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J Immunol (2005) 174 (8): 4718–4726.

<https://doi.org/10.4049/jimmunol.174.8.4718>

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T Regulatory-1 Cells Induce IgG4 Production by B Cells: Role of IL-10¹

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The study was aimed to find out whether T cells with a regulatory profile could regulate the secretion of IgG4. Using tetanus Ag we found that PBMC of healthy human donors responded to exogenous IL-10 by down-regulating IgG1 and increasing IgG4 secretion. IgE was not affected. To investigate the direct effect of IL-10-producing T cells on B cells, we generated T cell clones (TCC) with two different cytokine profiles: first, IL-10^{high}, IL-2^{low}, IL-4^{low} TCC, and second, IL-10^{low}, IL-2^{high}, IL-4^{high}. The T cell-dependent Ab secretion was measured by coculturing purified CD19⁺ B cells and the TCC. Interestingly, we found that IgG4 production in the coculture correlated with the TCC production of IL-10 ($r^2 = 0.352$, $p = 0.0001$), but not with IL-2, IL-4, nor IFN- γ . IgE showed only a trend with regard to IL-4. Further, there was decreased Ab secretion in the absence of T-B cell contact. IL-10 also induced IgG4 when added to a Th1 TCC-B cell coculture system. The present study thus shows that in T-B cell coculture, IL-10, if induced by the TCC or added to the system, down-regulates the immune response by inducing IgG4 secretion. This establishes a direct implication of IL-10 in humoral hyporesponsiveness, particularly in compartments where the T-B cell interplay determines the subsequent immune response. The correlation between IgG4 and IL-10 ($r^2 = 0.352$) indicates that IL-10 is an important but not the only factor for IgG4 induction. *The Journal of Immunology*, 2005, 174: 4718–4726.

Several studies have revealed that pathogen-specific T regulatory cells play an important role in the immune response against specific infection. Cholera toxin coadministered during immunization has surprisingly been found to induce T regulatory cells specific for bystander Ags (1). These T regulatory cells suppress a Th1 response. Specific T regulatory cells that recognized *Bordetella pertussis*, also suppressed Th1 responses (2) and probably prevent immunopathology (3). Recently, CD4⁺CD25⁺ T regulatory cells were shown both to suppress a Th1 response in colitis as well as a Th2 response induced by *Leishmania major* leading to resistance (4). It has been suggested that cytokines do not play the foremost role in CD4⁺CD25⁺ T regulatory cells (5, 6). However, colitis disease reversion by the CD4⁺CD25⁺ T regulatory cells was shown to be abrogated in mice when treated with anti-IL-10R or anti-TGF- β Ab (7). Ag-specific T regulatory cells, a different group of T regulatory cells, are mainly characterized by high IL-10 or IL-10 plus TGF- β production (2, 8).

In helminth infections production of high levels of IL-10, or IL-10 plus TGF- β (9, 10), and as recently reported, high levels of CTLA-4 expression (11) are associated with a status of little pathology and high worm load. Accordingly, Ag-specific T regulatory-1 (Tr-1)³ clones could be cloned from human tissue in high

frequency in onchocerciasis, a chronic helminth infection, in particular in patients having high worm load and little pathology (12).

The studies on T regulatory cells strongly suggest that the observed immunosuppression is controlled at least partly by IL-10, TGF- β , or CTLA-4 (7, 11), and is likely mediated by IL-10-producing T regulatory cells. These observations are in accordance with the growing evidence that IL-10 is not to be considered a Th2-type cytokine, but rather belongs to a distinct group of immunosuppressive factors (10, 13–15).

High levels of the noncomplement-fixing IgG4 Ab and the IgE Ab have been reported for hyporesponsive and hyperreactive disease forms in helminth infection, respectively. In onchocerciasis, the hyporesponsive form of the infection is characterized by a strong prevalence of the IgG4 Ig subclass compared with IgE and the complement-fixing IgG1, IgG2, and IgG3 Abs (16). This contrasts with the hyperreactive infection, which shows the highest IgE Ab response (17). Because IL-4 was thought to regulate induction of both IgG4 and IgE (18, 19), the question arose how a differential regulation of IgG4 and IgE was caused. Such regulation has been attributed to IL-12. In fact, IL-12 has been shown to selectively enhance IgG4 while inhibiting IgE production (20). This effect of IL-12 should not depend on IFN- γ , because IFN- γ level was earlier shown not to affect in vitro IgG4 production (21).

The uncoupling of IgG4 and IgE has been described in several other studies on bee venom atopy (22–24). In atopic diseases where cross-linking of IgE molecules and Ag on mast cells seems to play a major role (25), disease exacerbation has been shown to be inversely associated with increasing IFN- γ levels (24). In some cases, concomitant enhancement in IL-10 production was reported (24, 26). Although increase in IFN- γ production during the therapy of allergic diseases could always be attributed to a shift from a Th2 to a Th1 response (27), rising IL-10 production has recently been shown to originate from T regulatory cells (28). This indicated a role for these cells and the IL-10 they produce in the treatment of atopy. Although the induction of IgE and IgG4 by IL-4 has been shown, reports on successful treatments or sensitization in atopy described that IgG4 and IL-10 increased, whereas IgE levels decreased or did not change (26). In one study on asthma, the role of

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Received for publication February 12, 2004. Accepted for publication February 3, 2005.

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¹ This study was supported by the German Research Foundation (Ho 2009/1-3) and by the EU (ICA4-1999-1002). J.L. has received a scholarship from the German exchange service (Deutscher Akademischer Austauschdienst).

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³ Abbreviations used in this paper: Tr-1, T regulatory-1; TCC, T cell clone; hTCC, helper TCC.

IL-10 at inducing a differential regulation of IgG4 and IgE has been experimentally demonstrated in PBMC and in B cells using exogenous IL-10 as well as bee venom Ag-induced IL-10 (29). However, no study has been done in regard to the effect of T cell-derived IL-10 in an isolated T and B cell population.

We hypothesized that the high IgG4 levels could be related to Tr-1-like cells and their production of IL-10. IgG4 cannot properly bind the complement (30, 31) and therefore prevents Ab mediated complement activation, and pathogen uptake and destruction. The aim of the study was to find out whether IL-10 and IL-10-producing T cell clones (TCC) could directly regulate the secretion of IgG4 by B cells, by studying T-B cell coculture.

We used PBMC and generated TCCs from European blood donors directed against tetanus toxoid as model Ag. Our results show a correlation between IL-10 and IgG4 secretion, a direct effect of IL-10 on IgG4-secretion, establishing a new role for Tr-1-like cells and IL-10 in Ab-mediated hyporesponsiveness.

Materials and Methods

PBMC, APCs/feeder cells, human serum

All PBMC used in this study originated from healthy European donors and were kindly provided by C. Schwarz, and sera from healthy donors were provided by Dr. J. Hiller, both at the blood bank of the University Clinic Eppendorf (Hamburg, Germany). PBMC were separated from citrate venous blood by gradient centrifugation on Ficoll-Paque (density 1.077; Seromed Biochrom). APCs or feeder cells (used in the case of nonantigenic stimulation of TCC) consisted of autologous and nonautologous PBMC, respectively, which were irradiated (4000 rads). Cells were immediately used or cryopreserved.

