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A New Role of CTLA-4 on B Cells in Thymus-Dependent Immune Responses In Vivo

Dagmar Quandt, Holger Hoff, Marion Rudolph, Simon Fillatreau, and Monika C. Brunner-Weinzierl

The expression of CTLA-4 (CD152) on the cell surface of B cells and its consequences for the humoral immune response in vivo are unknown. We investigated the expression of CTLA-4 mRNA and protein in B cells in T cell-independent or -dependent ways. B cells in the presence of Ag-stimulated Th2 cells expressed mRNA of CTLA-4 and up-regulated intracellular CTLA-4 protein. Using a liposome-enhanced staining technique, we show for the first time, that surface CTLA-4 protein is expressed by 11–15% of B cells in a T cell-dependent culture system. To dissect the role of CTLA-4 on B cells in vivo, we used bone marrow chimera mice in which only B cells were CTLA-4 deficient. These mice showed that early B cell development and homeostasis is not influenced by CTLA-4 deficiency of B cells. Ag-specific responses after immunization of the chimera mice revealed elevated levels of IgM Abs in mice deficient for B cell CTLA-4. We propose that CTLA-4 signals on B cells determine the early fate of B cells in thymus-dependent immune responses. The Journal of Immunology, 2007, 179: 7316–7324.
vivo. Since surface CTLA-4 is expressed only at low levels on the cell surface and technically difficult to detect by conventional cytometric analysis, we clarified the expression on the surface of B cells using a unique, enhanced staining technique with fluorescein-filled liposomes (11, 12, 33). We identified surface CTLA-4-expressing B cells with a peak expression 48–72 h after stimulation in T cell-dependent culture systems. The CTLA-4 mRNA in highly purified B cells from those B cell-T cell cocultures was readily detectable at 24 h. To analyze the in vivo role of CTLA-4 on B cells, we generated bone marrow chimeric mice, in which the CTLA-4 deficiency was restricted to B cells. We discovered that development and homeostasis of B cells was independent of CTLA-4, as well as the production of natural Abs. Intriguingly, the Ag-specific IgM and temporal IgE Ab production after a TD immunization was down-regulated on CTLA-4-expressing B cells, even after a second antigenic challenge in vivo. These data are the first demonstrating a role for CTLA-4 on B cells in vivo.

Materials and Methods

Mice

Mice transgenic (tg) for the DO.11.10 αβ TCR (OVA-specific TCR) on a BALB/c background (a gift from D. Y. Loh, Washington University School of Medicine, St. Louis, MO), BALB/c, C57BL/6, CTLA-4−/− (a gift from J. P. Allison, University of California at Berkeley) and μMT (from Charles River Laboratories), the latter both on a C57BL/6 background, were bred and/or kept in the animal facility of the Bundesinstitut für Risikobewertung in
Representative data from three independent experiments are shown. mRNA is detectable in B cells 24 h after stimulation. Total RNA from B cells, T cells, and bone marrow-derived DCs were extracted and reversely transcribed for the indicated time points. Latex microspheres were coated as described in Methods. The thin black line represents the isotype control for CD69 staining. Representative data from two independent experiments are shown.

CTLA-4 ON B CELLS

CTLA-4 protein in B cells is not a result of molecule transfer from T cells and CTLA-4 mRNA is transcribed in primary B cells. A and B, CTLA-4 protein in B cells is not a result of molecule transfer from activated T cells. T cell-depleted B cell-containing splenocytes were obtained from CTLA-4<sup>+/−</sup> C57BL/6 or CTLA-4<sup>−/−</sup> C57BL/6 mice by depletion of CD90<sup>+</sup> cells with anti-CD90 beads by MACS. T cells were derived from CTLA-4<sup>−/−</sup> C57BL/6 mice via positive selection of CD4<sup>+</sup> splenocytes with anti-CD4 microbeads by MACS. The respective T cell-depleted B cell-containing splenocytes were incubated with T cells from CTLA-4<sup>−/−</sup> mice in a ratio of 3:1 and stimulated with 5 μg/ml Con A and 30 ng/ml IL-4 for 48 h. A, B cells from CTLA-4<sup>−/−</sup> or CTLA-4<sup>−/−</sup> mice are activated in T cell cocultures. The histogram shows the overlay of CD69 cells from CTLA-4<sup>−/−</sup> (gray shaded area) or CTLA-4<sup>+/−</sup> (thick black line) mice gated on CD19<sup>+</sup> cells. The thin black line represents the isotype control for CD69 staining. Representative data from two independent experiments are shown. B, B cells do not take up CTLA-4 from activated CTLA-4<sup>−</sup> T cells. After fixation, intracellular CTLA-4 was assayed by flow cytometry on CD19<sup>+</sup> B cells. The dot plots show the intracellular CTLA-4 and the numbers indicate CD19<sup>+</sup> CTLA-4<sup>−/−</sup> cells in cultures with CTLA-4<sup>−/−</sup> B cells and with CTLA-4<sup>−/−</sup> T cells. The specificity of the staining was controlled by using an isotype-matched Ab. Representative data from two similar independent experiments are shown. C and D, TCR<sup>αβ</sup>/Th2 effector cells were stimulated with T cell-depleted B cell-containing splenocytes in a ratio of 3:1 with 1 μg/ml OVA323-339 for 24 h. CD19<sup>+</sup> B cells were enriched using MACS and further purified by FACS technology to a purity of 99.9%. C, The dot plot represents the analysis of the enriched CD19<sup>+</sup> B cells. D, CTLA-4 mRNA is detectable in B cells 24 h after stimulation. Total RNA from B cells, T cells, and bone marrow-derived DCs were extracted and reversely transcribed into cDNA. CTLA-4 mRNA was amplified by PCR. The purity of the B cell preparation is controlled by amplification of LAT, which is only detectable in T cells. Representative data from three independent experiments are shown.

