

# CD83 regulates splenic B cell maturation and peripheral B cell homeostasis

Katja Lüthje<sup>1</sup>, Birte Kretschmer<sup>1</sup>, Bernhard Fleischer<sup>1,2</sup> and Minka Breloer<sup>1</sup>

<sup>1</sup>Department of Immunology, Bernhard-Nocht-Institute for Tropical Medicine, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany

<sup>2</sup>Institute for Immunology, University Medical Centre Hamburg-Eppendorf, 20246 Hamburg, Germany

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## Abstract

**The central function of murine CD83 that is expressed on thymic epithelial cells is to induce the progression of double-positive thymocytes to single CD4-positive T cells. Several lines of evidence suggest an additional role for CD83 in the regulation of peripheral T and B cell responses. Here we show that CD83 is expressed by immature B cells and regulates their further maturation and survival in the periphery. Employing mixed bone marrow chimeras, we compare wild-type, CD83 over-expressing and CD83-deficient B cells within the same host. CD83 over-expression on the immature B cells themselves led to an accumulation of transitional B cells and a reciprocally reduced maturation of follicular B cells that was strictly correlated to the intensity of CD83 over-expression. The absence of CD83 on B cells resulted in a decreased maturation of marginal zone B cells and conferred a mild selection advantage for B cell survival in the periphery. Consistent with these findings, the over-expression of CD83 specifically and dose dependently interfered with homeostasis of B cells while T cell survival was not affected by CD83 over-expression over a period of 30 weeks. Taken together, our data suggest that CD83 negatively regulates B cell maturation and survival.**

## Introduction

Murine CD83 is a glycosylated Ig-like type I transmembrane protein with 63% amino acid homology to human CD83 (1–3). It was first described as a maturation marker for dendritic cells (4) and several lines of evidence suggest a regulatory function of CD83 in the periphery. Different soluble CD83 species have been shown to inhibit lymphocyte proliferation *in vitro* (5–7) and to act immunosuppressive *in vivo* (8, 9). The most impressive example being the cure of experimental autoimmune encephalomyelitis in mice by application of the soluble extracellular domain of human CD83 (10, 11). It was suggested that human CD83 is a sialic acid-binding Ig-like lectin that interacts with neuraminidase-sensitive structures on HPB-ALL cells (12). However, the data concerning the nature of putative CD83 ligands are heterogeneous (5, 6, 12, 13) and the mechanism of CD83-mediated immune regulation remains enigmatic.

Recently, we provided evidence that CD83 is also involved in the regulation of B cell function *in vivo* (14) and *in vitro* (15). CD83 is rapidly up-regulated on activated B cells and premature transgenic (tg) CD83 surface expression on B cells themselves leads to a dramatically altered phenotype that is characterized by a defective or absent Ig response to

thymus independent (TI) and thymus dependent model antigen immunization *in vivo* and by a reduced calcium signaling, reduced Ig production but reciprocally increased IL-10 production *in vitro*. CD83 deficiency on the other hand leads to a mild increase in Ig production and decreased IL-10 release upon *in vitro* stimulation. Moreover, the *in vivo* application of anti-CD83 mAb induces a 10-fold increase in antigen-specific IgG1 upon TI-2 model antigen immunization of wild-type mice, thus confirming a genuine role for CD83 in the regulation of B cell responses (14).

The analysis of mouse strains deficient for CD83 through knockout (16) or mutation (17) techniques revealed that another central function of CD83 lies within the thymic selection of T cells. CD83 expressed on thymic epithelium seems to transmit a signal to double-positive thymocytes allowing their further maturation to CD4-positive T cells. CD83-deficient mice contained drastically reduced numbers of single CD4-positive thymocytes and CD4-positive T cells in the periphery. The remaining CD4-positive T cells displayed an impaired response to antigenic stimulation *in vivo* and *in vitro* (16, 17). In line with these results, the thymic maturation in the presence of ubiquitously expressed soluble

CD83lg fusion protein led to the generation of CD4-positive T cells in normal numbers but with an impaired response to antigenic stimulation *in vitro* and *Trypanosoma cruzi* and *Leishmania major* infections *in vivo* (18). This impaired function of CD83lg CD4 T cells was due to an intrinsic defect, acquired within the thymus, during thymic maturation and was not mediated by soluble CD83lg in the peripheral circulation (18).

In this context, the initial analysis of the lymphoid cell populations in CD83tg mice revealed a slight increase in CD4-positive T cells in the spleen but an otherwise unchanged T cell compartment (14, 19). Regarding the B cell development, however, an increase in immature transitional (TN1) B cells and a reciprocal decrease in mature follicular (FO) B cells in the spleen of CD83tg mice was observed, thus suggesting that the non-conditional over-expression of CD83 throughout the B cell development interfered with B cell maturation (14).

Here we thoroughly analyze the role of CD83 in the maturation of B cells. In line with recent data (20), we show that CD83 is expressed by immature wild-type B cells but not by pro- or pre-B cells. Employing mixed bone marrow chimeras, we demonstrate that CD83 over-expression on immature B cells themselves leads to their defective maturation resulting in an increase in TN1 B cells and a reciprocal decrease in mature FO B cells. The absence of CD83 on the developing B cells results in a decreased percentage of marginal zone (MZ) B cells. In contrast to its crucial role in the thymic T cell maturation (16, 17), the absence or over-expression of CD83 on bystander cells did not affect B cell maturation. Finally, performing long-term kinetic studies in mixed bone marrow chimeras, we provide evidence that CD83 negatively regulates B cell survival in the periphery.

## Methods

### *Mice and antibodies*

CD83tg founder 2, CD83tg founder 1 (19), CD83mu (17), CD83lgtg (18), C57BL/6, IgH<sup>a</sup>-congenic and CD45.1-congenic mice were bred in the facilities of the Bernhard-Nocht-Institute or of the University Hospital Hamburg-Eppendorf (Hamburg, Germany). Experiments employing mice described within this study are approved by the Federal Health Authorities of the State of Hamburg, the 'Amt für Gesundheit und Verbraucherschutz'. Antibodies were obtained from BD Pharmingen and Caltag Laboratories. The mAb to mouse CD83, michel-19 (rat IgG1), was described earlier (14).

### *Flow cytometry*

The FcRs of  $2 \times 10^5$  (or  $1 \times 10^6$ ; Fig. 1) *ex vivo* prepared bone marrow, spleen or peripheral blood lymphocytes were blocked with mouse serum (10% v/v) for 10 min on ice. Cells were stained with indicated mAb for 20 min on ice and analyzed on a Becton Dickinson FACSCalibur equipped with CellQuest Pro software. The following mAbs were used: FITC-anti-mouse CD83, clone michel-19; biotinylated anti-mouse CD83, clone michel-19; FITC-rat IgG1, clone R3-34; biotinylated rat IgG1, clone R3-34; PE-anti-mouse CD19, clone 1D3; FITC-anti-mouse CD21/CD35, clone 7G6; allophycocyanin (APC)-anti-mouse CD23, clone B3B4; PE-anti-

mouse B220, clone RA3-6B2; FITC-anti-mouse IgM<sup>a</sup>, clone DS-1; biotinylated anti-mouse IgM<sup>a</sup>, clone DS-1; biotinylated anti-mouse IgM<sup>b</sup>, clone AF6-78; Cy<sup>TM</sup>5-AffiniPure Goat anti-mouse IgM,  $\mu$ chain specific; FITC-anti-mouse IgD, clone 11-26c.2a; FITC-anti-mouse CD43, clone S7; PE-anti-mouse CD5, clone 53-7.3; FITC-anti-mouse-CD11b, clone M1/70; PE-anti-mouse CD3, clone 145-2C11; PE-anti-mouse CD4, clone 104; FITC-anti-mouse CD8, clone 53-6.7; biotinylated anti-mouse CD45.2, clone 104; APC-streptavidin and PerCP-streptavidin.