Reagents

Tetanus toxoid was kindly provided by Dr. C. Hungerer (Chiron Behring, Marburg, Germany). PHA was from Murex Biotech. Anti-CD3 was from OKT 3 hybridoma (American Type Culture Collection). Anti-CD28 (CD28.2) and anti-CD40 (5C3) Abs were from BD Pharmingen. Human rIL-2 was from Eurocetus. Human rIL-4 and human rIL-10 were from BD Pharmingen. For flow cytometry analysis, all Abs were purchased from Becton Dickinson Immunocytometry Systems, unless otherwise indicated. Abs consisted of anti-CD4-FITC and anti-CD8-PE, PerCP-conjugated anti-CD4, FITC-conjugated anti-CD3 and anti-CD14, and PE-conjugated anti-CD19. Cell culture conditions included the use of RPMI 1640 medium (PAA Laboratories), supplemented with glutamine 200 mg/ml, gentamicin 50 µg/ml, penicillin 100 U/ml, and streptomycin 100 µg/ml (Sigma-Aldrich) and incubator set at 37°C with 5% CO₂.

Generation of T cell lines and TCC

For generation of T cell lines, isolated PBMC were stimulated at 5×10^5 cells/ml with 10 µg/ml tetanus toxoid, alone or in the presence of human rIL-10 (20 ng/ml) to increase the occurrence of IL-10-producing cells, as described (8). PBMC were then cultured in 96-well round-bottom tissue culture plates (Greiner). The culture medium was complemented with 10% human serum. After 10 days, cells were restimulated with tetanus toxoid in the presence of irradiated autologous APCs (5×10^5 cells/ml) with or without IL-10. After another 10 days, before cloning, cells were prestimulated with PHA (1 µg/ml) for 72 h in the presence of human rIL-2 and feeder cells. The cloning was performed in Terasaki plates (Nunc) as described (12). TCC were picked after 10 days from plates with <10% positive wells and restimulated with PHA in 96-well round-bottom plates at 10- to 14-day intervals in the presence of rIL-2. The specificity of TCC was assessed by testing their proliferation to tetanus toxoid using 5×10^4 TCC and 1×10^5 autologous APCs. Cells were cultured in a total volume of 200 µl in 96-well round-bottom tissue culture plates for 4 days. Cultures were then pulsed with 0.2 mCi [³H]thymidine for the last 18 h of culture and the [³H]thymidine deoxyribose incorporation was measured by liquid scintillation spectrometry.

Isolation of CD19⁺ B cells, CD4-depleted cells, and CD14-depleted cells

Dynabeads CD19 (111.03), CD19 detachabeads (125.06), and Dynabeads CD4 (113.03), all from Dynal, were used to isolate CD19⁺ B cells per positive selection and CD4⁺-depleted cells per negative selection, respectively, according to the manufacturer's protocols. In addition, Dynabeads

CD14 was used to deplete monocytes from the PBMC. Briefly, 50 µl of Dynabeads were added to an Eppendorf tube of 1.5-ml size and washed twice with 1 ml of 2% FCS/PBS using the magnetic particle concentrator Dynal MPC (Dynal), and resuspended in RPMI 1640/10% FCS. Cryopreserved PBMC were adjusted to 1.2×10^7 cells/ml. One milliliter of the PBMC was added to the Dynabeads CD19 and incubated 20 min at 4°C under gentle vortexing. The rosetted cells were washed four times with 2% FCS/PBS and resuspended in 100 µl of RPMI 1640/10% FCS. CD19 detachabeads (20 µl) were added and incubated for 45 min at room temperature under gentle rotation. The suspension containing the detached beads was collected and the detached beads were washed twice. For the isolation of CD4⁺ or CD14-depleted cells, the CD4⁺ or CD14-depleted cell suspension was collected while the rosetted cells were adhered to the Dynal MPC. For all isolation procedures, cell suspensions and buffers were kept cold. Cells were adjusted to 1×10^6 cells/ml. To analyze the purity of the isolated cells, cells were double stained before and after isolation for surface expression of CD19 and CD4, CD3 and CD4, CD4 and CD8, or of CD19 and CD14. Fluorescence was measured using a FACSCalibur cytometer (Becton Dickinson) and gated for lymphocytes. CD19⁺ B cells were routinely obtained with a purity >99%.

In vitro cytokine production

For in vitro cytokine analysis culture medium contained 10% human serum. A total of 5×10^4 TCC were stimulated with tetanus toxoid (10 µg/ml) or with anti-CD3 and anti-CD28 (1 µg/ml and 2.5 µg/ml, respectively) in the presence of 1×10^5 irradiated autologous PBMC. TCC were then cultured in a total volume of 0.2 ml in 96-well tissue culture plates. Supernatants were harvested 24 h and 72 h after stimulation for IL-2 and IL-4, and IL-10 and IFN-γ measurement.

Cytokines were quantified using cytokine-specific sandwich ELISA. ELISA-plates (655061 Microton, high binding; Greiner) were coated with 50 µl/well of the appropriate anti-cytokine mAb (2 µg/ml; 8D4-8, JES3-9D7, and NIB42; BD Pharmingen; for IL-4, IL-10, and IFN-γ, respectively) diluted in 0.1 M NaHCO₃-Na₂HCO₃ buffer. Plates were incubated overnight at 4°C. After incubation, plates were washed four times with PBS containing 0.05% Tween 20 and blocked for 1 h at 37°C by adding 200 µl of 1% BSA, PBS to each well. Supernatants collected after 48 h (1:2 in 0.1% BSA, PBS) or recombinant cytokine standards (all from BD Pharmingen) were added at a volume of 50 µl/well. Plates were incubated overnight at 4°C, then washed, and the secondary Ab (1 µg/ml; MP4-25D2, JES3-268, and 4S.B3; BD Pharmingen; for IL-4, IL-10, and IFN-γ, respectively) was added at 100 µl/well. Plates were incubated for 2 h at room temperature. After washing, streptavidin-peroxidase complex (1:10,000; Roche Diagnostics) was added to each well at 100 µl/well. After 1 h of incubation at room temperature, plates were developed by adding 100 µl/well of TMB (Roth; dissolved at 6 mg/ml in DMSO). The reaction was stopped by adding 25 µl of 4 N H₂SO₄/well and plates were measured at 450 nm.

IL-2 cytokine measurement was performed as described above for IL-4; and IL-10 and IFN-γ with the difference that supernatants were collected after 24 h. Also, coating detection Abs and human rIL-2 were from OptEIA human IL-2-Set (BD Pharmingen) and were diluted according to the manufacturer.

The sensitivity of the ELISA was 31 pg/ml for IL-2 and IFN-γ, and 16 pg/ml for IL-4 and IL-10.

In vitro Ab production

For all Ab assays, the culture medium contained 10% FCS and cells were cultured for 14 days. Abs were measured in undiluted supernatants: 100 µl for IgG1 and 4 and 50 µl for IgE.

Ab production by PBMC. Cryopreserved, freshly isolated PBMC or CD14-depleted PBMC were cultured at 1×10^5 cells/well in a total volume of 0.2 ml (6 replicates), either in medium alone or stimulated with tetanus toxoid (10 µg/ml) or anti-CD3 and anti-CD28 (1 µg/ml and 2.5 µg/ml, respectively). For control of the B cell response, anti-CD40 and human rIL-4 (10 µl/ml and 100 µl/ml, respectively) were used to stimulate the PBMC as well. Each stimulus was added alone or with 20 ng/ml human rIL-10.