Abs and reagents

The following Abs against murine Ags were used: anti-CD19 (1D3; BD Biosciences), anti-CD40 Ab (3/23; BD Pharmingen), anti-trinitrophenol (A 19-3; BD Biosciences), anti-CD86 (GL1; BD Pharmingen), anti-Ha-IgG (Jackson ImmunoResearch Laboratories), anti-CD69 (H1.2F3; BD Pharmingen), anti-α (187.1), anti-CD3 (145-2C11), anti-CD4 (GK-1.5/4), anti-B220 (RA3.6B2), anti-MHC II (M5/114) and anti-CTLA-4 (UC10-4F10), anti-IgM (AF3), and anti-IgD (11.26C) were purified from hybridoma supernatants with protein G columns. Abs were used unconjugated for cell culture or in conjugates of FITC, PE, and Cy5. Abs were used unconjugated for cell culture or in conjugates of FITC, PE, and Cy5. Abs for ELISA were: coating Ab anti-mouse Ig (H + L) and detection Ab goat anti-mouse IgG, IgG2a, IgG1, IgM, and IgE all coupled to alkaline phosphatase and para-nitrophenyl phosphate substrate were purchased from Southern Biotechnology Associates. Magnetic microbeads anti-CD19, anti-CD4, anti-CD43, anti-CD90, anti-CD62 ligand, and anti-FITC multisort were purchased from Milenyi Biotec. CpG and oligonucleotide primer were obtained from ThermoBiol® LPS from Escherichia coli and Con A were purchased from Sigma-Aldrich. IL-4 was purified from supernatants of P3X63 myeloid cells as well as GM-CSF was purified from culture supernatants. Sulfate polystyrene latex microspheres of 5 ± 0.1-μm mean diameters were obtained from Interfacial Dynamics.

In vitro culture systems

B cells were isolated using MACS either by depletion of none B cells with anti-CD43 microbeads or via positive selection with anti-CD19 microbeads according to the manufacturers’ instructions. Isolated B cell were stimulated with CpG (1 μM), LPS (40 μg/ml), anti-CD19 (10 μg/ml), anti-CD40 (1 μg/ml), anti-κ (10 μg/ml), IL-4 (5 ng/ml), or anti-CD40 microspheres for the indicated time points. Latex microspheres were coated as described previously (11, 34) and B cells were stimulated at a ratio of 1:1 with Ab-coupled microspheres.

Three different B cell/T cell coculture systems were applied. At first B cells preincubated with LPS (40 μg/ml) or left untreated were stimulated with naive TCR<sup>αβ</sup>/Th4 cells (isolated with anti-FTTC multisort and CD62 ligand microbeads) in a ratio of 3:1 and 1 μg/ml OVA323-339 for the indicated periods. Second, B cell containing APCs were obtained by depletion of T cells with anti-CD90 microbeads and stimulated with TCR<sup>αβ</sup>/Th2 effector cells in a ratio of 3:1 with 1 μg/ml OVA323-339 for the indicated periods. Th2 effector cells were generated as described elsewhere (11). Third, for the coculture of T cell-depleted B cell-containing splenocytes from CTLA-4<sup>−/−</sup> or CTLA-4<sup>+/−</sup> mice with T cells from CTLA-4<sup>−/−</sup> mice, splenocytes were obtained by depletion of CD90<sup>+</sup> cells with anti-CD90 microbeads and T cells were sorted with anti-CD4 microbeads by MACS. The respective T cell-depleted B cell-containing splenocytes were incubated with T cells from CTLA-4<sup>−/−</sup> mice in a ratio of 3:1 and stimulated with 5 μg/ml Con A and 30 ng/ml IL-4 for 48 h. Total splenocytes were cultured with Con A (3 μg/ml) or CpG (1 μM). All the different types of cells were cultured in RPMI 1640 (PAA Laboratories) supplemented with 10% FCS (Sigma-Aldrich), 100 U/ml penicillin, 0.1 mg/ml streptomycin (both PAA Laboratories), and 10 μM 2-ME and were kept in a humidified incubator at 5% CO₂.