### *CD83-specific western blot*

Spleen cells ( $2 \times 10^7$ ) were lysed in 50  $\mu$ l lysis buffer [150 mM NaCl, 50 mM Tris, pH 7.4, 1% (w/v) CHAPS] supplemented with Complete EDTA-free Protease Inhibitor (Roche, Mannheim, Germany). For deglycosylation, 37  $\mu$ g protein of each sample were denatured in a total volume of 50  $\mu$ l with 1  $\mu$ l 10% SDS for 10 min at 70°C. Afterwards, 5  $\mu$ l 10% NP40 were added and samples were incubated with 0.5 U N-glycosidase F overnight. Twelve micrograms of protein were loaded in each slot, separated by SDS-PAGE on 10–20% PAA gradient gels (Anamed, Darmstadt, Germany) and blotted onto an immobilized-P polyvinylidene difluoride membrane (Millipore, Schwalbach, Germany). CD83 was detected by incubating the blocked membrane with a 1:10 000 dilution of polyclonal rabbit anti-mouse CD83 serum, followed by incubation with a 1:2000 dilution of HRP-conjugated goat anti-rabbit Ig (Dako, Glostrup, Denmark) and developed with ECL<sup>TM</sup> Western Blotting Detection Reagents (Amersham Biosciences, Buckinghamshire, UK).

### *Generation of bone marrow chimeras*

Recipient mice received 8 Gy of  $\gamma$  irradiation from a <sup>137</sup>Cs source. One day later, bone marrow was extracted from tibias and femurs of appropriate donor mice. Recipient mice received  $2 \times 10^6$  bone marrow cells by intravenous injection. Mixed chimeras were generated by injection of  $6 \times 10^6$  total bone marrow cells containing different amounts of C57BL/6-IgH<sup>a</sup>-congenic and CD83tg or CD83mu bone marrow. Chimeras were treated orally with 0.5‰ (v/v) Baytril (Bayer) in drinking water starting 1 week before transfer until 4 weeks after transfer.

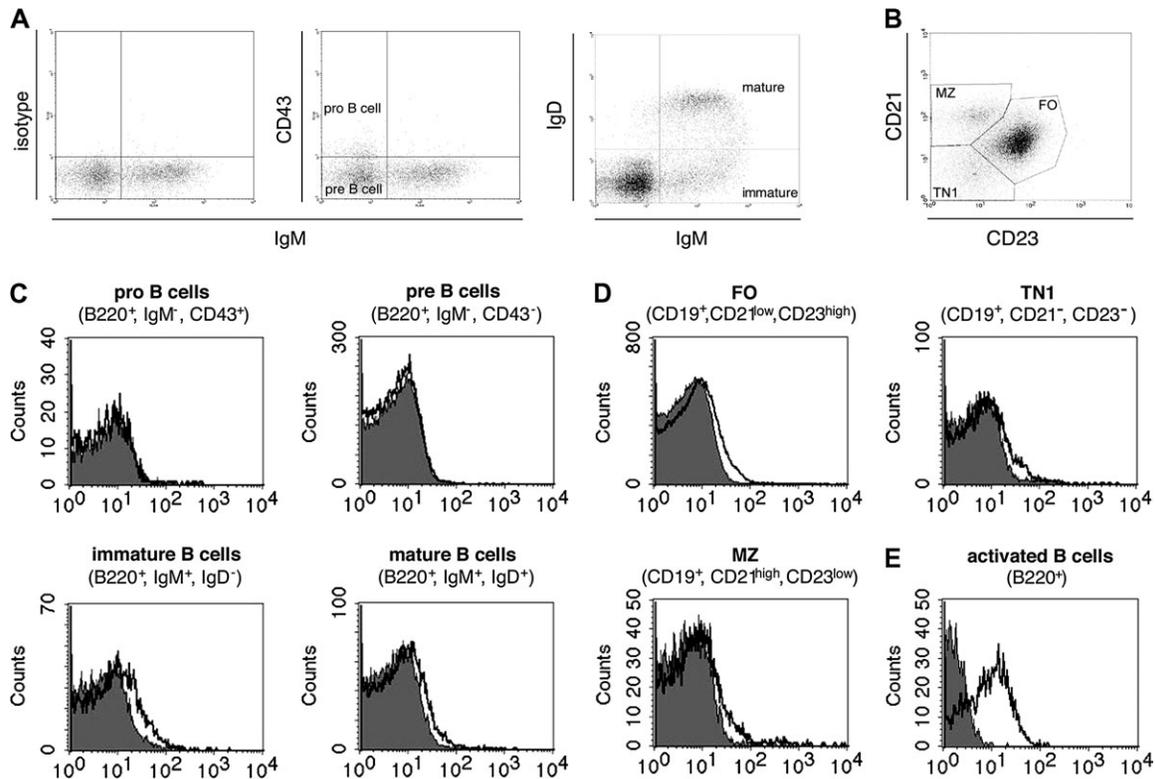
### *Adoptive transfer of mature spleen cells*

For adoptive transfer experiments,  $1.5 \times 10^7$  spleen cells of either C57BL/6 or CD83tg mice were adoptively transferred by tail vein injection into age- and sex-matched congenic (CD45.1<sup>+</sup>) recipients. The frequency of CD45.2<sup>+</sup> donor cells in blood of recipients was determined 7 days after cell transfer by flow cytometry.

## Results

### *CD83 expression on B cell subsets*

To investigate a possible role of CD83 in B cell maturation, we first analyzed the expression of CD83 throughout the development of wild-type B cells. We identified B cell subsets by the different expression pattern of surface molecules as shown in Fig. 1(A and B) and analyzed CD83 expression on



**Fig. 1.** CD83 surface expression on B cell precursors, naive mature B cells and activated B cells. (A) Bone marrow cells ( $2 \times 10^5$ ) were triple stained by B220 and IgM/CD43 or IgM/IgD. Gated on B220<sup>+</sup> cells, percentages of pro-B cells (CD43<sup>+</sup> and IgM<sup>-</sup>), pre-B cells (CD43<sup>-</sup> and IgM<sup>-</sup>), immature B cells (IgM<sup>+</sup>/IgD<sup>-</sup>) and mature B cells (IgM<sup>+</sup>/IgD<sup>+</sup>) were analyzed. (B) Spleen cells ( $2 \times 10^5$ ) were triple stained by CD19 and CD21/CD23. Gated on CD19<sup>+</sup> cells, percentages of immature TN1 (CD21<sup>-</sup> and CD23<sup>-</sup>), mature FO (CD21<sup>low</sup> and CD23<sup>high</sup>) and MZ B cells (CD21<sup>high</sup> and CD23<sup>low</sup>) were analyzed. (C) C57BL/6 bone marrow cells ( $2 \times 10^4$ ) were gated on pro-B cells (B220<sup>+</sup>, IgM<sup>-</sup> and CD43<sup>+</sup>), pre-B cells (B220<sup>+</sup>, IgM<sup>-</sup> and CD43<sup>-</sup>), immature B cells (B220<sup>+</sup>, IgM<sup>+</sup> and IgD<sup>-</sup>) or mature B cells (B220<sup>+</sup>, IgM<sup>+</sup> and IgD<sup>+</sup>) and analyzed for CD83 expression. (D) C57BL/6 spleen cells ( $2 \times 10^4$ ) were gated on FO (CD19<sup>+</sup>, CD21<sup>low</sup> and CD23<sup>high</sup>), TN1 (CD19<sup>+</sup>, CD21<sup>-</sup> and CD23<sup>-</sup>) and MZ (CD19<sup>+</sup>, CD21<sup>high</sup> and CD23<sup>low</sup>) B cells and analyzed for CD83 expression. (E) Lymph node cells ( $2 \times 10^4$ ) of a *Leishmania major* infected C57BL/6 mouse were gated on B cells (B220<sup>+</sup>) and CD83 expression was analyzed. Histograms show the expression level of CD83 (black lines) and the background staining by isotype control (solid histograms) of the gated cell population. Results are representative for at least three independent experiments.