T and B cell coculture for in vitro Ab induction. For the determination of the T cell-induced Ig secretion by B cells, purified CD19⁺ B cells were used at concentrations of 300, 500, 10,000, and 12,000 CD19⁺ B cells/well. CD4-depleted cells were used at 5000 CD4-depleted cells/well. Autologous CD19⁺ B cells or CD4-depleted cells were then cocultured with 5×10^4 TCC/well in the presence of tetanus toxoid, or anti-CD3 and anti-CD28.

Transwell assay. Ten-millimeter tissue culture inserts (8 µm; Nunc) were introduced in 24-well tissue culture plate (Greiner). IL-10-producing TCC were cocultured at 2.5×10^5 TCC/well (in the upper chamber) with $6 \times$

10^4 CD19⁺ B cells/well (in the lower chamber). TCC were stimulated with anti-CD3 and anti-CD28. The B cells were left either alone in the lower chamber or were added with an autologous Th1 TCC.

IgG subclass measurement. IgG1 and IgG4 were measured using IgG1 subclass and IgG4 subclass kits respectively (Bindazyme; The Binding Site) according to the instructions of the manufacturer. Detection ranges were 2.5–159.2 $\mu\text{g/L}$ and 1.9–121 $\mu\text{g/L}$ for IgG1 and IgG4, respectively.

IgE measurement. IgE was measured using sandwich ELISA as described above in *In vitro* cytokine production. Mouse monoclonal anti-human IgE Ab (1/2000 dilution, clone GE-1; Sigma-Aldrich), biotin-conjugated mouse anti-human IgE (1 $\mu\text{g/ml}$, clone G7-26; BD Pharmingen) were used for coating and detection, respectively. The PreciControl Universal (Elecsys; Roche Diagnostics) was used as standard to determine between 0.067 and 4.32 $\mu\text{g/L}$ IgE.

Statistical analysis

The Mann-Whitney *U* test was used for unpaired comparison and for correlation coefficients, regression plots were analyzed using Anova statistics with the Statview 5.0 program. $p < 0.05$ was considered to be significant. Box plot graphs used are mentioned whenever used and displayed horizontal lines representing the 90th (highest line, outside the box), 75th (upper box line), 50th (the median, inside the box), and the 25th (lower box line) percentiles of the values. Outliers above the 90th percentiles are displayed as circles.

Results

Exogenous IL-10 induces IgG4 secretion by PBMC

We set up a system with T cells from European blood donors and tetanus toxoid as model Ag. Due to the fact that the immune response to tetanus toxoid includes the prevalence of an IFN- γ -based Th1 response along with an IgG1 subclass response, IgG1 was measured in this system together with IgG4 and IgE Abs. To investigate the influence of IL-10 levels on IgG4 regulation, PBMC ($n = 11$) were stimulated with tetanus Ag in the presence or absence of exogenous IL-10 (20 ng/ml). The results expectedly showed IgG1 production following stimulation with tetanus toxoid. With exception of three PBMC, which already had high IgG4 values in nonstimulated wells, low amounts of IgG4 were detected in the absence of stimulation (Fig. 1A, right), nor did tetanus toxoid induce any significant IgG4 production ($p = 0.224$). Also, IgG1 was significantly induced upon stimulation with tetanus toxoid ($p = 0.011$), although already produced in the absence of stimulation (3–227 $\mu\text{g/L}$). However, upon addition of exogenous IL-10 there was an alteration of the tetanus toxoid-induced IgG1 and IgG4 profiles. In 10 of the 11 tested fresh PBMC, IL-10 induced an up-regulation of IgG4 production (Fig. 1A, right; $p = 0.0014$). The addition of IL-10 induced a reduction of IgG1 in 7 of 11 PBMC (Fig. 1A, left). These results show the induction of IgG4 by exogenous IL-10 in PBMC. We conclude that IL-10 induced an up-regulation of IgG4 and in some cases a down-regulation of IgG1 (Fig. 1A).

When cryopreserved PBMC were used, IgG1 was produced without antigenic stimulation (median value 77.91 $\mu\text{g/L}$, data not shown), and was enhanced after stimulation (median value 225.23 $\mu\text{g/L}$, data not shown). However, in contrast to the freshly prepared PBMC, IgG4 was not produced by the cryopreserved PBMC alone or in the presence of IL-10, although a constant reduction of IgG1 was observed in these batches of PBMC (median value from 225.23 to 35.04 $\mu\text{g/L}$, data not shown). These observations indicate that the effect of the exogenous IL-10 seen in the freshly prepared PBMC may have been indirectly due to a cell population that became impaired through freezing and thawing, like monocytes (32). Therefore, the contribution of the monocytes in regulating IgG4 in PBMC stimulated with Ag was tested. PBMC were depleted from CD14⁺ monocytes and assayed as described above. The depletion of the monocytes did not significantly affect the spontaneous IgG1 production in RPMI 1640 or after IL-10 addition ($p = 0.09$ and $p = 0.071$, respectively; Fig. 1B, left). How-

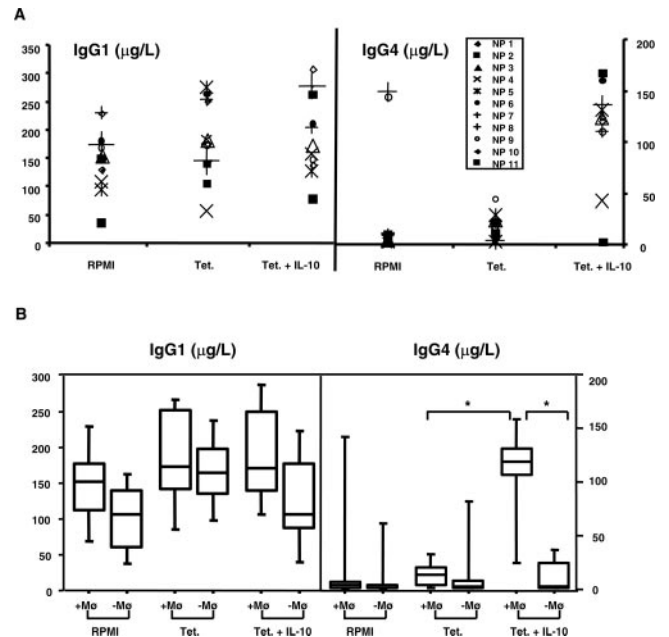


FIGURE 1. Exogenous IL-10 induces IgG4 production in PBMC. *A*, 1×10^5 PBMC were left alone or stimulated with tetanus toxoid (10 $\mu\text{g/ml}$), and IgG1 (left panel) and IgG4 (right panel) were measured in 14-day supernatants. *B*, IgG1 (left panel) and IgG4 (right panel) were measured as in *A* from PBMC with monocytes (+M ϕ) or depleted of CD14⁺ monocytes (-M ϕ). *, $p = 0.0014$. PBMC samples were from 11 healthy donors.

ever, IgG4 was significantly reduced after depletion of the monocytes from the PBMC ($p = 0.0014$; Fig. 1B, right). Being the main APCs of PBMC, monocytes seem to mediate the effect of IL-10 on Ab production. We concluded that monocytes are important for the regulation of IgG4 by IL-10 in PBMC.