Generation of bone marrow-derived dendritic cells (DCs)

Bone marrow cells of BALB/c mice were differentiated to DCs with GM-CSF and taken on day 6 for experiment.

FACS analysis

Surface and intracellular expression of CTLA-4 was detected using immunofluorescent liposomes as described elsewhere (35). Cytometric analyses were performed using a FACSCalibur (BD Biosciences) and CellQuest software (BD Biosciences). Dead cells were excluded by forward/side scatter gating and propidium iodide staining in live cell analysis.
mRNA analysis from purified B cells

B cells were purified from T cell cocultures 24 h after stimulation by depletion of T cells with anti-CD90 microbeads and were subsequently further purified by FACSort (Diva; BD Biosciences) to a purity of 99.9%. Total RNA from frozen cell pellets was extracted by using a RNeasy Mini Kit (Qiagen) and reversely transcribed into cDNA (Invitrogen Life Technologies). RT-PCR was performed using the following oligonucleotide primer: for CTLA-4, 5’-ACTCTGCTCCCTGAGGACCTCAG-3’ and 5’-GGATGGTGAGGTTC-ACTCTGC-3’; for linker for activation of T cells (LAT), 5’-GGATGGTGAGGTTC-ACTCTGC-3’; and for forrest, 5’-TGGAATCTGTGGCATCCATG 3’. Amplification was performed with 0.25 units of AmpliTaq DNA Polymerase (Perkin-Elmer), 200 μM of each deoxynucleotide triphosphate, 0.5 μM of each primer, and 1 μl of cDNA in a final volume of 50 μl. The cycling parameters were 95°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a final incubation at 72°C for 5 min. PCR products were separated on 0.8% agarose gels and stained with ethidium bromide. The gels were photographed with a Gel Doc 2000 system (BioRad). Gene expression was normalized to the expression of β-actin. Sequences of oligonucleotides used as primers were as follows: 5’TACATGAGTGCCAAACAGTGCTGG-3’ and 5’TTCCT-CTTCCTCTTCCTCC-3’. For statistical analysis, the Mann-Whitney U test was used.

Generation of chimeric mice and analysis of lymphocyte reconstitution

The bone marrow chimeras were generated as described elsewhere (36). In brief, recipient C57BL/6 mice were lethally irradiated with 10.5 Gy. Twenty-four hours thereafter, recipient mice were reconstituted with T cell-depleted bone marrow cells with a total of 3 × 10^6 cells i.v. By combination of bone marrow cells from different donors, three different groups of mice were generated: group 1, C57BL/6 B cells (80% μMT plus 20% C57BL/6); group 2, C57BL/6 B cells (80% μMT plus 20% C57BL/6-/-); and group 3, 80% C57BL/6 plus 20% C57BL/6-/- (80% C57BL/6 plus 20% C57BL/6-/-). Reconstitution of the recipient mice was checked 7.5–8 wk after cell transplantation. For that, blood from mice tail veins was examined by flow cytometry.

Immunoization and detection of natural and Ag-specific Abs by ELISA

Eight weeks after cell transplantation, the chimeric mice were immunized with 150 μg of DNP-keyhole limpet hemocyanin (KLH) in alum in 200 μl i.p. A secondary immunization with 150 μg of DNP-KLH was given 7 wk after primary immunization. For analysis of natural or Ag-specific Abs, blood was taken from the tail veins of mice after the indicated time points and serum was obtained by a double centrifugation step. Plates for analysis of natural Abs were coated with 1 μg/ml anti-mouse Ig (H + L) and for Ag-specific Ab analysis plates were coated with 50 μg/ml DNP-BSA overnight. After extensive washing, the serum was incubated with an 8-point serial dilution in PBS for 2 h at room temperature. After washing the plates, the respective Ab subtype was detected by goat anti-mouse IgG (IgG1, IgG2a, IgM, and IgE) coupled to alkaline phosphatase. As a substrate, paranitrophenyl phosphate was taken and the plates were read at 405 nm after individual incubation times. The relative titer was determined by using a positive serum as a standard on each plate. Specificity was controlled by a negative serum pooled from C57BL/6 mice or by taking preimmune serum.

