig class switching seen in responses induced in this way was typical of Th2 responses [20]. While several studies in mice support the thesis that IL-12 promotes the Ig class switching characteristic of Th1 responses and suppresses switching to IgG1 and IgE driven by the Th2 pathway, there are also circumstances where the reverse appears to be true [21, 22].

It has become apparent that B cells participating in TD responses can themselves manifest polarization along Th1 or Th2 pathway very soon after antigen challenge with the direction taken being dependent upon the nature of the antigen [23]. Thus it was shown that Cγ2a transcripts were produced in response to mouse mammary tumor virus or *Bordetella pertussis* while Cγ1 transcripts appeared in response to haptenated protein. These siwtching patterns were associated with mRNA for IFN-γ and IL-4, respectively. This differential switching occurred within 24 h of the onset of T cell priming.

In the present study, we have asked about the direct, early effects of IL-12 on the phenotypic deviation of resting human B cells engaging signals via the B cell receptor (BCR) and CD40, two essential components in the priming phase of TD B cell responses. The impact of prototype Th1- and Th2-directing cytokines, IFN-γ and IL-4, respectively, on this change has been assessed as has that of the immunomodulatory cytokine IL-10. The results are consistent with a progressive, hierarchical commitment of primed B cells to distinct, polarized pathways of differentiation that are dependent upon the Th subset cytokine environment established shortly after antigen challenge.

2 Results

2.1 Influence of IL-12 and Th subset cytokines on human B cells

IL-12 has previously been reported to act as a co-stimulatory factor for human B cells activated by SAC and IL-2 or anti-IgM immobilized onto beads [17, 18]. On stimulating $CD38^{low}$ B cells via an IgG1 mAb (AF6) to sIgM held on CD32-L cell transfectants, we found that IL-12 promoted a twofold increase in the low level of DNA synthesis induced through BCR alone (data not shown). Culturing resting B cells on CD32-L cells co-expressing CD40 ligand (CD40L) gave much higher levels of DNA synthesis both with and without co-ligation of sIgM but this was not influenced by the addition of IL-12 (data not detailed). Thus IL-12 does not appear to be a growth factor for B cells receiving a CD40 signal.

We next asked whether IL-12 could modify CD40- and BCR-driven phenotypic differentiation in resting B cells and what impact IFN-γ and IL-4 might have on this. CD5, CD23 and CD38 were highlighted for analysis. Co-ligation of CD40 and sIgM on resting B cells reduces the percentage of CD40-induced $CD23^+$ cells but increases the expression and proportion of $CD5^+$ cells generated. CD40 engagement also results in CD38 up-regulation that is further enhanced on sIgM co-ligation [24, 25]. From Table 1 it can be seen that the presence of IL-12 led to a reduction in the number of $CD23^+$ cells induced when resting B cells were cultured on L cells carrying CD40L. Almost complete down-regulation of CD23 due to IL-12 was seen where B cells had been co-stimulated via CD40 and sIgM. Similarly, IL-12 reversed the ability of sIgM/CD40 co-ligation to up-regulate CD5 expression on resting B cells (Table 1). IFN-γ was seen to promote a change identical to that of IL-12 in both CD5 and CD23 expression; moreover, the combined effect of IFN-γ and IL-12 was indistinguishable from that of either alone (Table 1). IL-4 has been reported to up-regulate CD23 and down-regulate CD5 expression on human B cells [26, 27] and this was confirmed here for all conditions of culture. Even for B cells receiving potent co-stimulation via CD40 and sIgM, IL-4 further enhanced CD23 expression and completely abolished CD5 induction (Table 1). IL-12 failed to impact significantly on these IL-4-driven alterations of CD40-dependent change.

With respect to CD38, none of the cytokines increased expression on unstimulated cells (data not detailed). For $CD38^{low}$ B cells triggered via sIgM, IFN-γ dramatically enhanced the number of cells expressing high level CD38 while IL-12 was less efficient in this regard (Fig. 1). IFN-γ and IL-12 each increased the level of CD38 promoted in cells stimulated via CD40: the outcome was essentially identical both with (data not detailed) and without concomitant sIgM signaling. Again, there was no additional effect with both cytokines together. IL-4 tended to diminish sIgM- and CD40-invoked increases in CD38 expression and suppressed the IL-12 enhancement of CD38 up-regulation following CD40 signals (Fig. 1).

Differential expression of CD80 and CD86 by APC has been implicated in directing Th polarization [28, 29] while immunoregulatory cytokines can influence co-stimulatory molecule expression [30]. We tested the influence of IL-12 on the expression of CD80 and CD86 and the capacity of IFN-γ and IL-4 to impact upon such change. Resting B cells expressed CD80 (14.8 ± 2 %; $n = 6$) and CD86 (9.7 ± 4 %;) at low level. Ligating sIgM

Table 1. Effect of IL-12, IFN-γ and IL-4 on CD5 and CD23 expression

	Resting B cells stimulated via[a]:							
	Control		sIgM		CD40		sIgM/CD40	
Cytokines added[b]	% cells positive (MFI) at day 3 for[c]:							
	CD5	CD23	CD5	CD23	CD5	CD23	CD5	CD23
CM	34 (48)	5 (52)	30 (64)	7 (70)	12 (81)	63 (256)	43 (184)	49 (128)
IL-12	27 (54)	3 (42)	25 (56)	3 (54)	9 (71)	44 (187)	17 (85)	16 (62)
IFN-γ	18 (51)	1 (31)	17 (57)	1 (54)	8 (71)	28 (129)	16 (91)	9 (70)
IFN-γ + IL-12	13 (43)	3 (23)	15 (57)	3 (35)	10 (84)	25 (136)	12 (76)	8 (66)
IL-4	15 (58)	59 (242)	10 (57)	54 (919)	4 (97)	79 (1203)	7 (77)	79 (1596)
IL-4 + IL-12	12 (69)	34 (235)	9 (55)	41 (706)	5 (74)	74 (728)	3 (56)	78 (820)

a) Resting B cells were cultured in the presence of non-transfected L cells (Control), anti-IgM antibody (0.5 μg/ml) cross-linked on CD32-transfected L cells (sIgM) and dual CD32/CD40L-transfected L cells without (CD40) and with anti-IgM antibodies (sIgM + CD40).
b) Cytokines IL-12 (1 ng/ml), IL-4 (100 ng/ml) and IFN-γ (1000 U/ml) were added at the beginning of the cell culture.
c) Data generated from two-color FACScan® analysis following staining of cells with CD23 mAb conjugated to FITC and CD5 mAb conjugated to PE-CY5 are reported as percentage positive cells with mean fluorescent intensities (MFI) for each marker shown in parentheses. Results are representative of four independent experiments.