IgG4 secretion is linked to the presence of Tr-1 or Tr-1-like cell-derived IL-10

The previous results indicated that IL-10 induced IgG4 production in stimulated PBMC depending on the presence of monocytes. Because IL-10-producing T cells are found in high frequency in conditions with high IgG4, for example in chronic helminth infection or after allergic desensitization, we hypothesized that the IL-10-producing CD4⁺ cells might directly interact with B cells to coincide the characteristically elevated IgG4 levels seen in these conditions. Those T-B cell interactions may in particular occur in draining lymph nodes where monocytes/macrophages are less frequent than in peripheral blood. Therefore, we generated TCC with two different cytokine profiles: first, IL-10^{high}, IL-2^{low}, IL-4^{low} thus resembling regulatory T cells, and second, IL-10^{low}, IL-2^{high}, high IL-4^{high} or IFN- γ ^{high} (Fig. 2A and Table I). The addition of human rIL-10 during the first two rounds of stimulation of the PBMC favored the differentiation of IL-10-producing TCC, as observed earlier (8). This gave rise to a significantly higher rate of TCC with the first cytokine profile ($p = 0.0325$, Fig. 2A) and lower rate of IL-4-producing TCC ($p = 0.0003$). However, addition of human rIL-10 had no influence on the differentiation of IL-2 and IFN- γ -producing TCC ($p = 0.95$, Fig. 2A).

CD19⁺ B cells were purified from autologous PBMC as described in *Materials and Methods* (for purity see Fig. 2B), and cultured alone or with the respective TCC shown in Table I. Experiments showed that significant levels of Abs were detected only when >500 CD19⁺ B cells were used or when CD4⁺-depleted cells were used at 5000 cells per well (data not shown). In subsequent experiments, 12,000 CD19⁺ B cells were used at a high

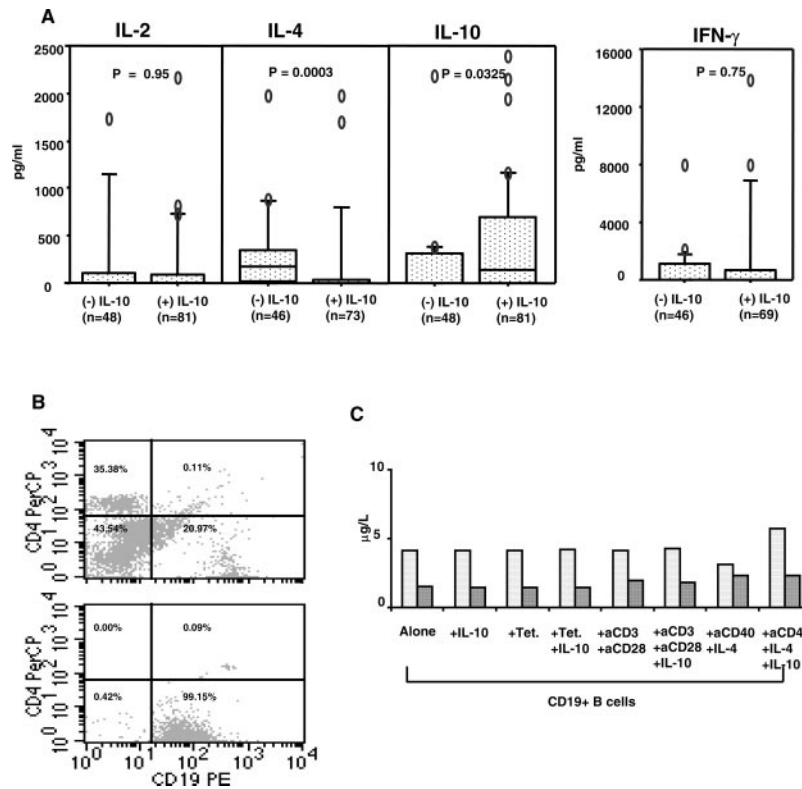


FIGURE 2. Cytokine profile of the TCC and isolation of CD19⁺ B cells from PBMC. *A*, Higher frequency of IL-10-producing TCC with the use of IL-10 during the first stimulations. TCC (5×10^4 TCC per well) obtained with IL-10 or without IL-10 during the first two rounds of stimulation of the PBMC as indicated on the figure, were stimulated with anti-CD3 and anti-CD28 ($10 \mu\text{g/ml}$ and $2.5 \mu\text{g/ml}$, respectively) in the presence of irradiated autologous PBMC (1×10^5). TCC were then cultured in a total volume of 0.2 ml in 96-well tissue culture plates. Supernatants were harvested 24 h and 72 h for measurement of IL-2 and IL-4, IL-10 and IFN- γ , respectively, after stimulation. The frequency of IL-10-producing TCC was higher when IL-10 was used during the first stimulations ($p = 0.03$). Inversely, the frequency of the IL-4-producing TCC was higher ($p = 0.0003$) in the absence of IL-10 during the first two rounds of stimulation of the PBMC. Cytokine production in the absence of stimulus was subtracted for each TCC. The number of TCC used is indicated in brackets. Statistical analysis was performed using the Mann-Whitney U test. The ovals of the box plots are outliers above the 90th percentiles. *B*, CD19⁺ B cell isolation. Dynabeads CD19 (111.03) and CD19 detachabeads (125.06) were used for the positive isolation of the CD19⁺ cells as indicated in *Material and Methods*. To control the purity of the isolated cells, 10^5 PBMC were labeled with CD4PerCp and CD19PE respectively before (*upper panel*) and after (*lower panel*) the isolation procedure. The figure shows one example of the purity of the isolated cells, routinely >99% CD19⁺ and <1% CD4⁺ cells. *C*, No inducible Ab in the absence of CD4⁺ T cells. Twelve thousand CD19⁺ cells were incubated alone, or stimulated in the presence or in the absence of IL-10 as indicated. IgG1 (light dots) and IgG4 (dark dots) were measured in supernatants after 14 days.

purity rate of >99% and <1% CD4⁺ (Fig. 2*C*). Very low Ab level was detected in the culture containing the CD19⁺ B cells alone (Fig. 2*C*) or CD4-depleted cells alone (data not shown), also the B cell alone produced no Ab in the presence of exogenous IL-10 (20 ng/ml, Fig. 2*C* or 40 ng/ml, data not shown). This indicated that the system measured the CD4⁺ T cell-dependent Ab production only. Abs were measured in the isolated TCC-B cell coculture after stimulation with anti-CD3 and anti-CD28 or with tetanus toxoid.

Due to the fact that cytokines and Ab production follow different kinetics, the TCC were tested for their cytokine profile using anti-CD3 and anti-CD28 or tetanus toxoid as stimulus in a separate but parallel (same round of stimulation for TCC and same batch of B

cells) assay to the Ab assay. Also, the SD calculated for the cytokine measurements was very low (< 5%, data not shown); this statistically excluded gross differences in the well where Ig were measured. The TCC produced higher levels of cytokine when stimulated by anti-CD3 and anti-CD28 compared with stimulation by tetanus toxoid (data not shown). A total number of 64 TCC (Table I) were cultured with the isolated B cells for 14 days. Ab were measured in the supernatants as described in *Materials and Methods*. Using regression plots and ANOVA, the correlation between the respective cytokines (Fig. 2*A*) and the Ab IgG1, IgG4, and IgE production by the B cell in the coculture was analyzed. Using anti-CD3 and anti-CD28 we found that the TCC-producing IL-10

Table I. Cytokine production by TCC used for the T cell-dependent IgG1, IgG4, and IgE Ab assays^a

IL-10 Expression	IL-10 ^{positive}	IL-10 ^{low}	Not Defined
Number of TCC	32	27	5 ^b
Profile	IL-2 ^{low} IL-4 ^{low} IL-10 ^{high}	IL-2 ^{high} IL-4 ^{high} IL-10 ^{low}	Mixed
Number of TCC	14	14	36

^a Cytokines were measured after anti-CD3/anti-CD28 stimulation. Total number of clones = 64; positive, cytokine values > 50 pg/ml; low, cytokine values ≤ 50 pg/ml; high, cytokine values of 200–2740 (for IL-2), 200–1986 (for IL-4), and 200–2388 (for IL-10).