Statistics

Statistical evaluation was performed using Prism version 3.0cx software. The Mann-Whitney U test was used for analyzing differences in DNP-specific Ab production of chimeric mice.

Results

T cell-dependent intracellular CTLA-4 protein expression in B cells in vitro

A limited number of publications suggest the expression of CTLA-4 by B cells and there is controversial discussion whether this expression is regulated by other immune cells, especially T cells. Therefore, we investigated T cell-dependent and -independent culture systems in vitro to clarify the conditions for CTLA-4 expression on B cells. To investigate T cell-dependent B cell
cultures for CTLA-4-expressing cells, we purified B cells from mouse splenocytes and cultured these cells with LPS, anti-κ (BCR cross-linking), anti-CD19, and anti-CD40 Abs, or anti-CD40 latex microspheres with or without further addition of IL-4. We were not able to detect any intracellular CTLA-4 expression in B cells (identified by CD19, B220, or CD86) under these culture conditions, although the B cells were activated, proliferated, and secreted Abs (Fig. 1A and data not shown). The intracellular CTLA-4 staining was controlled by intracellular CTLA-4 staining of CD4 cells activated with Con A in a splenocyte culture (Fig. 1A, top panel). There was also no intracellular CTLA-4 expression in the described B cell cultures at different time points (12, 24, 48, 96, or 120h) after stimulation (Fig. 1A and data not shown).

CpG oligonucleotides bind TLR9 on B cells and trigger them to differentiation and proliferation (37). No CTLA-4 expression in B cells was found on enriched B cells stimulated with CpG alone (Fig. 1A). But taking total splenocytes and stimulating them with CpG, T cells are probably activated in this setting, which has been shown in a system with autoreactive T cells through IL-12 produced by the APCs in response to CpG (38). When we stimulated total splenocytes (containing T and B cells) with CpG or Con A, up to 12% of all CD19+ B cells did show intracellular CTLA-4 expression 48 h after stimulation (Fig. 1B, upper and middle panels). When we used Th2 effector cells, polarized for 1 wk, and stimulated them with T cell-depleted B cell-containing splenocytes and the corresponding Ag for 48 h, we could detect intracellular CTLA-4 expression in up to 19% of all of the CD19+ B cells (Fig. 1B, lower panel). The kinetics of intracellular CTLA-4 expression in B cells revealed no CTLA-4 on B cells at 24 h after stimulation (Fig. 1C). The peak expression of CTLA-4 on B cells was observed between 48 and 72 h with 11.3 ± 3.7% and 14.1 ± 5.2% CTLA-4+CD19+ B cells, respectively (Fig. 1C). The expression of CTLA-4 on B cells decreased after 96 h to 7.1 ± 3.4% positive B cells (Fig. 1C). The kinetics of intracellular CTLA-4 expression in B cells is similar to the kinetics of intracellular CTLA-4 expression on T cells in the same cultures, but the overall expression of CTLA-4 on T cells is approximately four times higher with up to 80% (Fig. 1C). In T cells, there is still a substantial proportion of cells positive for intracellular CTLA-4 after 5 days, in contrast to B cells where the CTLA-4 is almost undetectable after 5 days of culture (data not shown). To summarize these results, we were able to detect intracellular CTLA-4 in B cells when stimulating them in the presence of activated T cells. The intracellular CTLA-4 expression is restricted to a proportion of ~20% of all B cells in the culture and the expression is transient with a peak expression of 48–72 h after stimulation.

B cells express mRNA and protein of CTLA-4

Since we were able to detect CTLA-4 protein in B cells only in the presence of T cells, the question arose whether this CTLA-4 protein was taken up from CTLA-4+ expressing T cells or whether the protein originates from the transcription of the CTLA-4 gene in the B cell itself. The possibility of molecule exchange between immune cells in close contact has been described previously (39, 40). Importantly when B and T cells are in close contact, only unidirectional molecule transfer from B cells to T cells has been found (41, 42). However, to exclude transfer of CTLA-4 molecules from T cells to B cells formally, we isolated B cell-containing APCs from either CTLA-4−/− or CTLA-4+/− mice and stimulated them with CD4+ T cells from CTLA-4+/− mice and Con A for 48 h. After the stimulation time, we found no difference in the up-regulation of CD69 expression of B cells from CTLA-4+/− and CTLA-4−/− mice (Fig. 2A), demonstrating that the activation of B cells from either genotype was similar in these cultures. When analyzing the expression of CTLA-4, there was no CTLA-4 expression detectable in B cells in the cultures with B cells from CTLA-4−/− mice as compared with 16% CTLA-4-positive B cells in the culture with CTLA-4−/− B cells (Fig. 2B). Since CTLA-4 was detectable on T cells of both cultures, no transfer of molecules from T cells to B cells has occurred (Fig. 2B). Similar results were obtained stimulating CTLA-4−/− T cells with Con A in cocultures of CFSE-labeled CTLA-4−/− B cells along with CTLA-4+/+ B cells (data not shown).