up-regulated CD80 and, particularly, CD86. CD40L delivered a more potent signal for CD80 and CD86 induction while co-ligating sIgM and CD40 led to further up-regulation of CD86 but not of CD80 (data not detailed). IL-12 modestly enhanced CD86, but not CD80, expression especially on cells stimulated through sIgM alone. An identical change was evoked with IFN-γ except that the increase in CD86 expression was observed earlier than with IL-12. In contrast to IL-12 and IFN-γ, IL-4 up-regulated both CD80 and CD86 on unstimulated B cells as well as on those stimulated through sIgM alone and increased CD80 expression on sIgM/CD40 co-

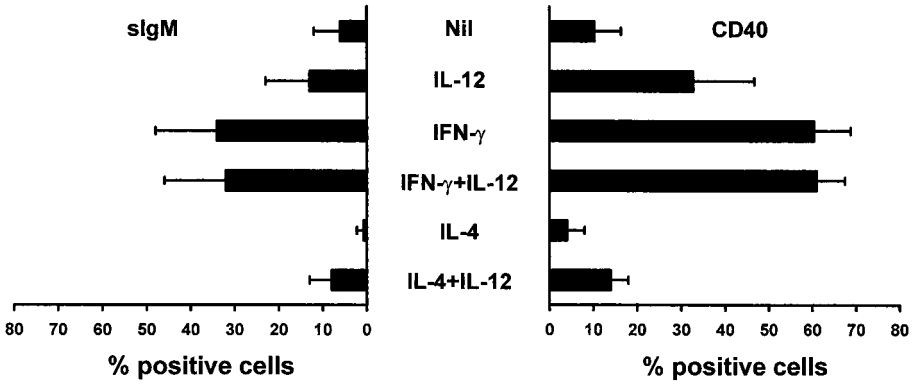

Figure 1. Effect of cytokines on CD40-dependent CD38 expression. sIgM- or CD40-stimulated B cells were cultured for 3 days in the presence or absence of IL-12, IFN-γ, and IL-4 and expression of CD38 was measured by FACScan. Data, expressed as the % positive cells, are the mean ± SD for four separate experiments.

stimulation. IL-4-dependent changes were left untouched by IL-12 (data not detailed).

2.2 IL-12-promoted change in B cells requires endogenous IFN-γ production

IL-12 has been shown to stimulate IFN-γ secretion by T cells, NK cells and B cell lines [21, 31]. Moreover, endogenous IFN-γ production in response to IL-12 has been implicated in the enhanced DNA synthesis occurring when IL-2 is added to human B cells stimulated by anti-IgM-coated beads [17]. However, some of the effects of IL-12 on the immune response, and particularly its antimicrobial activity, are present in IFN-γ knockout mice [32] and are therefore independent of IFN-γ activity. Neutralizing antibody to IFN-γ was found to block IL-12-dependent up-regulation of CD86 initiated by cross-linking sIgM; control antibody had no effect (Fig. 2). This was seen when neutralizing antibody was added at 100 μg/ml but was only partial with 10 μg/ml antibody (data not detailed). Neutralizing antibody to IFN-γ also completely reversed IL-12-driven change in CD40-stimulated B cells whether measured as a diminution in CD5 and CD23 expression or the up-regulation of CD38 (Fig. 2). Coupled with the qualitatively indistinguishable actions of IL-12 and IFN-γ on the phenotypic changes noted above, these data indicate that stimulated endogenous production of IFN-γ is responsible for the IL-12 effects registered in human B cells.

2.3 IL-10 dampens IL-12 function by suppressing IFN-γ production in human B cells

IL-10, an important immunoregulatory cytokine, has been shown both in mouse and human [33, 34] to prevent IFN-γ-mediated up-regulation of CD86 on monocytes and Langerhans cells. While dampening APC-dependent cytokine synthesis by Th0, Th1 and Th2 cells,