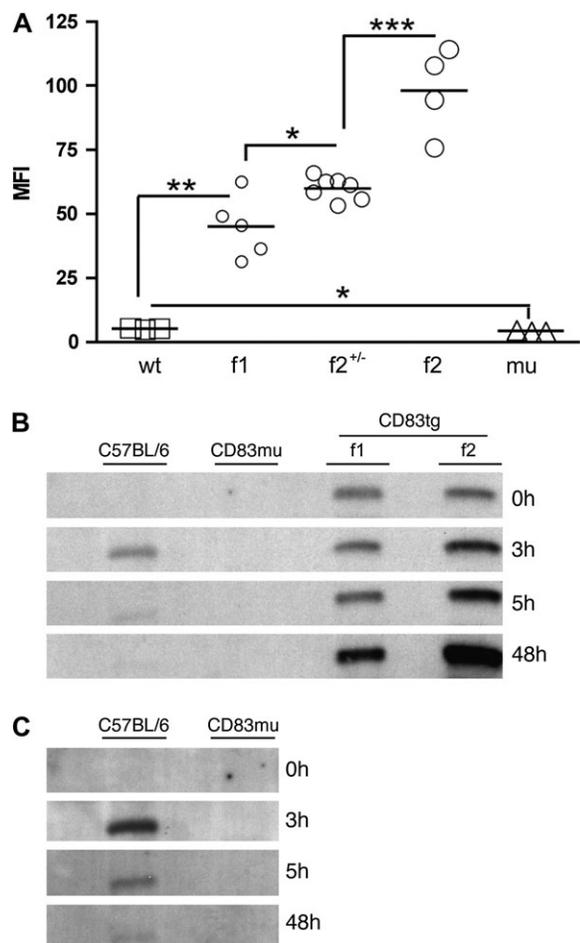
these B cell subsets from bone marrow (Fig. 1C) and spleen (Fig. 1D). Figure 1(C) shows that CD83 was not expressed by pro- or pre-B cells in the bone marrow but was constitutively present at low levels on immature B cells and recirculating mature B cells. In the spleen, comparable CD83 expression was detectable on immature TN1 B cells, MZ B cells and mature FO B cells (Fig. 1D). *In vivo* activation of lymphocytes during the course of an ongoing *L. major* infection induced strong up-regulation of surface CD83 specifically on B cells in the popliteal lymph node draining the site of infection (Fig. 1E) as we have shown before (14). Taken together, these data show that CD83 was not only expressed by activated B cells but also present on the cell surface of B cell precursors in bone marrow and spleen once they expressed a functional B cell receptor (BCR). Therefore, we decided to analyze the involvement of CD83 in the final progression of immature to mature B cells in the periphery.

#### Analysis of CD83 expression in CD83tg and CD83mu mice

To this end, we employed different mouse strains with manipulated CD83 expression levels. Figure 2(A) shows the constitutive surface expression of CD83 on splenic B cells

in naive wild-type mice, in two independently generated CD83tg strains (19), founder 1 and founder 2, as well as in the heterozygous F1 of CD83tg founder 2 crossed with C57BL/6 mice ( $f2^{+/-}$ ) and in CD83 mutant ( $\mu$ ) mice [CD83mu, termed LCD4.1 originally (17)]. While resting, wild-type B cells expressed CD83 at very low levels, B cells derived from CD83tg founder 1, heterozygous founder 2 and homozygous founder 2 displayed an incremental increase in constitutive CD83 expression. CD83mu mice on the other hand did not display any detectable CD83 expression on the cell surface as described (17).

Since CD83 was reported to reside in intracellular stores (21, 22), we performed also western blots with whole spleen cell lysates. Figure 2(B) shows that in resting spleen cells, only CD83tg founder 1 and founder 2 expressed detectable amounts of CD83. No intracellular CD83 was present in resting wild-type spleen cells, as we have shown before (15). Following stimulation by LPS, however, CD83 was rapidly expressed in wild-type but not in CD83mu cells (Fig. 2B and C), thus excluding the possibility that intracellular CD83 was present in the CD83mu cells at any time point. In summary, we have defined CD83tg and CD83mu mouse strains with either incrementally increased CD83 expression or



**Fig. 2.** CD83 expression in CD83tg and CD83mu mice. CD83 expression level of B cells (A) or spleen cells (B and C) in CD83tg founder 1 (f1), founder 2 heterozygote (f2<sup>-/-</sup>) and founder 2 homozygote (f2) as well as CD83mu mice was compared with CD83 expression level of wild-type (wt) B cells by FACS staining (A) or by western blot (B). (A) Spleen cells ( $2 \times 10^4$ ) were gated on CD19<sup>+</sup> cells and analyzed for CD83 expression. Every dot represents the result of an individual spleen. Asterisks indicate a significant difference of the mean (\* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ) employing Student's *t*-test. (B and C) Spleen cells  $\text{ml}^{-1}$  ( $1 \times 10^6$ ) were stimulated with LPS ( $10 \mu\text{g ml}^{-1}$ ) and lysed at the indicated time points. Lysates were deglycosylated and separated by SDS-PAGE. CD83 was detected by western blot with a polyclonal rabbit anti-CD83 serum exposed for 10 s (B) or 60 s (C).

absent CD83 expression as excellent tools to analyze the impact of CD83 on B cell maturation.

#### CD83 expression affects splenic B cell maturation

In a first step, we thoroughly analyzed the composition of B cell subsets in bone marrow and spleen in these different mouse strains. Figure 3(A) shows that altered CD83 expression levels did not lead to differences in the early B cell maturation in the bone marrow. Only the highest expression level of CD83 in the homozygous CD83tg founder 2 led to a reduction in pro-B cells but not in pre-B cells. The frequency of immature B cells in the bone marrow, however, was significantly increased in all CD83tg mice while the fre-

quency of recirculating mature B cells was reciprocally decreased in CD83tg founder 2 and founder 1. Neither the absence of CD83 in CD83mu mice nor the presence of soluble CD83Ig in CD83Igtg (18) mice did affect the composition of the bone marrow B cell compartment.

Figure 3(B) shows the composition of splenic B cell subsets in these mice. This analysis revealed a clear increase of immature TN1 B cells in the CD19-positive splenic B cell population of CD83tg mice that was reflected by a reciprocal decrease of mature FO cells as observed before (14). Strikingly, this accumulation of TN1 cells correlated positively with the CD83 expression level in the different CD83tg mouse strains and thus represents a dose-dependent effect. Re-analysis of immature and mature splenic B cells by differentiation into IgM/IgD double-positive mature and IgM<sup>high</sup> but IgD<sup>low</sup> immature B cells confirmed this result (Fig. 3B). While the absence of CD83 in CD83mu mice did not change the amount of TN1 and FO B cells, a highly significant decrease in MZ was observed (Fig. 3B). The presence of soluble CD83Ig in the circulation again did not change the splenic B cell composition.