To analyze the CTLA-4 mRNA in B cells, we performed RTPCRs from highly enriched B cells after stimulating them in a T cell-dependent culture system. We stimulated the B cells along with Th2 effector cells and cognate Ag (Fig. 1B) and enriched B cells from this culture 24 h after stimulation to a purity of 99.9% (Fig. 2C). The cDNA of these highly enriched B cells, of activated cells from this culture 24 h after stimulation to a purity of 99.9%, was detectable on T cells of both cultures, no transfer of molecules from T cells to B cells has occurred (Fig. 2B). Similar results were obtained stimulating CTLA-4−/− T cells with Con A in cocultures of CFSE-labeled CTLA-4−/− B cells along with CTLA-4+/+ B cells (data not shown).

Surface CTLA-4 expression on activated B cells in T cell-dependent culture systems

To interact with its ligands B7-1 and B7-2, CTLA-4 has to be expressed on the surface of B cells. Since the CTLA-4 molecule is
expressed only in very low numbers on the surface of T cells, we used an established staining-enhancing method based on fluorescent liposomes (11, 12, 33) to detect surface CTLA-4-positive B cells. We used total splenocytes (containing T and B cells) stimulated with Con A or Th2 effector cells stimulated with the cognate Ag and B cell-containing APCs. We were able to detect between 11 and 15% surface CTLA-4\textsuperscript{+}B cells with the liposome technique (Fig. 3A). Because the culture systems used so far contained also other cells, e.g., DCs and macrophages, although in very low numbers, we next analyzed CTLA-4 expression on B cells in exclusive B and T cell cocultures. We stimulated enriched (98%) B cells, either preincubated with LPS or ex vivo, along with purified T cells and the cognate Ag and analyzed CTLA-4 expression on B cells. We detected 9.7 ± 2.5% surface CTLA-4\textsuperscript{+}CD19\textsuperscript{+}B cells when using preactivated B cells (Fig. 3B). Preactivation of B cells with LPS did not influence the surface CTLA-4 expression.

**FIGURE 5.** Elevated DNP-specific IgM and temporal IgE response in CTLA-4\textsuperscript{−/−} B cell chimeric mice after primary and secondary TD immune response. C57BL/6 mice were lethally irradiated and reconstituted with 80% bone marrow from μMT mice plus 20% bone marrow from C57BL/6 mice (C57BL/6 B cells (□)) or reconstituted with 80% bone marrow from μMT mice plus 20% bone marrow from CTLA-4\textsuperscript{−/−} (C57BL/6 background) mice (CTLA-4\textsuperscript{−/−} B cells (●)). Each square in the diagrams represents data from a single mouse. The relative titer was calculated as described in Materials and Methods. A, Natural IgM and IgG titers are unaltered in CTLA-4\textsuperscript{−/−} B cell chimeric mice as compared with C57BL/6 B cell chimeric mice. The serum of these chimeric mice was analyzed for natural IgM and IgG titers by ELISA 8 wk after reconstitution. Representative data from three independent experiments are shown. B, Elevated DNP-specific IgM Ab titer in CTLA-4\textsuperscript{−/−} B cell chimeric mice after primary and secondary immunization. The primary immunization was done with 150 μg of DNP-KLH in alum i.p. 8.5 wk after reconstitution. A secondary immunization with DNP-KLH (150 μg) was given 7 wk after primary immunization. The serum of the chimeric mice was analyzed for DNP-specific IgM, IgG1, IgG2a, and IgE Ab production by ELISA at the indicated time points. Data from two independent experiments were combined. C, The elevated DNP-specific IgM Abs are not controlled by the 20% CTLA-4\textsuperscript{−/−} T cells. A third group of chimeric mice was generated by lethal irradiation of C57BL/6 mice and reconstitution with 80% bone marrow from C57BL/6 mice plus 20% bone marrow from CTLA-4\textsuperscript{−/−} mice (80% C57BL/6 20% CTLA-4\textsuperscript{−/−} (●)). The serum of the chimeric mice was analyzed for natural IgM and IgG titers by ELISA 8 wk after reconstitution. Representative data from three independent experiments are shown. The chimeric mice were immunized as described in B and 2 wk after secondary immunization DNP-specific IgM Abs were analyzed by ELISA. Data from two independent experiments were combined.
of B cells, since we observed 9.05 ± 2.1% surface CTLA-4+ CD19+ B cells without preactivation of the B cells before the coculture with T cells (Fig. 3B). However, phenotypical analysis of the activation status of surface CTLA-4+ as compared with surface CTLA-4+ B cells, 48 h after stimulation with Th2 effector cells and cognate Ag, revealed no difference with regard to expression of surface receptors that are induced (CD69) or altered in their expression (MHC II, CD40, and CD86) after activation (Fig. 3C). Both populations showed a clear induction of CD69 and up-regulation of CD86 on B cells after 48 h of culture, whereas CD40 remained unchanged and MHC II expression was down-regulated (Fig. 3C). Likewise, surface CTLA-4+ expressing T cells show similar activation marker expression to CTLA-4+ T cells. Taken together, surface CTLA-4+ B cells are clearly detectable in T cell-dependent culture systems using the staining-enhancing liposome technique and they up-regulate expression of activation-induced genes after activation.