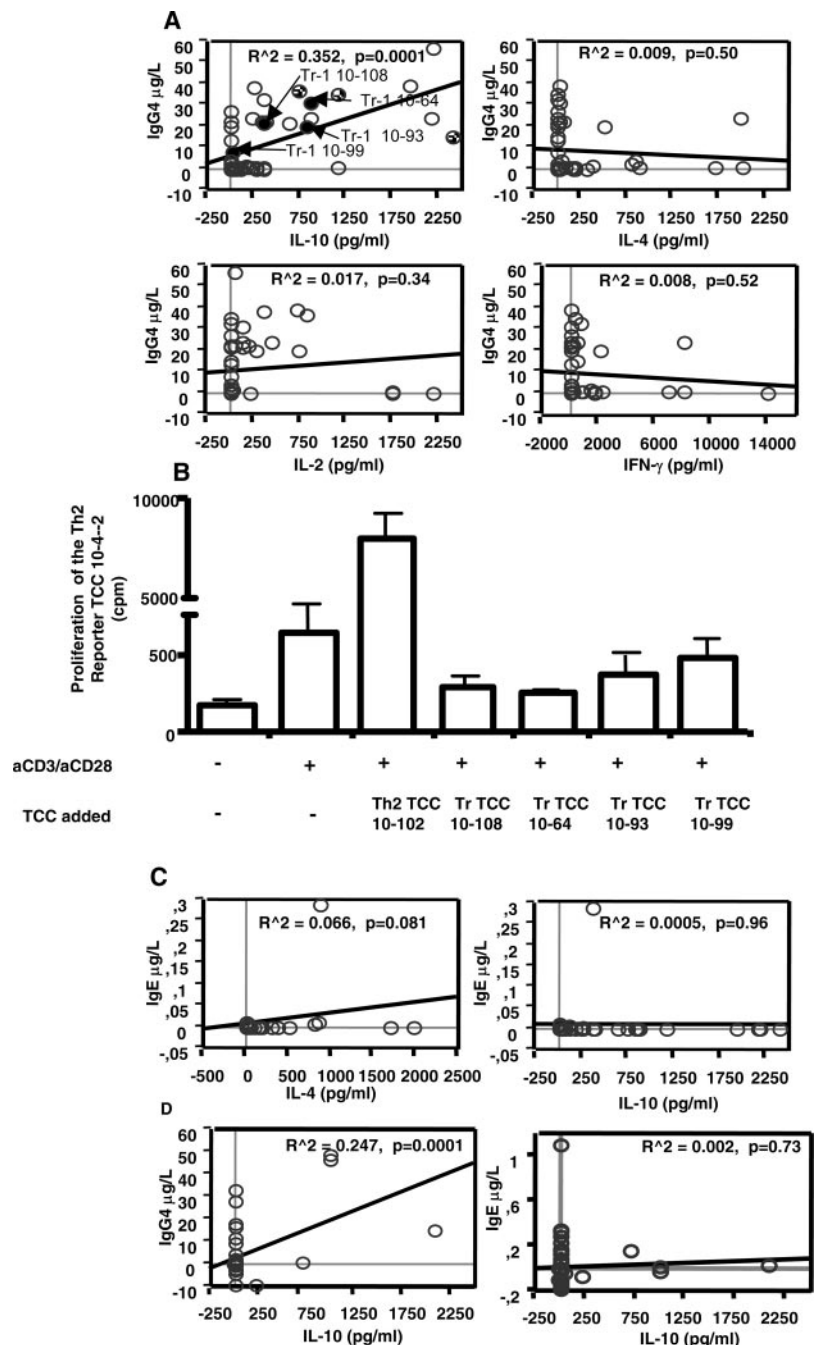
^b TCC not defined for all cytokines.

also induced IgG4 production when cocultured with CD19⁺ B cells. There is a moderate but significant correlation between the presence of IL-10-producing cells and the induction of IgG4 (Fig. 3A, $r^2 = 0.352$, $p = 0.0001$). Interestingly, neither IL-2, IL-4, nor IFN- γ was found to correlate with IgG4 as IL-10 did (Fig. 3A). The TCC with a regulatory cytokine profile we described in the present study are poorly proliferative and not always responsive even to IL-15 (data not shown). In our earlier studies, such Tr-1 cells were shown to suppress the proliferation of Th1 and Th2 cells (12). In this study, four representative TCC with a Tr-1 profile (three TCC with IL-10^{high}, IL-2^{low}, IL-4^{low}, and one TCC with IL-10^{positive}, IL-2^{low}, IL-4^{low}, Table I) yet inducing IgG4 production (Fig. 3A, ●) were therefore tested for suppression of the proliferation of a Th2 reporter TCC (TCC 10-4-2) (Fig. 3B). Interestingly, we found that four of four of the tested TCC were suppressive compared with the Th2 control TCC used (Fig. 3B,

compare third bar to fourth through seventh bar). These TCC therefore appear to be Tr-1 cells. They consistently induced IgG4 in the T-B cell coculture (Fig. 3A, ●, *first graph left*). The IL-10^{positive}, IL-2^{low}, IL-4^{low} TCC induced little IgG4 (8–9 $\mu\text{g/L}$) and was found to be a weak inhibitor (Fig. 3, A and B). These results (Fig. 3A, ●, and Fig. 3B) show that four of four TCC characterized as Tr-1 cells induced IgG4 production in a T-B cell coculture. The remaining TCC used in this study were not tested for suppression, but showed equivalent cytokine profiles to the Tr-1 TCC and were therefore designated in this study as Tr-1-like TCC. Three (3) Tr-1-like TCC (Fig. 3A, dotted circles, *first graph left*) were used for the subsequent experiments (Figs. 4 and 5).

In contrast to IgG4, IgG1 induction showed no correlation with the cytokines IL-2, IL-4, IL-10, and IFN- γ (data not shown). IgE, detected at very low level compared with IgG1 and IgG4, also did not correlate with IL-10 ($r^2 = 0.0005$, $p = 0.96$, Fig. 3B), IL-2, or