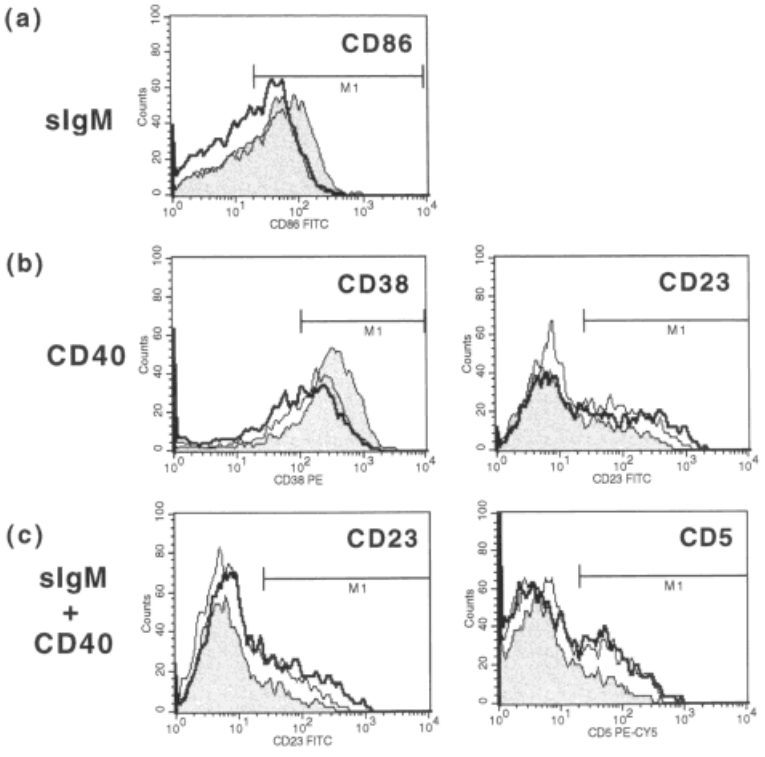

Figure 2. Neutralizing anti-IFN-γ reverses IL-12-promoted phenotypic changes. Neutralizing anti-IFN-γ (100 μg/ml) reversed the effect of 1 ng/ml IL-12 on: (a) CD86 expression following sIgM signaling; (b) CD38 and CD23 expression after CD40 ligation and; (c) CD23 and CD5 expression after sIgM and CD40 co-ligation. Histograms represent the staining profiles for sIgM-, and/or CD40-stimulated B cells (thin line), with profiles obtained when 1 ng/ml IL-12 was added to the cells at the beginning of the cell culture (shadowed histogram) and when neutralizing anti-IFN-γ was added together with IL-12 (heavy line). The range (M1) for CD38 is set at the point that distinguishes lower level of CD38 expression by non-germinal center B cells from high level of expression by germinal center B cells, and for CD86, CD23 and CD5 with an irrelevant isotype-matched fluorescent conjugate. Results are representative of two independent experiments. FACScan® data are represented as $\log_{10}$ fluorescence.

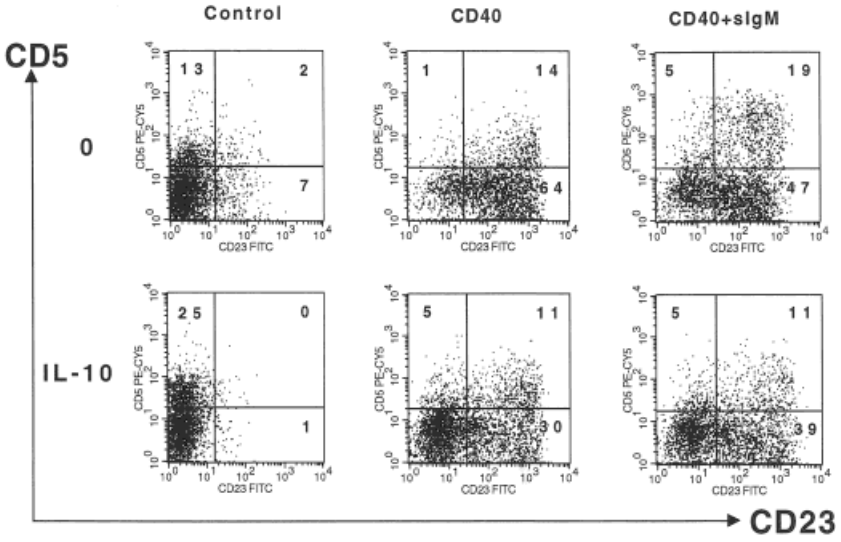

Figure 3. Effect of IL-10 on phenotypic changes in stimulated B cells. Resting B cells were cultured in the presence of non-transfected L cells (Control) and dual CD32/CD40L-transfected L cells without (CD40) and with anti-IgM antibody (sIgM + CD40) with or without 10 ng/ml IL-10. Two-color FACScan® analysis shows staining of the cells with CD23 mAb conjugated to FITC (x-axis) and CD5 mAb conjugated to PE-CY5 (y-axis); the horizontal and vertical lines are set with an irrelevant isotype-matched fluorescent conjugate. The percentage of positive cells is indicated for each quadrant. Results are representative of three independent experiments.

it also exerts direct effects on the growth and differentiation of human B cells [35, 36]. We asked how IL-10 might directly influence changes in B cells engaging TD signals and whether it impacts upon the IL-12-driven alterations observed.

From Fig. 3 it can be seen that IL-10 reduces the level of CD23 induced on engaging CD40, either alone or together with sIgM. The amount of CD5 induced on co-ligating sIgM and CD40 was also diminished by IL-10. IL-10 had no influence on the expression of CD80 and CD86 or of CD38 under any conditions of activation (data not shown). When assessed for its influence on IL-12-promoted change, IL-10 reversed completely the up-regulation of CD86 on sIgM stimulation and partially the enhancement of CD38 expression on CD40-stimulated cells (Fig. 4). IL-10 caused an approximate 50 % reversal of the IL-12-driven CD38 enhancement seen in B cells co-stimulated via sIgM and CD40 (data not detailed). By contrast, the identical changes induced by IFN-γ remained untouched by the presence of IL-10 (Fig. 4). IL-10 was tested over a range of concentrations with all effects being maximal at 10 ng/ml. As IL-10 itself reduces CD5 and CD23 expression in B cells engaging TD signals, there was no further impact upon the same changes induced by IL-12.

We next asked whether the reversal of IL-12-promoted change by IL-10 might be due to its ability to suppress endogenous IFN-γ production. Importantly, the populations remained < 1 % $CD3^+$, $CD14^+$, $CD56^+$ and > 98 % $CD19^+$ throughout culture (data not detailed). In the absence of IL-12, no IFN-γ could be detected in the supernatants of these highly pure B cell cultures even when potent signaling was being delivered through sIgM/CD40 co-ligation (Table 2). By contrast, the inclusion of IL-12 resulted in production of IFN-γ that was most apparent from cells receiving CD40 signals, especially in conjunction with sIgM-delivered signals. Although the amount of IFN-γ promoted by IL-12 could vary between experiments, the presence of IL-10 always led to a reduction in the level of IFN-γ detected (Table 2). Despite a substantial diminution by IL-10 in the amount of IL-12-dependent IFN-γ produced on CD40 ligation, residual levels could still be significant probably accounting for the failure of IL-10 to reverse completely IL-12-promoted CD38 up-regulation under these conditions. To establish fully that the IL-12-promoted IFN-γ production was from B cells and not due to a minor non-B cell contaminant, we determined intracellular IFN-γ content in $CD19^+$ cells by flow cytometric analysis. Fig. 5 shows that B cells stimulated via sIgM and CD40 developed intracellular IFN-γ only when co-cultured with IL-12. As with the ELISA determinations, the inclusion of IL-10 led to a significant reduction in the amount of IFN-γ detected. Specificity of the staining was confirmed by its abolition on pre-incubation with rIFN-γ (Fig. 5).