Normal B and T cell homeostasis and development in mice with a CTLA-4 deficiency of B cells

So far there is no established role for CTLA-4 expressing B cells in vivo. There are suggestive data claiming that CTLA-4 might influence isotype class switching to IgG1 and IgE in vitro (25, 45). B cells from the CTLA-4−/− mice display an activated phenotype and all Ab classes show elevated titers compared with CTLA-4+/+ animals (31, 32). The B cell phenotype in CTLA-4−/− mice could either be B cell intrinsic or a secondary resort of CTLA-4-deficient activated T cells. To analyze the role for CTLA-4 on B cells in vivo, we generated bone marrow chimeric mice, in which substitution of the irradiated animals, the peripheral B cell as well as T cell pool of the chimeric mice was analyzed by FACS. There was no significant difference in the frequencies of peripheral B cells in CTLA-4−/− B cell chimeric mice as compared with C57BL/6 B cell chimeric mice, respectively (Fig. 4A). In addition, we discriminated the pool of total B cells into developmental B cell stage T1 (CD19+IgMintIgDlow) and the mature B cell stage (CD19+IgMmmIgDhigh) in accordance with the study of Carsetti et al. (46). As for the total frequency of CD19+ cells, there was no difference in the CTLA-4−/− B cell chimeric mice as compared with C57BL/6 B cell chimeric mice (Fig. 4A). The peripheral T cell pool, divided into CD4+ and CD8+ cells, is as well reconstituted in the CTLA-4−/− B cell chimeric mice (Fig. 4B). Furthermore, we analyzed the natural Ab production, mainly produced by B1 B cells, in the CTLA-4−/− B cell chimeric mice. No difference in the Ab titer of natural IgM and total IgG Abs could be observed between the CTLA-4−/− B cell chimeric mice and the C57BL/6 B cell chimeric mice (Fig. 5A). Thus, B and T cell development and homeostasis are normal in mice with a CTLA-4 deficiency of B cells.

Elevated DNP-specific IgM and temporal IgE response in CTLA-4−/− B cell chimeric mice after primary and secondary TD immune responses

Because we discovered a T cell-dependent CTLA-4 induction on B cells, we used a TD immunization protocol with 150 μg of DNP-KLH in alum to analyze the role for CTLA-4 on B cells for Ab production. We immunized the chimeric mice and determined DNP-specific Ab titers of IgM, IgG1, IgG2a, and IgE subclasses at 1 and 3 wk after primary immunization by ELISA. We found a significantly higher DNP-specific IgM Ab production at 1 and 3 wk postimmunization in the CTLA-4−/− B cell chimeric mice as compared with the C57BL/6 B cell chimeric mice (Fig. 5B). The DNP-specific Ab titers of the IgG1 and IgG2 subclasses did not show any difference between the groups of chimeric mice (Fig. 5B). Concerning the DNP-specific IgE Ab response, we found a significantly higher Ab titer 3 wk postimmunization in the CTLA-4−/− B cell chimeric mice as compared with the C57BL/6 B cell chimeric mice (Fig. 5B). To study the B cell memory response, we immunized the chimeric mice 7.5 wk after primary immunization for a second time using 150 μg of DNP-KLH. We analyzed again the DNP-specific Ab titers 2 wk after the boost of the above-mentioned subclasses. Like for the primary response, we did find a significantly elevated DNP-specific IgM response in mice deficient for CTLA-4 in the B cell compartment, whereas all other Ab classes remained unchanged (Fig. 5B).