FIGURE 3. IgG4 production correlates with the presence of IL-10 production by the Tr-1 and Tr-1-like TCC. For the determination of the T cell-induced Ig secretion by the B cells, purified CD19⁺ B cells were used at 12,000 CD19⁺ B cells/well. Autologous CD19⁺ B cells were cultured with 5×10^4 TCC/well in the presence of anti-CD3 and anti-CD28 (1 $\mu\text{g/ml}$ and 2.5 $\mu\text{g/ml}$ respectively; A and B) or tetanus toxoid (10 $\mu\text{g/ml}$, C). Supernatants were collected 14 days after stimulation. IgG1, IgG4, and IgE Ab were quantified using IgG-subclass kits for IgG1 and IgG4 and a sandwich ELISA for IgE. A and B, correlation between Ab and cytokines using anti-CD3 and anti-CD28 as stimulus. A, Correlation between IgG4 and the cytokines IL-10, IL-4, IL-2, and IFN- γ ; *first graph left*, correlation between IgG4 and IL-10; filled circles indicate the suppressive Tr-1 TCC shown in B; dotted circles indicate the Tr-1-like TCC used for Transwell assay (see Fig. 4 and Fig. 5). B, Tr-1 cells induced IgG4 and suppressed the proliferation of the Th2 reporter TCC 10-4-2. A total of 4×10^4 of the Th2 reporter TCC/well was stimulated with anti-CD3, anti-CD28, alone or together with 2×10^4 of autologous control Th2 TCC or Tr-1 TCC as indicated in the presence of 1×10^5 irradiated autologous PBMC. The graph shows cellular proliferation, as cpm; results are from one of two representative experiments. C, Correlation between IgE and the cytokines IL-4 and IL-10. D, Correlation between IgG4, IgE, and IL-10 using tetanus Ag as stimulus. There is a positive and significant correlation between IgG4 and IL-10 ($r^2 = 0.352$, and $r^2 = 0.247$, respectively, with anti-CD3 and anti-CD28 and tetanus toxoid, $p = 0.0001$). Values in the absence of stimulation were subtracted. In some cases, stimulation led to a reduction of Ab in the well and hence to the negative values in A, C, and D. In vitro cytokine analysis has already been described in Fig. 2A, except that for correlation analysis, cytokine and Ab production assays were conducted in two parallel experiments, because Abs and cytokines have different kinetics.



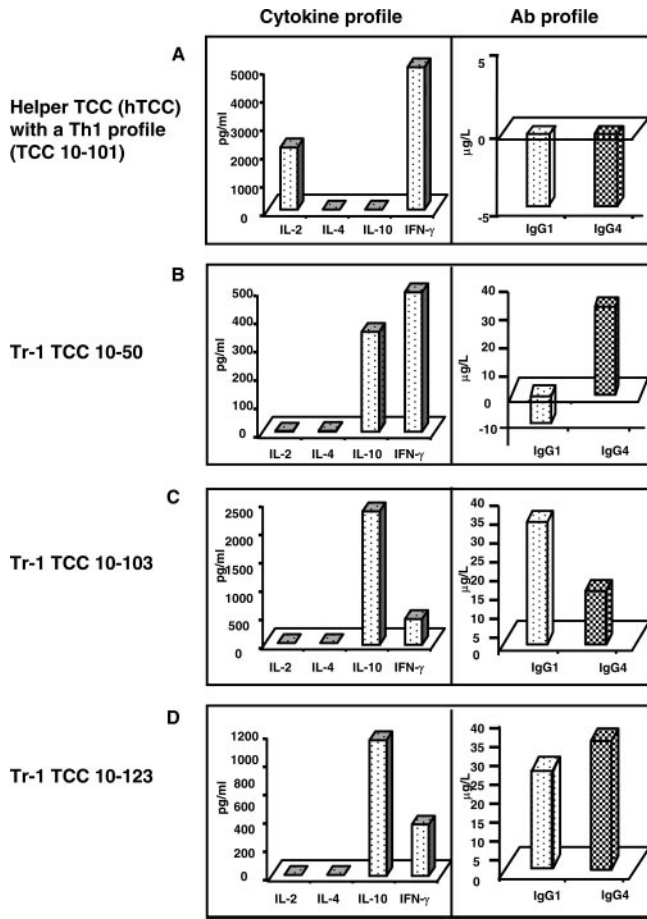


FIGURE 4. Cytokine and Abs profile of the TCCs used in the subsequent experiments (Fig. 5 and 6). IL-2, IL-4, IL-10, and IFN- γ , *left graphs*, and IgG1 (light dots) and IgG4 (dark dots), *right graphs*, were measured as already described in Fig. 3. Values in the absence of stimulation were subtracted. In some cases, stimulation led to a reduction of Ab in the well and hence to the negative values in A and in B. A, A typical Th1 clone induced neither of the Abs. Therefore, it was used in the experiments of Fig. 5 and 6, Tr-1-like clones induced either IgG4 alone or IgG4 and IgG1.

IFN- γ (data not shown), though there was a trend to a weak correlation with IL-4 ($r^2 = 0.066$, $p = 0.081$, Fig. 3B).

These results could be extended to cytokine production after stimulation with tetanus toxoid Ag. IL-10 was found again to correlate with IgG4 ($r^2 = 0.247$, $p = 0.0001$, Fig. 3C), while not correlating with IgE ($r^2 = 0.002$, $p = 0.73$, Fig. 3C). Also using tetanus toxoid, we could not see any correlation between IgG1 or IgE with none of the analyzed cytokines (data not shown). We concluded that the IL-10 released by the TCC induced the purified B cells to produce IgG4 while having no effect on the induction of IgG1 or IgE. These results show a novel mechanism by which T cell-derived IL-10 might modulate the immune response via induction of IgG4.

The optimal induction of IgG4 by IL-10 requires T-B cell contact

The correlation obtained between IL-10 and IgG4 production, $r^2 = 0.352$ with stimulation by anti-CD3 and anti-CD28 and 0.247 for the stimulation by tetanus toxoid (see *IgG4 secretion is linked to the presence of Tr-1 or Tr-1-like cell-derived IL-10*), also indicates that additional factors may be required for IL-10 to induce IgG4 production in the coculture. To find out whether T and B cell contact was an important factor, we used Transwell chambers to separate the IL-10-producing TCC compartment from the B cells.

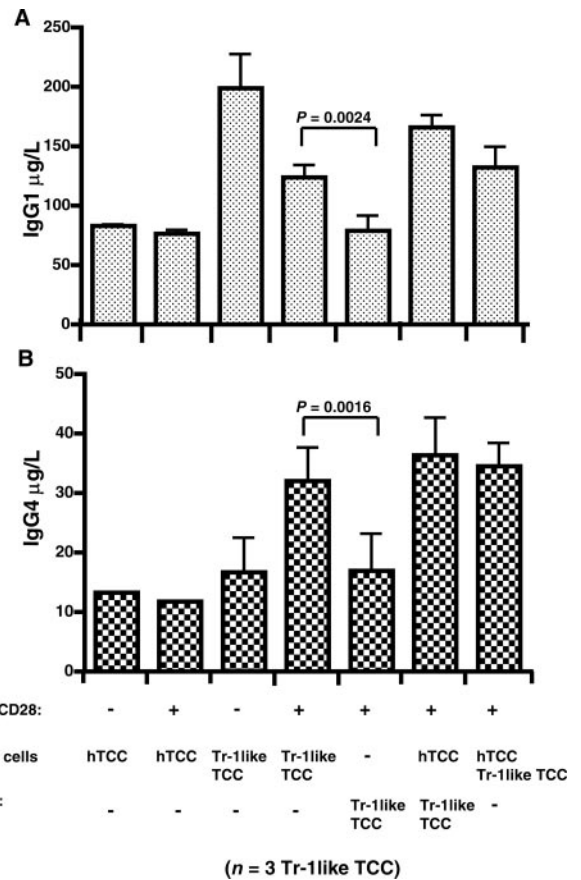
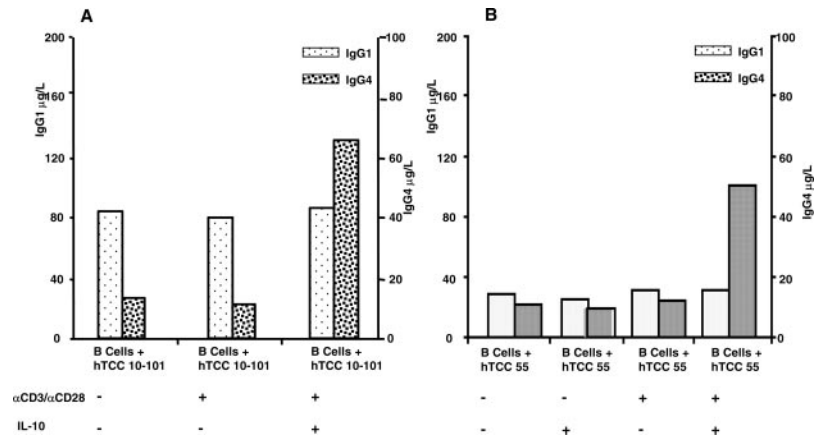