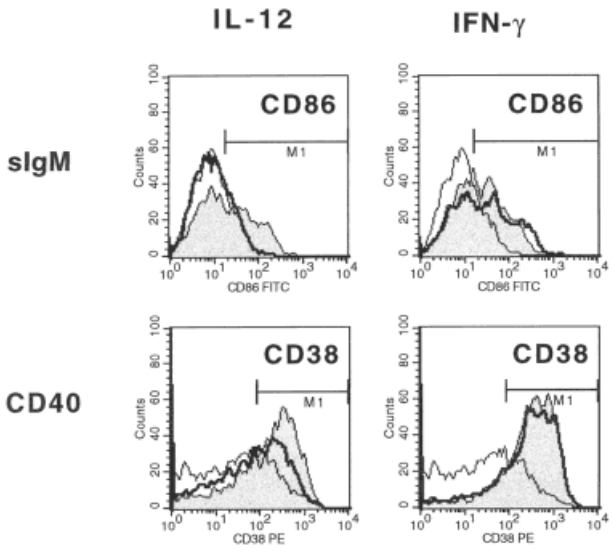

Figure 4. IL-10 reverses the effect of IL-12-, but not of IFN-γ- promoted changes. Histogram profiles show expression of CD86 and CD38 following activation of resting B cells by sIgM- (sIgM) and CD40-ligation (CD40) (thin lines) when 1 ng/ml IL-12 or 1000 U/ml IFN-γ was added to cells at the beginning of a 3-day culture either without (shadowed histogram) or together with 10 ng/ml IL-10 (heavy line). The range (M1) for CD38 is set as in Fig. 2 and for CD86 with an irrelevant isotype-matched fluorescent conjugate. Results are representative of three independent experiments.

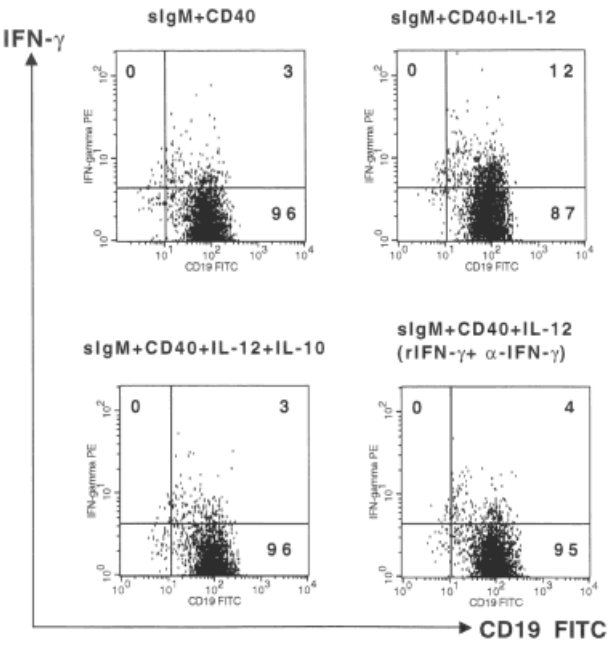

Figure 5. IL-12-dependent IFN-γ production from human B cells and its suppression by IL-10 demonstrated by intracellular staining. Resting B cells were cultured for 3 days in the presence of dual CD32/CD40L-transfected L cells and anti-IgM antibody (sIgM + CD40) with either no added cytokines, 10 ng/ml IL-12 alone, or IL-12 + 10 ng/ml IL-10. Monensin was added during the last 15 h of culture. Cells were harvested and stained for surface CD19 and intracellular IFN-γ. Two-color FACScan analysis shows staining of the cells with CD19 mAb conjugated to FITC (x-axis) and anti-IFN-γ mAb conjugated to PE (y-axis); specificity controls were performed by preincubation of the anti-IFN-γ mAb with saturating concentrations of rIFN-γ prior to addition to the permeabilized cells. The horizontal and vertical lines are set with an irrelevant isotype-matched fluorescent conjugate. The percentage of positive cells is indicated for each quadrant. Results are representative of three independent experiments.

3 Discussion

We have shown that a major DC product, IL-12, alters the phenotype induced in B cells on engaging BCR and CD40. The change was indistinguishable from that evoked by the Th1-promoting cytokine IFN-γ and characterized by a diminution of CD5 and CD23 expression and the hyper-induction of CD38. By contrast, the Th2-promoting cytokine IL-4 encouraged increased CD23 expression and down-regulated CD5 while leaving CD38 expression unchanged. Importantly, where B cells had been exposed to both IL-12 and IL-4, the actions of the latter were dominant. The possibility of DC influencing B cells has been highlighted by recent studies in both human and rat [15, 16, 19, 20]. Whilst DC-derived IL-12 is typically associated with the development of Th1 responses, its influence upon Ig isotype production is ambiguous. Some studies implicate it in the development of Th1-associated isotypes while others demonstrate it favoring the production of antibody subclasses characteristic of a Th2 pathway [37, 38]. In the rat, the influence of DC on B cells appears to be in the development of antibody responses associated with the Th2 arm though a contribution from IL-12 has yet to be established in this system [20]. Our findings reconcile these observations by providing a model whereby the influence of DC-derived IL-12 on the direction taken by B cells being recruited into TD responses can be appropriately countered – or encouraged – by the products of polarizing Th cell responses once they are established.

Teleologically, it would seem inappropriate for the initial DC encounter – through, for example, the production of IL-12 – to fix inexorably the direction of the B cell response. The outcome should ultimately be under the guidance of the Th cell, with the route that it is taking determining the B cell's fate. Nevertheless, the manifestation of Th cell polarization has been considered a relatively late feature in ongoing immune responses that would make the hierarchical model proposed here for Th subset control of early B cell differentiation seemingly

Table 2. IL-10 suppresses IL-12-dependent IFN-γ production by human B cells

IFN-γ (pg/ml) detected in supernatant from B cells stimulated with[a)]												
	Control			sIgM			CD40			sIgM/CD40		
Experiment no.	CM	IL-12	IL-10 + IL-12	CM	IL-12	IL-10 + IL-12	CM	IL-12	IL-10 + IL-12	CM	IL-12	IL-10 + IL-12
1	0	31	0	0	62	35	0	275	69	0	417	125
2	0	257	95	0	251	112	0	437	166	0	602	250
3	0	0	0	0	16	0	0	16	0	0	16	0

a) Resting B cells were cultured in the presence of non-transfected L cells (control), with anti-IgM antibody cross-linked on CD32-transfected L cells (sIgM), and on dual CD32/CD40L-transfected L cells without (CD40) and with anti-IgM antibody (sIgM/CD40) with either no added cytokines (CM), 10 μg/ml IL-12 alone, or IL-12 10 ng/ml + IL-10. IFN-γ (pg/ml) was determined after 3 days of culture in supernatants analyzed in duplicate by ELISA as described in Sect. 4.6.