The observed elevated Ag-specific IgM and IgE Ab response cannot be attributed to the fraction of 20% CTLA-4-deficient T cells that are present in the B cell CTLA-4-deficient chimeric mice. This possibility was excluded by a third chimeric group of mice, with 20% of the T cells being CTLA-4-deficient and the majority of the B cells being CTLA-4 competent (Fig. 5C). DNP-specific IgM Ab titers after immunization of these mice did not show the elevated IgM response as observed in the CTLA-4−/− B cell chimeric mice, but rather a significantly lower DNP-specific IgM response (Fig. 5C). Thus, CTLA-4 on B cells can indeed negatively regulate the Ag-specific IgM and IgE response in TD immune responses.

Discussion

The importance of the CTLA-4 molecule for T cell differentiation and effector functions is well established. In contrast, the expression and potential relevance of expression of endogenous CTLA-4 for the differentiation of B cells has not been documented. In this study, we describe a T cell-dependent, activation-induced, and transient expression of CTLA-4 on murine B cells. Expression of CTLA-4 on the cell surface of B cells was detected by liposome-enhanced immunofluorescence and flow cytometry. The in vivo importance for CTLA-4 expressing B cells could be shown by the generation of chimeric mice, in which the CTLA-4 deficiency was restricted to the B cell compartment. Immunization of these chimeric mice with a TD protocol revealed that CTLA-4 on B cells controls the Ag-specific IgM responses of primary and secondary immune reactions in vivo.

We could show that isolated B cells stimulated with anti-CD40, anti-κ L chain plus anti-CD40, anti-κ L chain plus anti-CD19, LPS, and CpG with or without IL-4 do not up-regulate CTLA-4 in B cells. On the contrary (25, 45) and in line with earlier data for human B cells (26), we detected CTLA-4 on B cells cultured with activated T cells. Intracellular CTLA-4 in B cells is only detectable after stimulation, with a peak expression of 15–20% of all B cells at 48–72 h (Fig 1C). Surface CTLA-4+ CD19+ cells were only detectable using the staining-enhancing liposome technique (11, 12, 33), thus showing that the frequency of surface CTLA-4 expressing B cells was hardly different than intracellular CTLA-4 expression in B cells (Fig. 3A), with up to 15% CTLA-4+ CD19+ B cells. Equal expression of surface and intracellular CTLA-4 protein expression was suggested for B cells taken from the blood of patients with B cell chronic lymphocytic leukemia (27). This expression profile differs strikingly from CTLA-4 expression in T cells where one finds nearly all cells CTLA-4+ intracellularly, whereas only up to 12% of the T cells are also surface CTLA-4 positive, at least in a primary peptide stimulation (12). This shows that the way of intracellular storage of CTLA-4 in vesicles described for T cells is different for B cells, even tighter restricted, probably controlled by T cells, because B cells only express CTLA-4 when T cells do so. Because we excluded uptake of
CTLA-4 protein from T cells, which is in line with data showing that T cells mainly take up B7 molecules and MHC from APCs and that this molecule transfer from APCs to T cells is usually unidirectional (39, 47), we assume that other molecules of T-B interactions are the trigger for CTLA-4 expression of B cells; the receptor ligand pathway CTLA-4/B7 could possibly perform this function. Alternatively, a certain subpopulation of B cells, such as B1, B2, or a subclass of B2, the marginal zone B cells, could differentially express CTLA-4. Since CTLA-4 on splenic B cells is only expressed when activated T cells are present, it is most likely that CTLA-4-expressing B cells belong to the follicular B cells, since these cells, in contrast to marginal zone B1 and B2 cells, interact with T cells in TD immune responses (48, 49). Still, one has to keep in mind that marginal zone B cells are able to interact with naive T cells, inducing a strong proliferation and cytokine production in them (50). No less interesting is the finding that follicular B cells have the potential to convert into marginal zone B cells (48). Further studies are needed to clarify the affiliation of the CTLA-4-expressing B cells to one or the other B cell subset.