FIGURE 5. IL-10 required T-B cell contact for optimal Ab induction. Transwell assay, 10-mm inserts were placed in 24-well tissue culture plates. IL-10-producing TCC were cocultured at 2.5×10^5 TCC/well in the upper chamber (Transwell) with 6×10^4 CD19 $^+$ B cells/well in the lower chamber (bottom). TCC were stimulated with anti-CD3 and anti-CD28. In some cases, the Tr-1 TCC and/or the Th1 hTCC were added to the B cells as indicated. IgG1 (light dots) (A) and IgG4 (dark dots) (B) were measured as already described in Fig. 3. In the absence of T-B cell contact, IgG4 but also IgG1 is significantly reduced, $p = 0.0016$ and $p = 0.0024$, respectively. The experiment was conducted in duplicate for each of the three Tr-1-like TCC (Fig. 4, B–D). Abs were measured in pooled supernatant for each TCC; results were analyzed using Student’s *t* test.

Because no Ab could be induced in the absence of CD4 $^+$ T cells (Fig. 2C), a well-defined Th1-TCC (TCC 10-101) that did not produce any IL-10 nor induced any IgG1 or IgG4 and therefore named helper TCC (hTCC) (Fig. 4A) was included in the B cell compartment (lower chamber). Each one of the three well-characterized Tr-1 TCC mentioned before (Fig. 3A, dotted circles, and Fig. 4, B–D) were incubated in the upper chambers. In additional wells, the Tr-1 TCC were left together with the B cells (with or without the Th1 hTCC) in the same compartment.

The results showed no difference in IgG1 and IgG4 production between the wells where IL-10-producing Tr-1 TCC and B cells were together and the wells where the Th1 hTCC was added to both Tr-1 TCC and B cells (compare fourth and seventh bars, Fig. 5, A and B). However, there was an overall reduction in IgG4 but also in IgG1 production in the absence of direct T-B cell contact (see fifth bars, Fig. 5, A and B). It is noteworthy that anti-CD3/anti-CD28-stimulation of this Th1 hTCC alone with the B cells did not induce any IgG4 production (compare first and second bars) as

FIGURE 6. Th1 TCC-induced IgG4 production in the presence of exogenous IL-10. *A*, The Th1 TCC described in Fig. 4A was stimulated with anti-CD3 and anti-CD28 in the presence of 20 ng/ml human rIL-10 as indicated. The TCC was cultured with CD19⁺ B cells for 14 days. *B*, A second Th1 TCC was tested as for *A*, except that cells incubated with IL-10 alone were included as control. No IgG1 was induced by the Th1 TCC with or without exogenous IL-10. However, IgG4 was strongly induced upon IL-10 addition.



also shown in the previous experiment (Fig. 4A, here the Ab production without stimulation was subtracted), indicating that cell-cell contact and T cell stimulation alone in the absence of IL-10 did not induce production of IgG1 and IgG4.

The results therefore indicate that T-B cell contact is necessary for an optimal IgG induction in our system. Moreover, it indicates that contact does not need to be necessarily provided by the IL-10-producing TCC because the Th1 hTCC could provide the necessary cell-cell contact as long as a Tr-1 TCC was present in the upper chamber, resulting in maximal IgG4 Ab induction in this system (compare fourth and sixth bars, Fig. 5, *A* and *B*). Equivalent results were also obtained using heterologous B cells (data not shown).

Exogenous IL-10 directly induces IgG4 production by purified B cells in the presence of a Th1 TCC

The previous results indicated that a cell-cell contact with B cells was necessary for optimal IgG4 induction. However, the results also indicated that this contact did not need to be necessarily provided by the IL-10-producing TCC. It seemed sufficient to provide a T-B cell-cell contact and secreted productions (containing IL-10) from a Tr-1-like TCC. Additionally, our experiments demonstrated a correlation between IgG4 and IL-10 (Fig. 3A). To confirm the importance of these two factors, we used the Th1 TCC that did not induce Ab production when used as hTCC in direct coculture (Fig. 4A) and in the Transwell assays (Fig. 5, *A* and *B*), to find out whether addition of exogenous IL-10 to the Th1/B cells would be sufficient for IgG4 production. The Th1 TCC was therefore stimulated and cocultured with the CD19⁺ B cells, and the IgG1 and IgG4 was measured in the presence or the absence of exogenous IL-10. The Th1 TCC induced no IgG1 before and after IL-10 addition. Interestingly, IgG4 was strongly induced upon IL-10 addition (Fig. 6A). The same experiment was conducted using a second Th1 TCC, to confirm this observation and to additionally determine whether IL-10 alone was able to induce IgG4 production in the absence of T cell stimulation (Fig. 6B). The results confirmed that exogenous IL-10 was sufficient to induce IgG4 Ab in a Th1-B cell coculture. Additionally, it indicated that stimulation of the Th1 TCC is a condition for the induction of IgG4 by IL-10 to take place. These results further confirm that optimal induction requires IL-10 and direct contact between CD14⁺ TCC and B cells.

Taken together, while results show the requirement of monocytes in PBMC for optimal IgG4 production, the interesting finding in this study is that monocytes are not necessary in isolated T-B cell populations. In this study, we show that IgG4 induction required Tr-1/Tr-1-like T cell or IL-10, T cell to B cell contact, as well as stimulation of the T cell providing the contact.

Discussion

Unlike other IgG subclasses, IgG4 is found in conditions where IL-10 is increased, as in allergic diseases after immunotherapy (24, 26, 33) or chronic helminth infections (10, 34, 35). In the present study, we investigated whether IgG4 could be related to Tr-1 and Tr-1-like cells and their production of IL-10. We found, at a clonal level, a significant correlation between IL-10 secretion by Tr-1 TCC and their ability to induce B cells to produce IgG4. Moreover, we found that cell-cell contact is required for optimal Ab induction. Also, there is a direct effect of IL-10 on IgG4 production, because a well-defined Th1 TCC can induce IgG4 production in the presence of exogenous IL-10. In conclusion, the present study provides experimental evidence that IL-10 up-regulates the secretion of IgG4, that this up-regulation does not happen with B cells alone but requires, in a two-cell-system, the presence of T cells. However, IgG4 can also be induced in the presence of Th1 cells, provided that IL-10 is added. This implies that IgG4 is not affected by the presence of IFN- γ or Th1 cells as long as IL-10 is present.