redundant. A recent study, however, has revealed that Th1 and Th2 characteristics start to develop during initial T cell priming and impact upon the direction taken by the responding B cells within 24 h [23]. For several cell types, IL-12 seems to exert its actions through invoking autocrine IFN-γ production [21]. This includes the IL-12-dependent enhancement of DNA synthesis in human B cells stimulated with anti-IgM-coupled beads [18]. By contrast, Caux and colleagues [16], were able to inhibit IL-2-dependent IgM production in B cells cultured with DC and CD40L on adding anti-IL-12 but not with neutralizing antibody to IFN-γ. In our study, the phenotypic change driven by IL-12 in B cells receiving TD signals was both accompanied by IFN-γ production and abolished in a dose-dependent manner by anti-IFN-γ. Moreover, IL-10 dampened IL-12-promoted IFN-γ production while tempering the development of an IL-12-dependent phenotype. Importantly, IL-10 failed to impact upon the same change where evoked by exogenous IFN-γ. Thus, under some conditions of stimulation, B cells appear to secrete Th1-promoting cytokines. Indeed, it should be noted that IL-12 was first identified as a product of transformed B cells [39]. Although not observed in the mouse [40], it was recently reported that human B cells produce bio-active IL-12 on CD40 ligation [41]. The ability in our study to modulate further the phenotype of CD40-activated B cells with exogenous IL-12 implies that any such endogenous IL-12 must be limiting.

The phenotype of CD23 hyper-expression promoted by IL-4 is in keeping with previous observations on the actions of this cytokine on human B cells and indicates that it may be a robust marker of early B cell skewing along a Th2-associated pathway [26, 42]. Of interest is the finding that both Th1- and Th2-polarizing cytokines down-regulate CD5 on B cells signaled through BCR and CD40. The significance of this is unclear although it should be noted that B cells entering germinal center (GC) responses are typically $CD5^-$ [24, 25]; they are also strongly $CD38^+$ [43]. That both IL-12 and IFN-γ drive a phenotype of high CD38 expression with an accompanying loss of CD23 raises the possibility that these may be early features of B cells being guided along a Th1 pathway. Interestingly, IL-4 and IL-10 each down-regulated the IL-12-enhanced expression of CD38 following BCR or CD40 engagement. In addition, IL-10 (but not IL-4) reversed the CD40-dependent up-regulation of CD5 and CD23 consistent with its role as a general dampener of an immune response including those situations where Th2 cells are influencing B cell development [44].

High level CD38 is also a feature of plasma cells and we could consider CD38 hyper-expression as marking the commitment of BCR/CD40-recruited B cells to terminal differentiation outside of follicles [45]. This could be driven by DC-derived IL-12 (and encouraged by CD8 T cell-produced IFN-γ?) in B cells engaging Th cells prior to Th subset polarization. Subsequent divergence of Th cells to IL-4 production could then establish recruitment of primed B cells into the longer-lived GC response as was suggested by recent findings in the mouse [46]. This would provide a mechanism for the B cell bifurcation into extrafollicular and follicular pathways observed in TD responses [47].

While APC expression of CD86 has been linked to the development of Th2-polarizing responses [28, 29, 48] we found that both IFN-γ and IL-4 resulted in its up-regulation on either resting B cells or B cells stimulated via sIgM. They could each enhance the already high expression of CD86 promoted on co-engaging sIgM and CD40 and certainly did not lead to its down-regulation under these conditions. By contrast, only IL-4, and not IFN-γ, promoted increases in CD80 and this was seen

under all conditions of B cell triggering. The effects of IL-12 on co-stimulatory molecule expression followed that of IFN-γ but with a delay of 24 h, consistent with the actions of IL-12 being dependent upon induced IFN-γ. IL-10 had no direct effect on either CD80 or CD86 expression but was able to reverse the IL-12-dependent, but not the IFN-γ-dependent, induction of CD86, again consistent with IL-10 dampening early DC-dependent, but not Th1-driven, change in responding B cells.

In conclusion, by focusing on early changes in differentiation markers and co-stimulatory molecule expression, we have shown that IL-12 directs a shift in phenotype indistinguishable from that established with the Th1-driving cytokine IFN-γ in B cells engaging signals via BCR and CD40. Indeed, the actions of IL-12 in this regard appear to be wholly dependent upon its ability to promote endogenous IFN-γ production. The IL-12 skewing of the B cell phenotype is mutable, being dampened by IL-10 through the latter's ability to suppress the induction of IFN-γ by the former. Where supplied exogenously, the phenotypic skewing of the B cell by IFN-γ remains unchecked by IL-10. Moreover, when IL-4 is provided to the B cell engaged in TD signaling, the potential Th1-like deviation engendered by IL-12 is overridden in favor of a Th2-associated phenotype. The findings point to, and are consistent with, a progressive and hierarchical commitment of B cells to polarized pathways of development dominated by the early skewing of Th cells in the priming phase of the response and not at the level of the DC or its products.

4 Materials and methods

4.1 Reagents

Human IL-4, IFN-γ, IL-10 and IL-12 were purchase from R&D Systems Ltd (Oxford, GB) as were neutralizing polyclonal goat anti-human IFN-γ and control goat IgG antibodies. OKT3 (anti-CD3), 61D3 or UCHM-1 (anti-CD14), and OKT10 (anti-CD38) mAb were produced from hybridomas in the Department of Immunology, University of Birmingham and purified by ion exchange chromatography on DE52 (Whatman Ltd, Maidstone, GB). For FACS analysis we used FITC-conjugated IgD, CD19, CD23, CD40, CD56, and CD14 and PE-conjugated CD2 (Dako Ltd, High Wycombe, GB), PE-conjugated CD3, CD80 and CD38, and PerCP-conjugated CD20 (Becton Dickinson, Oxford, GB), PE- and FITC-conjugated CD86 (PharMingen, San Diego, CA) and PE-Cy5-conjugated CD5 (Immunotech, Marseille, France).