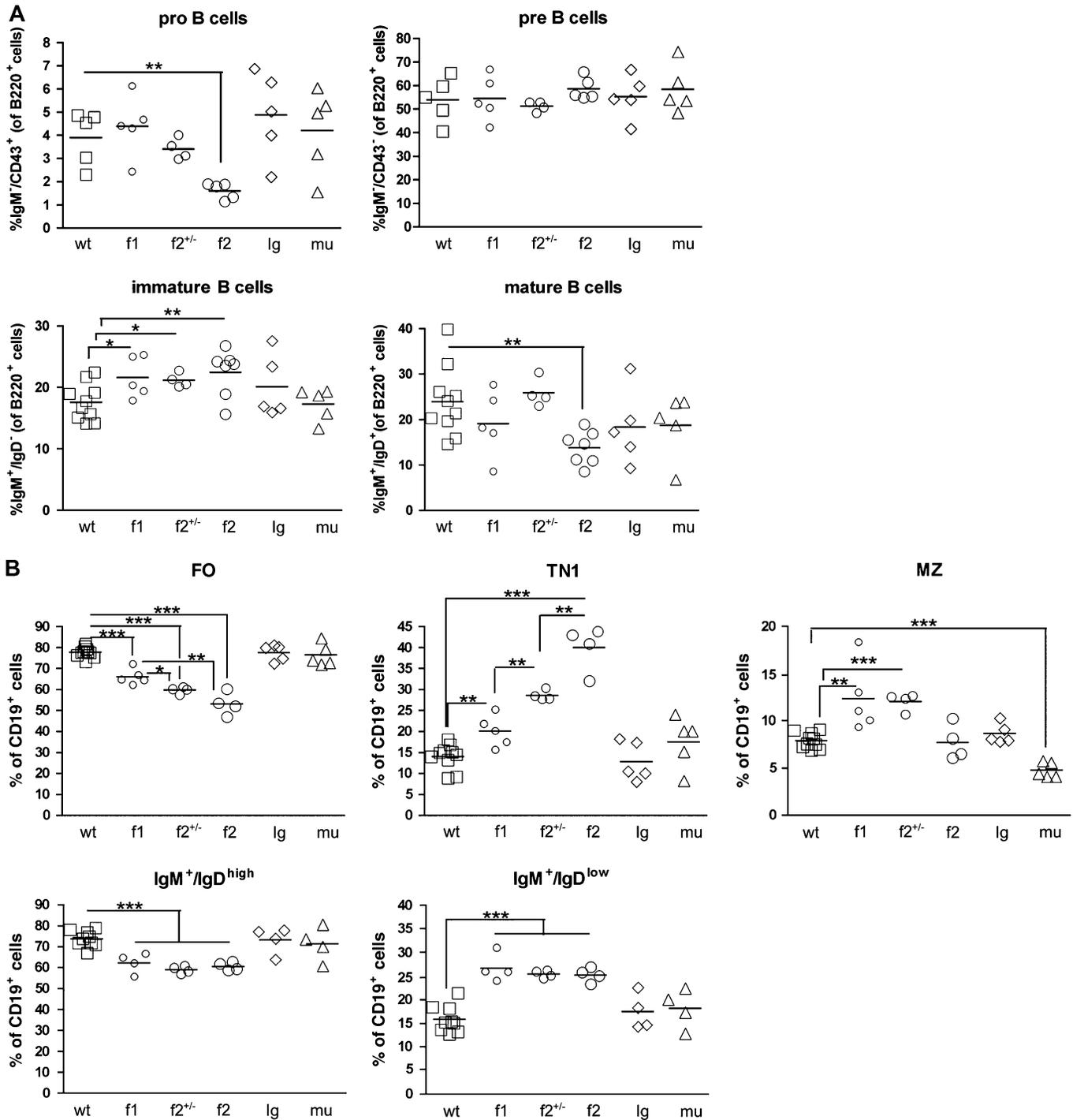
Taken together, these analyses show that over-expression of CD83 *in vivo* resulted in a partial arrest of late B cell maturation that correlated positively with the intensity of CD83 expression level. The absence of CD83, however, led to a diminished MZ B cell maturation. In context with our previous finding that CD83 is expressed by immature B cells in bone marrow and spleen (Fig. 1C and D), it is tempting to speculate that CD83 on immature B cells regulates their further maturation. Since neither the CD83 over-expression under the control of a MHC-I promoter nor the CD83 deficiency due to a point mutation of the CD83 gene was only restricted to the B cell population, it remains possible that the described phenotype of these mice was due to altered CD83 expression levels on environmental cells.

#### CD83 expression on B cells themselves affects splenic B cell maturation

In order to address this question, we generated bone marrow chimeras by transplanting wild-type bone marrow in CD83tg and CD83mu hosts and vice versa. Figure 4 shows that wild-type bone marrow that was transplanted into CD83tg mice matured exactly like wild-type bone marrow transplanted into wild-type hosts. The percentage of FO, TN1 and MZ B cells in the spleen (Fig. 4A) was equal in both chimeras. CD83tg bone marrow that matured in wild-type hosts on the other hand displayed the same reduced maturation of FO B cells and accumulation of immature TN1 B cells in the spleen that we observed in non-chimeric CD83tg mice (Fig. 4A).

The comparison of founder 1 and founder 2 bone marrow grafts showed a clear correlation of CD83 expression level on the cells of hematopoietic origin and the severity of the developmental B cell defect. The distribution of CD4<sup>+</sup> and CD8<sup>+</sup> T cells was normal in these chimeras, thus ruling out a generalized maturation defect of CD83 over-expressing bone marrow (data not shown).

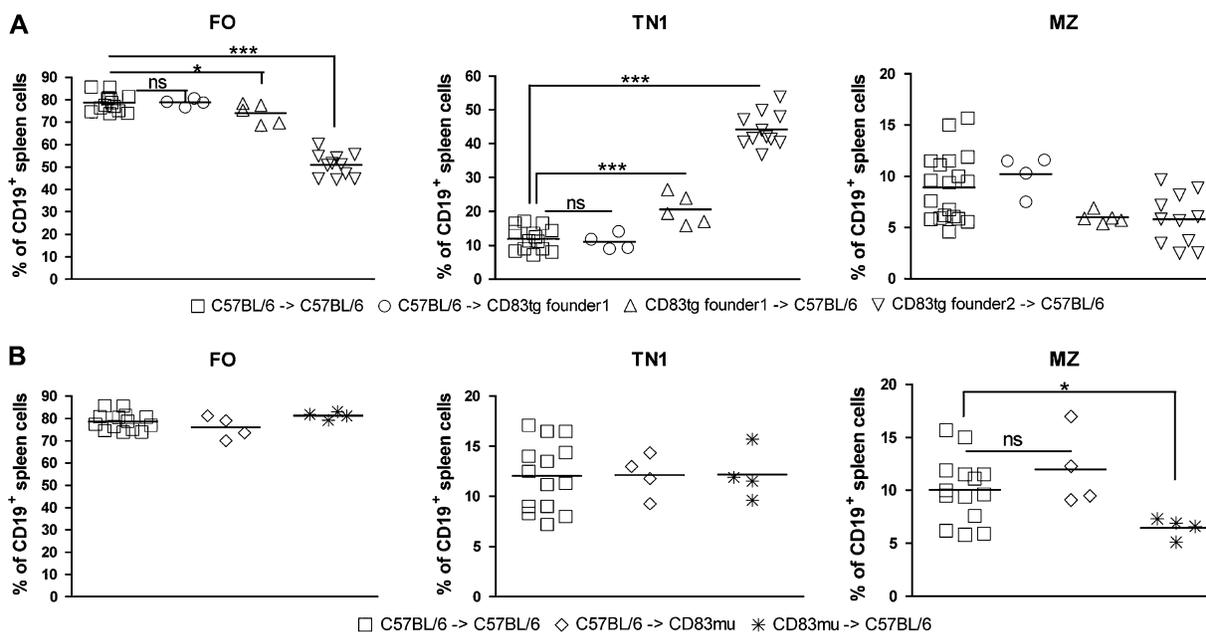
Similar experiments were performed with bone marrow grafts derived from CD83mu mice. As shown in Fig. 4(B),