IL-10 was for some time thought to be a representative of Th2 responses, although earlier results show its production also by Th1 cells (36–38). Increasing data from several studies brought up the evidence that in inflammatory Th2 responses, like allergic conditions or inflammation in helminth infection with pathology, IL-10 has a down-regulatory role and counteracts Th2 responses (39–41). IL-10 is also produced by Tr-1 cells (8, 13, 42, 43), by Th3 cells (44, 45), and by macrophages (46). In helminth infections, including schistosomiasis (47), onchocerciasis, and lymphatic filariasis, IL-10 is associated with several regulatory mechanisms that favor high parasite loads but mild disease. In onchocerciasis, the role of IL-10 has been shown to contrast that of IL-4 (10). Thus, patients with a typical Th2 cytokine response to nematode Ag successfully control worm load, but present severe symptoms (10, 35, 48). It has also been shown for human filariasis and other helminth infections that the asymptomatic carriers have higher IgG4 levels than the patients with chronic disease (10, 16, 34).

In the present study, we confirm that IL-10 induces IgG4 production by PBMC as described earlier using exogenous IL-10 and bee venom Ag-induced-IL-10 (29), and we extend these results by our finding that in PBMC, monocytes are important for IgG4 induction. We found that responses of frozen PBMC to exogenous IL-10 were reduced compared with the fresh PBMC (data not shown). Because monocytes are known to suffer from cryopreservation (32), we tested the contribution of this cell population by depleting the CD14⁺ monocytes from the PBMC. This resulted in clearly lower IgG4 production. At present, it is unclear whether

monocytes are required for IgG4 production only in response to antigenic stimulation, or also in response to other stimuli, e.g., TLR ligands. Given the fact that recently, murine regulatory T cells have been shown to express TLR as well (49), a detailed study should be set up to investigate the contribution toward IgG4 induction of the diverse cell subtypes of PBMC upon nonspecific stimuli.

In the present study, we also found that exogenous IL-10 was effective at inducing IgG4 when used at 20 ng/ml. Interestingly, however, the induction of IgG4 in PBMC significantly decreased in three of five tested samples when higher concentrations of exogenous IL-10 were used (40 and 50 ng/ml were tested, data not shown). This suggests a negative feedback regulation by IL-10 in IgG4 induction. Such feedback effect is known for TGF- β 1, which induces, in a dose-dependent manner, its endogenous inhibitor Smad7 (50, 51).

However, our particularly novel finding for IgG4 induction is that monocytes are not necessary. This will be relevant in a compartment where T-B cell interaction but not monocytes is determinant, like in the course of eliciting an adaptive memory response in lymph nodes, T cell-derived IL-10 as well as contact between T and B cells are the main factors for the induction of IgG4, a subclass which is associated with immunosuppression in allergy and helminth infection. The data thus demonstrate an additional mechanism by which IL-10 may down-regulate Th2-mediated inflammatory processes.

Direct *in vitro* evidence of IL-10 correlating with IgG4 production was consistently observed using anti-CD3 and anti-CD28 for T cell stimulation ($r^2 = 0.352$, $p = 0.0001$), but also using tetanus toxoid ($r^2 = 0.247$, $p = 0.0001$). However, the intermediate degree of correlation indicated that the induction of IgG4 also involved other factors, such as cell contact, specific surface receptors like CTLA-4 and ICOS, and other soluble mediators of the IL-10 or the TGF- β family. The expression of CTLA-4 is common to several T regulatory cells including the CD4⁺CD25⁺ Tr. In a murine colitis model, the effect of T regulatory cells has been shown to be completely abolished if recipient mice were treated by anti-IL-10, anti-TGF- β , or anti-CTLA-4 (7). Also, the recently defined Th1-like T regulatory cells express ICOS, which was required for their function (52).

We have shown in our experiments that T cells with a regulatory profile induce polyclonal IgG4 production from B cells. However, Ag-specific IgG4, although not detectable probably because of low frequency of Ag-specific B cells present in the assay, would be expected to be a more potent immunoregulator on an equimolar basis, or in other words, even very small amounts of Ag-specific IgG4 would have an immunoregulatory effect comparable to higher amounts of polyclonal IgG4. Because there is no good reason to assume that Ag-specific B cells would be selectively exempt from receiving signals from regulatory T cells *in vivo*, as suggested by earlier reports (26, 29), we assume that a part of the immunoregulatory function of IgG4 *in vivo*, in addition to polyclonal IgG4, is mediated by small amounts of Ag-specific IgG4 produced by a small number of Ag-specific B cells that have interacted with IL-10-secreting regulatory T cells.

Although IgG4 correlated with IL-10 production by TCC, it did not correlate with IL-2 or IFN- γ . Also, there was no correlation between IgG4 and IL-4. IL-10 did not affect IgE Ab production. However, we did find a trend of IgE correlating weakly with IL-4 ($r^2 = 0.066$, $p = 0.081$). This underscores the uncoupling of IgG4 and IgE already described in several systems (20, 24). The present results now extend the role of IL-10 in cellular hyporesponsiveness to the regulation of the humoral response as a result of T-B cell interaction.

Our results show that IL-10 induces B cells to produce IgG4. This is in concordance with the function IL-10 seems to play in

allergic diseases. Interestingly, patients monitored for 70 days during specific immunotherapy had increased IgG4 and IgA levels, which coincided with production of IL-10 and TGF β , respectively (28). Our results are also consistent with earlier report on bee venom phospholipase, where it has been shown that the high IL-4-producing T cells induced IgE, while T cells producing low amounts of IL-4 and IFN- γ induced IgG4 (22). However, the authors did not investigate whether IL-10 was also produced, and thus, IFN- γ -producing T cells were characterized as Th1. In a recent report, the Th1 immune response with a dampening function on a Th2 proinflammatory airway hyperreactivity was shown in mice to include a component of T regulatory cells that coproduce IFN- γ and IL-10, and which express the Th1 transcription factor T-bet. These T regulatory cells were therefore named Th1-like T regulatory cells (52).

Our results also showed that the Ab response induced by the Tr-1 cells was not influenced by the presence of the Th1 TCC (Fig. 6). Interestingly, this implies that IFN- γ does not interfere with IgG4 and may explain why the T regulatory cells can make IFN- γ , probably helping to suppress the Th2 response at the level of cytokine regulation. After specific immunotherapy in allergy, there is an increase in both IFN- γ and IL-10 (41, 53, 54) and decreased release of histamine (55) at protein and RNA levels (56). Because until recently T regulatory cells were difficult to characterize, their involvement in the changes after specific immunotherapy, although hitherto not clearly documented, is likely. Furthermore, the induction of a Th1 response during such immunotherapy did not cause additional inflammation in the treated patients (26). Hence, the results indicate that the main dampening role was played by IL-10 (41, 57) and that IFN- γ might help control the Th2 response by down-regulating IL-4. It cannot be excluded that T regulatory cells, at least in part, are the source of IFN- γ measured after immunotherapy. In a recent study on house dust mites allergy, specific IgG4 was increased in patients after specific immunotherapy, while the total number of IL-4-expressing CD4⁺ cells decreased, in contrast to IFN- γ -expressing CD4⁺ or CD8⁺ cells (58).

Several observations had already led to the hypothesis that T regulatory-derived IL-10 induces many regulatory functions including IgG4 secretion (41, 59). The present study brings experimental data to support these assumptions.

Acknowledgments

We are indebted to C. Schwarz at the University Clinic of Eppendorf and Dr. J. Hoch at the University Clinic (Bonn, Germany) for continually providing us with buffy coats.

Disclosures

The authors have no financial conflict of interest.

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