4.2 Isolation of resting B cells from human tonsils

Tonsils were obtained from patients undergoing routine tonsillectomy. Cells were extracted by dissection and dispersal in RPMI 1640 (Gibco Ltd, Paisley, Scotland). Mononuclear cells were layered onto Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) and centrifuged at 450 × g for 20 min at room temperature. Interface cells were washed in RPMI 1640 and T cells were depleted by E-rosetting with aminoethyl isothiouronium bromide-treated SRBC with rosettes removed by centrifugation on Ficoll-Paque. Resting B cells were prepared from non-rosetting cells by negative depletion on a magnetic cell separator (VarioMACS, Miltenyi BiotecGmbH, Bergisch Gladbach, Germany) using depletion columns. Briefly, to remove remaining T cells and monocytes, as well as $CD38^+$ B cells, non-rosetting cells were cultured with 20 μg/ml anti-CD3 (OKT3), anti-CD14 (61D3 or UCHM-1) and anti-CD38 (OKT10) mAb for 15 min at 4 °C. After washing with PBS containing 5 % BSA and 5 mM EDTA (MACS buffer), cells were incubated with goat anti-mouse IgG Microbeads® (Miltenyi Biotec) for an additional 15 min at 4 °C. After washing with MACS buffer, magnetically unlabeled cells were collected as "resting B cells", their purity being assessed by immunofluorescence labeling with CD3, CD19, CD20, CD56 and CD14 as well as with double labeling with IgD and CD38 mAb. For preparations used in this study the purity of resting B cells ($CD38^{low}$) was > 98 %.

4.3 Culture of tonsillar B cells

Resting B cells (10^6/ml) were cultured in flat-bottom 96-well microtiter plates to measure DNA synthesis or in 48-well plates (Becton Dickinson Labware, Oxford, GB) to determine changes in phenotype, in a total volume of 200 μl or 0.5 ml, respectively, in RPMI 1640 containing 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Gibco, Grand Island, NY) and 10 % FCS (Sera Lab Ltd, Crawley Down, GB) at 37 °C in a humidified incubator in 5 % CO_2. Where indicated, mouse L cells transfected with the human CD32 gene (CD32-L cells), co-transfected with CD32 and CD40L (CD32/CD40L-L cells), or non-transfected mouse L cells were used. Adherent L cells cultured in RPMI 1640 with 10 % FCS and antibiotics (complete medium, CM) were recovered using 0.02 % disodium EDTA in PBS, pH 7.2, resuspended in CM and γ-irradiated (7 000 rad) before addition to B cells at the ratio 1 : 10 (L cells: B cells). The influence of sIgM ligation without or with ligation of CD40 was assessed by using CD32-L cells and CD32/CD40L-L cells, respectively, and 0.5 μg/ml anti-IgM antibody AF6 (IgG1). IL-4 (10 and 100 ng/ml), IFN-γ (100 and 1000 U/ml), IL-10 (1–100 ng/ml) and IL-12 (1 and 10 ng/ml) were added at the beginning of the cell culture where indicated. In some experiments, neutralizing polyclonal goat anti-human IFN-γ antibodies (1–100 μg/ml) or control goat anti-human IgG antibodies were added to cultures where indicated.

4.4 Flow cytometric analysis

The cells were harvested after different periods of culture by incubation for 5 min with 0.02 % disodium EDTA to disperse aggregates, washed with RPMI 1640 and than stained prior

to analysis on a FACScan® flow cytometer (Becton Dickinson, Mountain, View, CA). Cell suspensions were stained using standard direct two- or three-color immunofluorescence staining methods as already described [49]. Briefly, after harvesting, cells were washed in PBS supplemented with 5 % goat serum (Harlaan Sera-Lab Limited, Loughborough, GB) and 0.1 % sodium azide (Sigma, Poole, Dorset, GB) and then incubated using at least 2×10^5 cells per sample with previously determined optimal concentrations of mAb conjugated to different fluorochromes (FITC, PE and PE-CY5 or FITC, PE and PerCP) for 15 min in the dark at room temperature. Cells were then washed and subsequently resuspended in 0.5 % formaldehyde (Sigma) in PBS containing 5 % goat serum and 0.1 % sodium azide and analyzed within 24 h of staining on a flow cytometer.

4.5 Determination of IFN-γ production

Released IFN-γ was measured in duplicate supernatants of cultured cells using Quantikine® human IFN-γ immunoassay (R&D systems, Oxon, GB) following the manufacturer's instructions. The detection limit of the kit was 3.0 pg/ml. Intracellular IFN-γ was detected by flow cytometry using a PE-labeled anti-IFN-γ mAb (4S.B3; PharMingen). B cells were cultured as described in Sect. 2.3. An inhibitor of protein secretion, monensin (2 μM; Sigma), was added during the last 16 h of culture. Harvested cells were washed with PBS and processed for surface and intracellular staining as previously described [50]. Briefly, cells were first stained with FITC-conjugated anti-CD19 antibody (Becton Dickinson) for 15 min in the dark at room temperature, washed and fixed for 20 min at 4 °C with 4 % paraformaldehyde in PBS. Cells were then permeabilized with 0.1 % saponin in PBS for 10 min prior to addition of PE-conjugated anti-IFN-γ antibody or isotypic control (PharMingen) and then incubated for a further 30 min at 4 °C. Cells were then washed in PBS containing 0.1 % saponin to wash out unbound antibody, resuspended in PBS, and analyzed immediately on a flow cytometer. In some experiments, the specificity of intracellular IFN-γ staining was controlled by preincubation of cells with a molar excess of recombinant IFN-γ.

Acknowledgments: A.G. undertook this work whilst in receipt of a Wellcome Travelling Fellowship. J.G. is a Medical Research Council Non-Clinical Professor and this work is funded in part by a MRC (U.K.) Programme Grant. We wish to thank Ian MacLennan and Peter Lane for their critical reading of this manuscript.

5 References

1 **Steinman, R. M.,** The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 1991. **9:** 271–296.

2 **O'Garra, A.,** Cytokines induce the development of functionally heterogenous T helper cell subsets. *Immunity* 1998. **8:** 275–283.

3 **Macatonia, S. E., Hosken, N. A., Litton, M., Vieira, P., Hsieh, C. Y., Culpepper, J. A., Wysocka, M., Trinchieri, G., Murphy, K. M. and O'Garra, A.,** Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. *J. Immunol.* 1995. **154:** 5071–5079.