**Fig. 3.** B cell subsets in mice with aberrant CD83 expression level. B cell subsets of CD83<sup>tg</sup> [founder 1 (f1), founder 2 heterozygote (f2<sup>-/-</sup>) and founder 2 homozygote (f2)], CD83<sup>lg</sup> (lg), CD83<sup>mu</sup> and wild-type (wt) mice were analyzed by FACS staining of  $2 \times 10^5$  cells. Every dot represents the result of one individual mouse; the bar indicates the mean. Asterisks indicate a significant difference of the mean (\* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ) employing Student's *t*-test. (A) Analysis of  $2 \times 10^4$  B220<sup>+</sup> bone marrow cells: pro-B cells (IgM<sup>-</sup> and CD43<sup>+</sup>), pre-B cells (IgM<sup>-</sup> and CD43<sup>-</sup>), immature B cells (IgM<sup>+</sup> and IgD<sup>-</sup>) and mature B cells (IgM<sup>+</sup> and IgD<sup>+</sup>); (B) Analysis of  $2 \times 10^4$  CD19<sup>+</sup> spleen cells—upper panels: FO (CD21<sup>low</sup> and CD23<sup>high</sup>), TN1 (CD21<sup>-</sup> and CD23<sup>-</sup>) and MZ (CD21<sup>high</sup> and CD23<sup>low</sup>) B cells; lower panels: mature (IgM<sup>+</sup> and IgD<sup>high</sup>) and immature (IgM<sup>+</sup> and IgD<sup>low</sup>) B cells.

the CD83 deficiency on somatic tissue did not interfere with wild-type B cell maturation but led to a defective maturation of CD4<sup>+</sup> T cell as described (17) (data not shown). The re-

duced percentage of MZ B cells in the spleen observed in CD83<sup>mu</sup> mice occurred only upon transplantation of CD83<sup>mu</sup> bone marrow grafts into wild-type hosts.



**Fig. 4.** Altered splenic B cell maturation in CD83tg and CD83mu mice is due to CD83 expression on hematopoietic cells. (A) Lethally irradiated C57BL/6 (open square) and CD83tg founder 1 (open circle) mice were reconstituted with  $2 \times 10^6$  IgH<sup>a</sup>-congenic C57BL/6 bone marrow. Lethally irradiated C57BL/6-IgH<sup>a</sup>-congenic mice were reconstituted with CD83tg founder 1 (open triangle) or founder 2 (open reverse triangle) bone marrow, respectively. (B) Lethally irradiated C57BL/6 (open square) and CD83mu (open diamond) mice were reconstituted with  $2 \times 10^6$  IgH<sup>a</sup>-congenic C57BL/6 bone marrow. Lethally irradiated C57BL/6-IgH<sup>a</sup>-congenic mice were reconstituted with CD83mu (star) bone marrow, respectively. B cell development in spleen of bone marrow chimeras was analyzed by FACS staining 10–12 weeks after bone marrow transfer. Spleen cells ( $2 \times 10^4$ ) were analyzed for FO (CD19<sup>+</sup>, CD21<sup>low</sup> and CD23<sup>high</sup>), TN1 (CD19<sup>+</sup>, CD21<sup>-</sup> and CD23<sup>-</sup>) and MZ (CD19<sup>+</sup>, CD21<sup>high</sup> and CD23<sup>low</sup>) B cells. Every dot represents the result of one individual mouse; the bar indicates the mean. Asterisks indicate a significant difference of the mean (\* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ) employing Student's *t*-test.

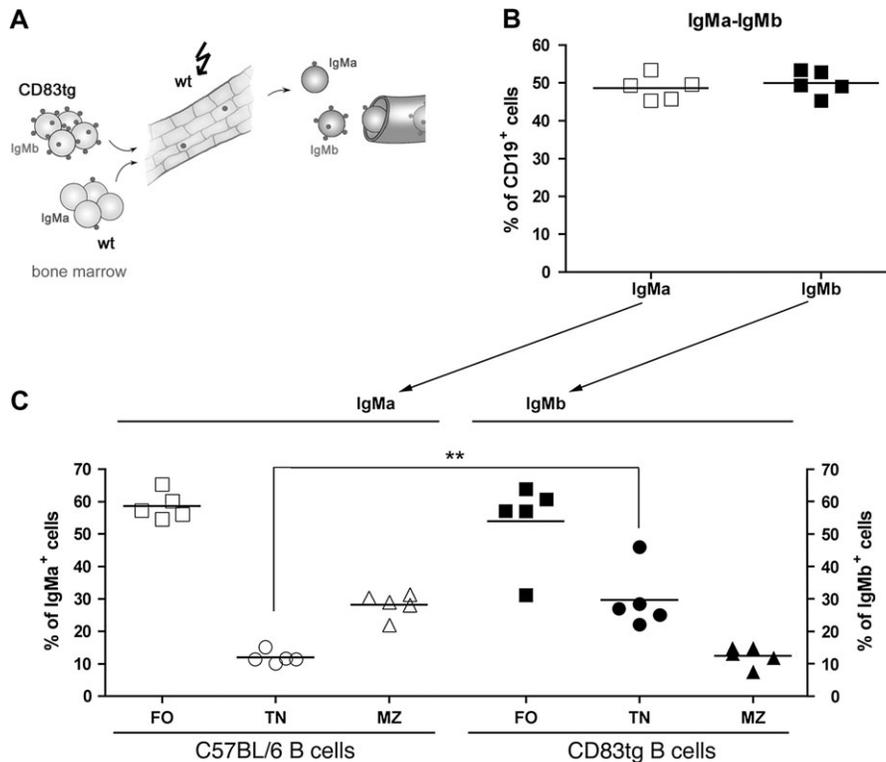
These studies analyzing B cell development in bone marrow chimeras show that altered CD83 expression on hematopoietic but not on somatic cells interfered with B cell maturation. Since all hematopoietic cells that matured from CD83tg or CD83mu bone marrow grafts displayed either increased or absent CD83 expression, we cannot distinguish whether the altered B cell maturation observed in the chimeras was due to altered CD83 expression on the precursor B cell itself or due to altered CD83 expression on other cell types of hematopoietic origin. In order to address this question, we generated bone marrow chimeras by transplanting a mixture of wild-type and CD83tg bone marrow cells into the same host. In these mixed bone marrow chimeras, CD83tg and wild-type B cells simultaneously develop in a wild-type environment including radio-resistant somatic cells. In this situation, CD83 is only over-expressed by one part of the transferred hematopoietic cells. Any interference with B cell maturation due to the CD83 over-expressing bystander cells of hematopoietic origin, however, would also interfere with the maturation of wild-type B cells within the same mixed chimera (Fig. 5A). Analysis of splenic B cells after successful engraftment revealed that 50% of the cells originated from wild-type bone marrow and 50% of the cells originated from CD83tg bone marrow (Fig. 5B). While the B cells of wild-type origin contained the normal 10% of TN1 cells, the CD83 over-expressing bone marrow matured to 30%

cells in the spleen. This result clearly shows that the partial arrest in the TN1 stage of B cell maturation in CD83tg mice was due to CD83 over-expression on the immature B cell itself.