To signal, CTLA-4 expressed on B cells requires cell-cell contact to cell types that express its ligand B7-1 or B7-2, such as DCs, macrophages, T cells, or even B cells themselves. There are a number of reports showing the expression and function of B7-1 and B7-2 molecules on T cells, which not only challenges the cell type-specific costimulator expression but also gives the opportunity of interaction of CTLA-4 on B cells with its ligand B7 on T cells (15, 16, 19, 51, 52). The CTLA-4/B7 pathways are even more complex, since engagement of B7 molecules on T cells is able to manipulate T cell functions; e.g., B7-1-deficient T cells show a significantly higher IL-4 production than wild-type T cells and B7-1/2−/− T cells are not controlled by regulatory T cells anymore depending on the cytoplasmic domain of the B7 molecule (19, 52). This possible criss-cross regulation of CTLA-4 and its ligand between T-T, T-B, and B-B lymphocytes shows that the traditional view is too simple. CTLA-4 on B cells might well interact with B7 molecules on other B cells, according to our data probably in the B cell zone, where B cells are migrating to, after activation with T cells in the T cell zone.

We show here that CTLA-4 on B cells inhibits B cell effector functions. Since recently, the traditional view of CTLA-4 inhibitory effects on T cell responses was merely attributed to its inhibitory effect of inducing cell cycle arrest (12, 34, 53). New data now demonstrate that CTLA-4 signaling can indeed down-regulate effector molecules such as IFN-γ of individual CD8 T cells (35). One or both functions of CTLA-4 mentioned could explain the enhanced Ab production caused by the genetic inactivation of CTLA-4 on B cells (12, 34, 54, 55). Alternatively, different actions of receptors depending on the cell type specific expression are shown, which might also hold true for CTLA-4 on B cells. This is exemplified by B7-2 molecules on T cells, (16) or CD28 on plasma cells (22, 56). As our research has demonstrated that already intracellular trafficking of CTLA-4 to the cell surface differs between T and B cells, we favor the notion that initiated signaling pathways of CTLA-4 might differ also between T and B cells.

Perhaps most importantly, we have been able to identify a new role for CTLA-4 on B cells in vivo in TD immune responses. Altered development or homeostasis of B cells in B cell CTLA-4−/− chimeric mice was ruled out, hence the effects in Ab production are not due to different cell numbers of Ag unexperienced B cells. Furthermore, analysis of natural IgM and total IgG titers in the chimeric mice before immunization revealed no difference in the CTLA-4−/− chimeric mice as compared with CTLA-4+/+, stressing the point that only T cell-dependent actions of B cells lead to CTLA-4 expression in B cells (natural Abs are T cell independent) (48). In a TD immune reaction, T cell and B cells meet in the T cell zone, where CTLA-4 on B cells could be induced. As early as 7 days after immunization with DNP-KLH, we observed a significantly higher Ag-specific IgM production in sera of CTLA-4−/− B cell chimeric mice as compared with wild type. These Abs are probably generated by Ab-forming cells of the primary focus, since Abs from the germinal centre are only detectable from about day 10 on after immunization (49). CTLA-4 on B cells might regulate the number of B cells in the primary focus by either dampening the proliferation at place, or by influencing the mobility of the B cells migrating from the primary focus to the B cell primary follicle. Further studies are needed to analyze the number of B cells in the early germinal center, determined by peanut agglutinin high cells (57), to clarify a role for CTLA-4 in B cell migration. The early Abs produced in a TD immune response are not only for immediate protection of the host, but also give rise to Ag-Ab complexes on follicular DCs and may regulate the long-term Ab response. Three weeks after immunization, we still found significant elevated levels of Ag-specific IgM Abs in the CTLA-4−/− B cell chimeric mice. Since T cells are able to express CTLA-4 at all differentiation steps (10–12), we assume that CTLA-4 on B cells is able to down-modulate “late IgM” Abs originating from unswitched Ab-forming cells of the germinal center reaction (58), but at the same time also regulate IgM Abs produced by terminal differentiated long-lived plasma cells of the primary focus (59, 60).

Apart from the elevated Ag-specific IgM titers, we also found elevated Ag-specific IgE titers at least 3 wk after primary immunization in the CTLA-4−/− B cell chimeric mice. This implies another regulatory checkpoint for CTLA-4 on B cells in the germinal center reaction. Studies in vitro suggested a role for CTLA-4 on B cells activated by anti-CD40 plus IL-4 in the IgE production via inactivation of STAT6 and NF-kB, which we could not confirm (Fig. 1A and Ref. 25). Because reduced IgE production in vivo is not seen after boosting, we assume an indirect regulation (Fig. 5B). In a secondary Ab response, we again detected significant elevated Ag-specific IgM Abs. Whether these derive from naive B cells or from the recently identified IgM-positive memory B cell pool has to be determined in the future (61). A role for CTLA-4 for differentiation or maintenance of memory cells is intriguing, as a fraction of CTLA-4−/− B cells could get survival signals, similar to a fraction of CTLA-4-expressing T cells (11), and could therefore take part in the memory formation.

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