4 **Hilkens, C. M. U., Kalinski, P., de Boer, M. and Kapsenberg, M. L.,** Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood* 1997. **90:** 1920–1926.

5 **Mosmann, T. R., Cherwinski, H., Bond, M. H., Giedlin, M. A. and Coffman, R. L.,** Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 1986. **136:** 2348–2357.

6 **Mosman, T. R. and Coffman, R. L.,** TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* 1989. **7:** 145–173.

7 **Snapper, C. M. and Paul, W. E.,** Interferon-γ and B-cell stimulatory factor I reciprocally regulate Ig isotype production. *Science* 1987. **236:** 944–947.

8 **Coffman, R. L., Lebman, D. A. and Rothman, P.,** Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* 1993. **9:** 229–270.

9 **Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J. and Paul, W. E.,** Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science* 1995. **270:** 1845–1847.

10 **de Waal Malefyt, R., Haanen, J., Spits, H., Roncarolo, M., te Velde, A., Figdor, C., Johnson, K., Kastelein, R., Yssel, H. and de Vries, J. E.,** Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via downregulation of class II mamor histocompatibility complex expression. *J. Exp. Med.* 1991. **174:** 915–924.

11 **Fiorentino, D. F., Zlotnik, A., Vieira, P., Mosmann, T. R., Howard, M., Moore, K. W. and O'Garra, A.,** IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J. Immunol.* 1991. **146:** 3444–3451.

12 **Del Prete, G., De Carli, M., Almerigogna, F., Giudizi, M. G., Biagiotti, R. and Romagnani, S.,** Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J. Immunol.* 1993. **150:** 353–360.

13 **Schanden, L., Alonso-Vega, C., Willems, F., Gerard, C., Delvaux, A., Velu, T., Devos, R., de Boer, M. and Goldman, M.,** B7-CD28-dependent IL-5 production by human resting T cells is inhibited by IL-10. *Immunology* 1994. **152:** 4368–4374.

14 **Muraille, E. and Leo, O.,** Revisiting the Th1/Th2 paradigm. *Scand. J Immunol.* 1998. **47:** 1–9.

15 **Dubois, B., Vanbervliet, B., Fayette, J., Massacrier, C., van Kooten, C., Briére, F., Banchereau, J. and Caux, D.,** Dendritic cells enhance growth and differentiation of CD40-activated B lymphocytes. *J. Exp. Med.* 1997. **185:** 941–951.

16 **Dubois, B., Massacrier, C., Vanbervliet, B., Fayette, J., Briére, F., Banchereau, J. and Caux, C.,** Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J. Immunol.* 1998. **161:** 2223–2231.

17 **Jelinek, D. F. and Braaten, J. K.,** Role of IL-12 in human B lymphocyte proliferation and differentiation. *J. Immunol.* 1995. **154:** 1606–1613.

18 **Li, L., Young, D., Wolf, S. F. and Choi, Y. S.,** Interleukin-12 stimulates B cell growth by inducing IFN-γ. *Cell. Immunol.* 1996. **168:** 133–140.

19 **Kushnir, N., Liu, L. and MacPherson, G. G.,** Dendritic cells and resting B cells form clusters in vitro and in vivo: T cell independence, partial LFA-1 dependence, and regulation by cross-linking surface molecules. *J. Immunol.* 1998. **160:** 1774–1781.

20 **Wykes, M., Pombo, A., Jenkins, C. and MacPherson, G. G.,** Dendritic cells interact with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. *J. Immunol.* 1998. **161:** 1313–1319.

21 **Trinchieri, G.,** Interleukin-12: A proinflammatory cytokine with immunoregulatory functions that bridge innate resistance and antigen-specific adaptive immunity. *Annu. Rev. Immunol.* 1995. **13:** 251–276.

22 **Wynn, T. A., Jankovic, D., Hieny, S, Zioncheck, K., Jardieu, P., Cheever, A. W. and Sher, A.,** IL-12 exacerbates rather than suppress T helper 2-dependent pathology in the absence of endogenous IFN-γ. *J. Immunol.* 1995. **154:** 3999–4009.

23 **Toellner, K. M., Luther, S. A., Sze, D. M. Y., Choy, R. K. W., Taylor, D. R. and MacLennan, I. C. M.,** T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. *J. Exp. Med.* 1998. **187:** 1193–1204.

24 **Galibert, L., Burdin, N., de Saint-Vis, B., Garrone, P., Van Kooten, C., Banchereau, J. and Rousset, F.,** CD40 and B cell antigen receptor dual triggering of resting B lymphocytes turns on a partial germinal center phenotype. *J. Exp. Med.* 1996. **183:** 77–85.

25 **Wheeler, K. and Gordon, J.,** Co-ligation of surface IgM and CD40 on naive B lymphocytes generates a blast population with an ambigous extrafollicular-germinal centre cell phenotype. *Int. Immunol.* 1996. **8:** 815–828.

26 **Defrance, T., Aubry, J. P., Rousset, F., Vanbervliet, B., Bonnefoy, J. Y., Arai, N., Takebe, Y., Yokota, T., Lee, F., Arai, K., de Vries, J. and Banchereau, J.,** Human recombinant interleukin 4 induces Fcε receptors (CD23) on normal human B lymphocytes. *J. Exp. Med.* 1987. **165:** 1459–1467.

27 **Defrance, T., Vanbervliet, B., Durand, I. and Banchereau, J.,** Human interleukin-4 down-regulates the surface expression of CD5 on normal and leukemic B cells. *Eur. J. Immunol.* 1989. **19:** 293–299.

28 **Freeman, G. J., Boussiotis, V. A., Anumanthan, A., Bernstein, G. M., Ke, X. Y., Rennert, P. D., Gray, G. S., Gribben, J. G. and Nadler, L. M.,** B7-1 and B7-2 dot not deliver identical costimulatory signals, since B7-2 but not B7-1 preferentially costimulates the initial production of IL-4. *Immunity* 1995. **2:** 523–532.

29 **Kuchroo, V., Prabhu Das, M., Brown, J. A., Ranger, A. M., Zamvil, S. S., Sobel, R. A., Weiner, H. L., Nabavi, N. and Glimcher, L. H.,** B7-1 and B7-2 costimulatory molecules differentially activate the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 1995. **80:** 707–718.