#### *CD83 expression level on the B cells themselves affects peripheral B cell survival in vivo*

Upon screening mixed bone marrow chimeras, we observed differences in the survival of CD83tg bone marrow-derived B cells in direct competition to wild-type bone marrow-derived B cells (14), suggesting an impact of CD83 expression on the longevity of B cells. To analyze this phenomenon, we generated mixed chimeras of CD83 over-expressing or CD83-deficient bone marrow grafts with wild-type bone marrow grafts and monitored the distribution of wild-type and CD83tg- or CD83mu-derived lymphocytes in the peripheral blood over time.

Figure 6(A) shows that transplantation of equal amounts of wild-type and CD83tg bone marrow resulted in an equal distribution of wild-type and CD83tg B cells in the peripheral blood 4 weeks after transplantation. Tracking of the peripheral B cells of different origin over time revealed that the CD83tg B cell pool contracted while the wild-type B cell pool increased until the proportion was shifted to 20% CD83tg and 80% wild-type B cells 20 weeks after transplantation. When the initial input of CD83tg bone marrow was increased to 80%, the initial distribution of B cells in the blood 4–6



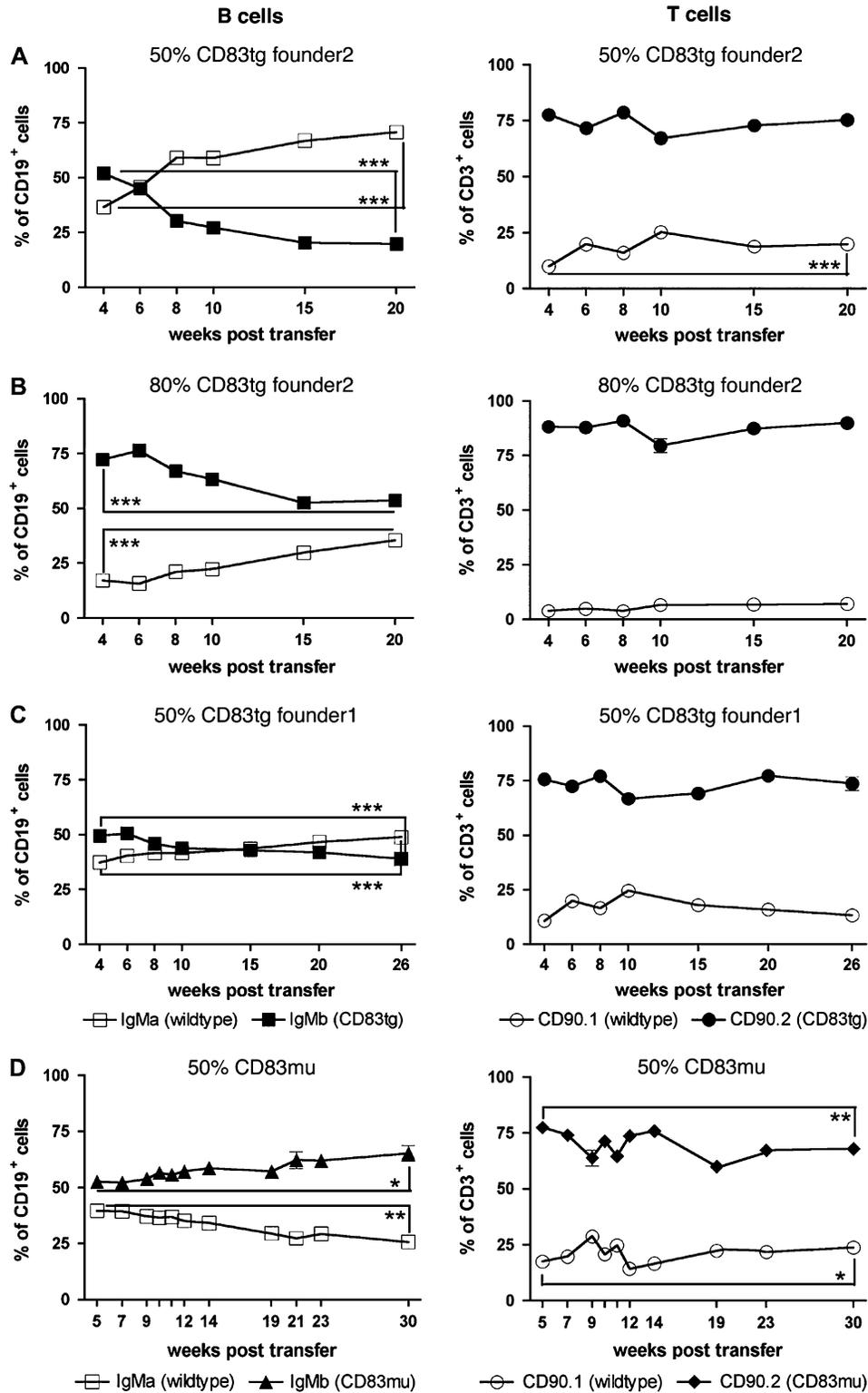
**Fig. 5.** Impaired splenic B cell maturation is due to CD83 over-expression on the B cells themselves. (A) Lethally irradiated C57BL/6 mice were reconstituted with  $1.2 \times 10^6$  C57BL/6-IgH<sup>a</sup> and  $4.8 \times 10^6$  CD83tg-IgH<sup>b</sup> tg bone marrow cells. (B and C) Spleen cells were analyzed for B cell composition 21 weeks after transfer. Every dot represents the result of an individual mixed chimera and the bar indicates the mean. (B) Percentages of C57BL/6-derived B cells (IgM<sup>a+</sup>) and of CD83tg-derived B cells (IgM<sup>b+</sup>) were measured by FACS and are shown as percentage of total B cells. (C) Percentages of FO (CD21<sup>low</sup> and CD23<sup>high</sup>), TN1 (CD21<sup>-</sup> and CD23<sup>-</sup>) and MZ (CD21<sup>high</sup> and CD23<sup>low</sup>) B cells of C57BL/6 or CD83tg origin were analyzed by gating on IgM<sup>a+</sup> (left axis) or IgM<sup>b+</sup> (right axis) cells.

weeks after transplantation reflected the grafted proportion of 80% CD83tg and 20% wild-type bone marrow (Fig. 6B). Regardless of this increased proportion of CD83tg cells in the beginning, the amount of B cells derived from CD83tg bone marrow again decreased over time while the wild-type B cells displayed a reciprocal increase. Strikingly, this reduced fitness of CD83tg lymphocytes was B cell specific, since the percentage of CD83tg T cells remained stable in the peripheral blood over the time monitored (Fig. 6A and B). Moreover, engraftment of equal amounts of CD83tg and wild-type bone marrow led to preferred maturation of CD83tg T cells representing 70% of the peripheral T cells to 30% wild-type T cells (Fig. 6A). Repetition of these experiments with bone marrow derived from CD83tg founder 1 showed that T cell pools again were stable while the B cells also displayed a reduced fitness in direct comparison to wild-type B cells (Fig. 6C). This impaired survival of CD83tg founder 1-derived B cells was less severe than the impaired survival of CD83tg founder 2-derived B cells, strongly suggesting a positive correlation between impaired survival and CD83 expression level because CD83tg founder 2-derived B cells express more surface CD83 than CD83tg founder 1-derived B cells (Fig. 2A and B).

It should be noted that in order to establish the system, we analyzed the kinetics of the irradiation-induced loss of host-derived T and B cells in the peripheral blood employing single

chimeras, whereby bone marrow graft and host differed in congenic markers. These studies revealed that host-derived B cells completely disappeared from the peripheral blood 4 weeks after transfer confirming that the distribution of B cells beginning week 4 after transfer exclusively represented graft-derived lymphocytes. Host-derived T cells, however, still represented 20% of peripheral T cells 8 and 12 weeks after transfer (data not shown). Therefore, it should be pointed out that in the mixed bone marrow chimera, one cannot distinguish between T cells of CD83tg origin and residual host-derived T cells since they share the congenic marker CD90.2. This may lead to an overestimation of the percentage of CD83tg T cells in the mixed chimera. The re-analysis of wild-type and CD83tg-derived T cells by staining CD3<sup>+</sup>CD83<sup>+</sup> versus CD3<sup>+</sup>CD83<sup>-</sup> cells confirmed the results obtained by CD3<sup>+</sup>CD90.1<sup>+</sup> versus CD3<sup>+</sup>CD90.2<sup>+</sup> staining and even suggested expansion of CD83tg T cells over time (data not shown). Therefore, despite this experimental constriction, the stability of the CD83tg T cell pool as measured until week 26 after transfer clearly shows that CD83 over-expression did not reduce the survival of T cells in the periphery.

Finally, we monitored the survival of CD83-deficient B and T cells in direct competition with wild-type lymphocytes. Figure 6(D) shows that CD83 deficiency did not interfere with the survival of B cells in the periphery. In contrast, the initial equal distribution of CD83mu and wild-type B cells



**Fig. 6.** CD83 expression level influences peripheral B and T cell survival. (A–C) Irradiated C57BL/6 mice were reconstituted with  $3 \times 10^6$  IgH<sup>a</sup>-congenic C57BL/6 and  $3 \times 10^6$  CD83tg founder 2 bone marrow cells or with  $1.2 \times 10^6$  IgH<sup>a</sup>-congenic C57BL/6 and  $4.8 \times 10^6$  CD83tg founder 2 bone marrow cells as indicated in the headline ( $N = 5$ ). (D) Irradiated C57BL/6 mice were reconstituted with  $3 \times 10^6$  IgH<sup>a</sup>-congenic C57BL/6 and  $3 \times 10^6$  CD83mu bone marrow cells ( $N = 4$ ). The distribution of wild-type (open square and circle) and CD83tg (closed square and circle) or CD83mu (closed triangle and diamond) bone marrow-derived B and T cells in the peripheral blood of chimeras was analyzed by FACS staining. B cells were identified by staining of CD19-IgM<sup>a</sup>/IgM<sup>b</sup> double-positive cells and T cells by staining of CD3-CD90.1/CD90.2 double-positive cells at indicated time points after bone marrow transfer. Asterisks indicate a significant difference of the mean (\* $P < 0.05$ , \*\* $P < 0.005$ , \*\*\* $P < 0.0005$ ) employing Student's *t*-test; error bars show standard error of the mean.

changed slightly to an increased percentage of CD83<sup>mu</sup> B cells 30 weeks after transplantation, suggesting a selection advantage of CD83-deficient B cells in comparison to wild-type B cells that constitutively express low levels of CD83 (Fig. 1C and D). CD83-deficient T cells on the other hand were engrafted successfully 5 weeks after transplantation but slightly decreased over time until percentages shifted to 65% CD83<sup>mu</sup> and to 25% wild-type T cells.

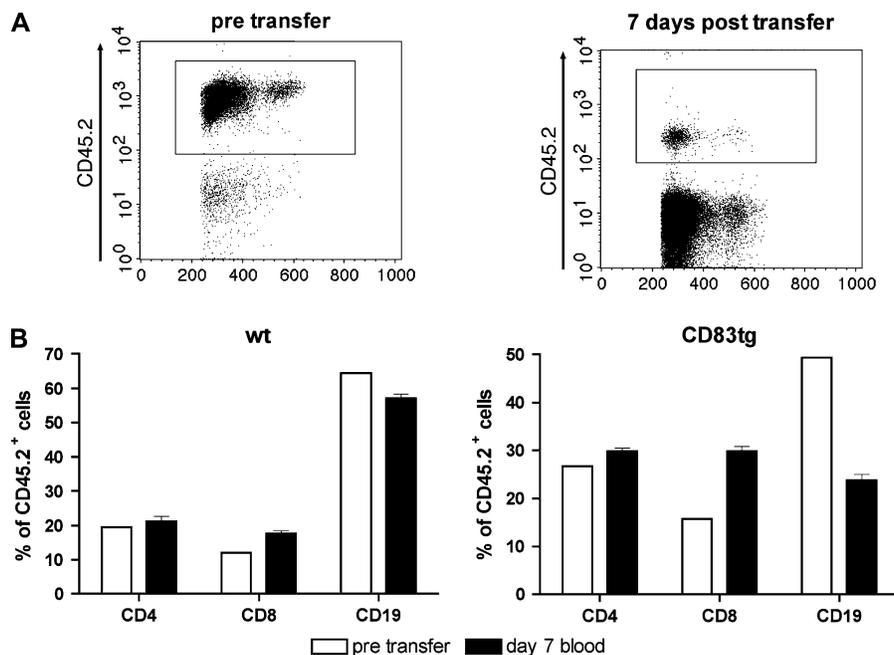
We have shown that CD83 over-expression on B lineage cells already interfered with B cell maturation (Figs 4 and 5). Therefore, it is possible that delayed and reduced maturation of CD83<sup>tg</sup> B cells contributed to the observed contraction of the CD83<sup>tg</sup> B cell pool in comparison to the wild-type B cell pool within the mixed bone marrow chimeras. To selectively observe peripheral B cell survival, we transferred mature CD83<sup>tg</sup> and wild-type spleen cells into immune-competent congenic hosts. One week later, the transferred cells were identified employing a congenic marker (Fig. 7A) and the frequencies of CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells were compared with the frequencies at the time of transfer (Fig. 7B). CD19<sup>+</sup> B cells accounted for circa 50% of the cells in the CD83<sup>tg</sup> spleen cell population at the time of transfer. One week after transfer, however, the CD83<sup>tg</sup> B cell population had contracted accounting only to 25% within the transferred CD83<sup>tg</sup> spleen cell pool. In contrast, the composition of T and B cells within the transferred wild-type spleen cells was not changed dramatically as CD19<sup>+</sup> B cells accounted for the major cell population before and after transfer. This result suggests that CD83 over-expression specifically interferes with B cell survival *in vivo*.

In summary, the experiments performed show that CD83 over-expression specifically and dose dependently impaired B cell survival in the peripheral blood while showing no interference with T cell survival. CD83 deficiency on the other hand conferred a mild selection advantage to B cells over a period of 30 weeks but slightly interfered with T cell survival.