30 **Lenschow, D. J., Walunas, T. L. and Bluestone, J. A.,** CD28/B7 system of T cell costimulation. *Annu. Rev. Immunol.* 1996. **14:** 233–258.

31 **Sartori, A., Ma, X., Gri, G., Showe, L., Benjamin, D. and Trinchieri, G.,** Interleukin-12: An immunoregulatory cytokine produced by B cells and antigen-presenting cells. *Methods: A Companion to Methods Enzymol.* 1997. **11:** 116–127.

32 **Taylor, A. P. and Murray, H. W.,** Intracellular antimicrobial activity in the absence of interferon-γ: effect of interleukin-12 in experimental visceral leishmaniasis in interferon-γ gene-disrupted mice. *J. Exp. Med.* 1997. **185:** 1231–1239.

33 **Willems, F., Marchant, A., Delville, J. P., Gerard, C., Delvaux, A., Velu, T., de Boer, M. and Goldman, M.,** Interleukin-10 inhibits B7 and intracellular adhesion molecule-1 expression on human monocytes. *Eur. J. Immunol.* 1994. **24:** 1007–1009.

34 **Kawamura, T. and Furue, M.,** Comparative analysis of B7.1 and B7.2 expression on Langerhans cells: differential regulation by helper type 1 and T helper type 2 cytokines. *Eur. J. Immunol.* 1995. **25:** 1913–1917.

35 **Rousset, F., Garcia, E., Defrance, T., Peronne, C., Vezzio, N., Hsu, D. H., Kastelein, R., Moore, K. H. and Banchereau, J.,** Interleukin 10 is a potent growth and differentiation factor for activated human B lym-

phocytes. *Proc. Natl. Acad. Sci. USA* 1992. **89:** 1890–1893.

36 **Levy, Y. and Brouet, J. C.,** Interleukin-10 prevents spontaneous death of germinal center B cells by induction of the Bcl-2 protein. *J. Clin. Invest.* 1994. **93:** 424–428.

37 **Buchanan, J. M., Vogel, L. A., Van Cleave, V. H. and Metzger, D. W.,** Interleukin-12 alters the isotype-restricted antibody response of mice to hen egg lysozyme. *Int. Immunol.* 1995. **7:** 1519–1528.

38 **Kiniwa, M., Gately, M., Gubler, U., Chizzonite, R., Fargeas, C. and Delespesse, G.,** Recombinant interleukin-12 suppresses the synthesis of immunoglobulin E by interleukin-4 stimulated human lymphocytes. *J. Clin. Invest.* 1992. **90:** 262–266.

39 **Sharma, M. D., de Moraes, M. L., Zavala, F., Pontoux, C. and Papiernik, M.,** Induction and inhibition of CD40-CD40 ligand interactions: a new strategy underlying host-virus relationships. *J. Immunol.* 1998. **161:** 5357–5365.

40 **Guéry, J.-C., Ria, F., Galbiati, F. and Adorini, L.,** Normal B cells fail to secrete interleukin-12. *Eur. J. Immunol.* 1997. **27:** 1632–1639.

41 **Schultze, J. L., Michalak, S., Lowne, J., Wong, A., Gilleecee, M. H., Gribben, J. G. and Nadler, L. M.,** Human non-germinal center B cell interleukin (IL)-12 production is primarily regulated by T cell signals CD40 ligand, interferon γ, and IL-10: role of B cells in the maintenance of T cell responses. *J. Exp. Med.* 1999. **189:** 1–12.

42 **Gordon, J.,** B cell signalling via the C type lectins CD23 and CD72. *Immunol. Today* 1994. **15:** 411–417.

43 **Arpin, C., Dechanet, J., Van Kooten, C., Merville, P., Grouard, G., Briere, F., Banchereau, J. and Liu, Y. J.,** Generation of memory B cells and plasma B cells in vitro. *Science* 1995. **268:** 720–722.

44 **Jeannin, P., Lecoanet, S., Delneste, Y., Gauchat, J.-F. and Bonnefoy, J. Y.,** IgE versus IgG4 production can be differentially regulated by IL-10. *J. Immunol.* 1998. **160:** 3555–3561.

45 **Liu, Y. J., Johnson, G. D., Gordon, J. and MacLennan, I. C. M.,** Germinal centres in T cell-dependent antibody responses. *Immunol. Today* 1992. **3:** 17–21.

46 **Flynn, S., Toellner, K. M., Raykundalia, C., Goodall, M. and Lane, P.,** CD4 T cell cytokine differentiation: The B cell activation molecule, OX40 ligand, instructs CD4 T cells to express interleukin 4 and upregulates expression of the chemokine receptor, blr-1. *J. Exp. Med.* 1998. **188:** 297–304.

47 **MacLennan, I. C. M., Gulbranson-Judge, A., Toellner, K. M., Casamayor-Palleja, M., Chan, E., Sze, D. M. Z., Luther, S. A. and Acha-Orbea, H.,** The changing preference of T and B cells for partners as T-dependent antibody responses develop. *Immunol. Rev.* 1997. **156:** 53–66.

48 **Jeannin, P., Delneste, Y., Lecoanet-Henchoz, S., Gauchat, J.-F., Ellis, J. and Bonnefoy, J. Y.,** CD86 (B7-2) on human B cells. A functional role in proliferation and selective differentiation into IgE- and IgG4-producing cells. *J. Biol. Chem.* 1997. **272:** 15613–15619.

49 **Gagro, A. and Rabatic, S.,** Allergen-induced CD23 on CD4+ T lymphocytes and CD21 on B lymphocytes in patients with allergic asthma: evidence and regulation. *Eur. J. Immunol.* 1994. **24:** 1109–1114.

50 **Gagro, A., Rabatic, S., Ivancic, I., Bendelja, K., Jelacic, J., Sabioncello, A., Misulic, J., Buneta, D. and Dekaris, D.,** Detection of intracellular cytokines in human lymphocytes and monocytes at the single cell level by flow cytometry. *Period. Biol.* 1999. **101:** 17–26.

Correspondence: John Gordon, The Medical Research Council Centre for Immune Regulation, The University of Birmingham, The Medical School, Vincent Drive, Birmingham B15 2TT, GB
Fax: +44-121-414 3599
e-mail: j.gordon@bham.ac.uk