## Discussion

In the current study, we describe a novel function of CD83: in addition to its well established role in thymic selection of CD4-positive T cells (16, 17), we show that CD83 is also involved in the maturation and homeostasis of B cells in the periphery. In contrast to the development of CD4-positive T cells, where CD83 is needed on the thymic epithelium and not on the double-positive thymocytes (23), the further progression of immature to mature B cells and their survival in the periphery is mediated by CD83 expressed on the B cells themselves. We draw this conclusion because of the following evidence:

We detected low levels of CD83 on developing B cells beyond the pre-B cell stage, i.e. once they expressed a functional BCR (24) which is in line with another study published recently (20). Over-expression of CD83 led to a decreased maturation of FO B cells and a reciprocal accumulation of immature TN1 cells in the spleen that was strictly correlated to the intensity of CD83 over-expression, as we showed employing different CD83<sup>tg</sup> mouse strains. This partial arrest in B cell maturation was not due to CD83 over-expression on



**Fig. 7.** Impaired peripheral survival of mature CD83<sup>tg</sup> B cells. Spleen cells ( $1.5 \times 10^7$ ) derived from wild-type or CD83<sup>tg</sup> mice were transferred to CD45.1 congenic recipients intravenously. (A) Example for the detection and gating of CD45.2 spleen cells before transfer and 7 days after transfer into CD45.2 congenic hosts from the peripheral blood. (B) The frequency of CD4<sup>+</sup>, CD8<sup>+</sup> and CD19<sup>+</sup> cells within the transferred CD45.2<sup>+</sup> spleen cell pool was determined by FACS analysis before transfer (open bars) and in the peripheral blood 7 days after transfer (closed bars). Bar graphs represent the mean of recovered CD45.2 cells of four individual mice; error bars show standard error of the mean.

environmental cells since it was not observed upon transplantation of wild-type bone marrow into CD83tg hosts but in contrast occurred in chimeras consisting of wild-type hosts that had received CD83tg bone marrow. Furthermore, the impaired maturation and homeostasis was due to CD83 expression on the B cell precursors themselves since it was restricted to B cells of CD83tg origin and did not apply to wild-type B cells within mixed bone marrow chimeras. Analyses with CD83-deficient mice on the other hand revealed a reciprocal slightly increased survival of CD83mu B cells in direct comparison to wild-type B cells and a reduced development of MZ B cells but unchanged maturation of FO B cells in the spleen.

It is well known that the intensity of BCR-mediated signals contributes to the decision into what B cell subtype an immature B cell will finally mature. Strong signals are thought to favor the development of FO, whereas weak signals favor the development of MZ B cells (25–27). Reporting a defective Ig response of CD83tg B cells *in vivo* (14) and an altered phenotype *in vitro* (15), we suggested that CD83 might represent an additional regulatory receptor such as CD22 (28, 29) that is up-regulated upon B cell activation (14, 15, 20) and contributes to the regulatory mechanisms that prevent over-stimulation of the B cell population (30–32). In this context, the observed increase in TN1 and reciprocal decrease in FO B cells when CD83 was over-expressed on immature B cells may be understood as the result of an increased negative regulation of BCR-mediated signals by over-expression of a negative regulator that is only present at low levels in wild-type TN1. The correlation of this effect with the level of CD83 expression is in line with the hypothesis that increased CD83 expression confers increased susceptibility for the reception of negative signals that dampen the BCR-mediated positive signals received during splenic maturation. The absence of such putative CD83-mediated negative regulation in the CD83mu B cells would explain the observed reduction of MZ as weak signals are increased.

Several examples for similar effects on B cell maturation by altered expression of BCR co-receptors have been described. Mice lacking the positive co-receptor CD21 were shown to possess reduced amounts of FO B cells and increased numbers of MZ B cells as the BCR signals were weaker in these mice (33). CD22-deficient mice on the other hand displayed a decrease in MZ B cell numbers as expected for mice lacking a negative regulator of the BCR and thus receiving increased BCR-mediated signaling (34).

Since the survival in the periphery also depends on BCR-mediated signals (35), the observed reduced fitness of CD83 over-expressing B cells and the slight selection advantage of CD83-deficient B cells may again reflect the consequence of increased or absent negative co-regulation. It should be stressed that the impact of CD83 over-expression on survival was correlated to the intensity of CD83 over-expression on the B cells themselves as we showed comparing two different CD83tg founders in mixed chimeras. Furthermore, the impact of CD83 over-expression was strictly restricted to the B cell compartment since survival of CD83 over-expressing T cells was not affected. In contrast, the CD83tg T cell pool displayed increased engraftment and remained stable over a period of up to 26 weeks, thus ruling

out a generalized and artificial alteration in CD83tg lymphocyte survival. We cannot formally exclude that the impaired maturation of CD83tg B cells within irradiated hosts contributed to the observed contraction of the CD83tg B cell pool in the peripheral circulation of mixed bone marrow chimeras. Transferring mature spleen cells into immune-competent hosts, however, we could show that frequencies of mature CD83tg B cells decreased in the peripheral circulation with respect to CD4- and CD8-positive CD83tg T cells while the frequencies of mature wild-type B cells remained stable. Regarding CD83mu T cells, we observed, in concordance with a recent study employing CD83<sup>-/-</sup> mice (20), a reduced survival of CD83-deficient T cells in comparison to wild-type T cells. In contrast to our findings, however, Prazma *et al.* (20) also observed a reduced survival of CD83-deficient B cells. These results however are difficult to compare with our bone marrow chimera generated results since the authors transferred mature spleen cells and monitored the rapid loss of CD83<sup>-/-</sup> spleen-derived B220<sup>+</sup> cells from the circulation. Furthermore, as there was no CD83<sup>-/-</sup> cell population that showed normal survival and thus provided an intrinsic positive control, it cannot be excluded that these results reflected a non-specific generalized defect of the CD83<sup>-/-</sup> lymphocytes.

By comparing maturation and homeostasis of wild-type B cells that constitutively express low levels of surface CD83 to CD83-deficient B cells, we analyzed the impact of naturally expressed CD83 on the fate of wild-type B cells. Therefore, we would like to stress that the reduced peripheral survival observed for wild-type B cells in direct competition to CD83-deficient B cells was mediated by CD83 expressed at physiologic levels. The reciprocal approach, employing CD83tg B cells, creates an admittedly artificial situation because the naive CD83tg B cells express CD83 at intensities that are comparable to activated wild-type B cells [Fig. 2B and (15)]. Nevertheless, we argue that analyzing the dramatic impact of tg CD83 over-expression also yields relevant information about the weaker impact of wild-type CD83 constitutively expressed at low levels on resting B cells. In line with this reasoning, CD83 over-expressing and CD83-deficient B cells displayed reciprocal phenotypes compared with wild-type B cells within mixed bone marrow chimeras. Taken together, within this study, we provide strong evidence that CD83 is not only involved in the regulation of B cell activation and function (14, 15) but, by expression on the B cells themselves, also plays a role in B cell maturation and survival.

Regarding the mechanism of CD83-mediated modulation of maturation and survival, it was reported that soluble CD83, released by shedding or other means from CD83-positive dendritic cells suppressed mixed lymphocyte reaction *in vitro* (36). Since addition of recombinant soluble CD83 was shown to dramatically affect T cell response *in vivo*, one may speculate that CD83 over-expressing mice release soluble CD83 that contributes to the phenotype described in this study. Wild-type B cells co-transferred with CD83tg B cells into mixed bone marrow chimeras, however, responded normally to model antigen immunization (14) and displayed normal maturation and survival as we showed in this study. This observation clearly rules out such

a suppressive mechanism since any soluble CD83 possibly present in these chimeras would act on wild-type and CD83tg lymphocytes equally. Moreover, the tg expression of soluble CD83lg fusion protein leading to serum concentrations of 20 ng ml<sup>-1</sup> did not change the cellular composition of the B cell population as shown in this study and did not interfere with B cell response (18), thus further arguing against an impact of soluble CD83 on B cell maturation. Alternatively, as specifically the B cells that over-express CD83 display impaired function *in vivo* and *in vitro* (14, 15), CD83 may deliver negative signals into the very B cell it is expressed on, as we have suggested (32). Since the intracellular domain of CD83 does not contain tyrosine residues, there is no evidence of direct signal transduction through immunoreceptor tyrosine-based inhibitory motif (ITIM) motives and the molecular mechanism of putative CD83-mediated signaling still needs to be elucidated.

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### Abbreviations

APC	allophycocyanin
BCR	B cell receptor
FO	follicular B cell
ITIM	immunoreceptor tyrosine-based inhibitory motif
mu	mutant
MZ	marginal zone B cell
tg	transgenic
TI	thymus independent
TN1	transitional B cell

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