Peritoneal cavity B-1a cells promote peripheral CD4⁺ T-cell activation

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Innate-like murine B-1a cells are well known for their ability to secrete natural IgM. Their non-Ab mediated functions, including Ag presentation to CD4⁺ T cells, are less well explored. Using combined adoptive transfer experiments with peptide-pulsed peritoneal cavity (PerC)-derived B-1a cells and CFSE-labeled T cells, we show that B-1a cells present Ag to CD4⁺ T cells from the periphery in vivo. In vitro characterization, using co-cultures in which B-1a or splenic B cells presented whole OVA protein to OVA-specific Tg T cells, shows that B-1a cells differentially promote intracellular cytokine-expressing T cells. PerC-derived B-1a cells increase the percentage of IL-10-producing T cells along with IL-4- and IFN-γ-producing CD4⁺ T cells. These data suggest that B cells in the PerC have the potential to influence peripheral immune responses without the necessity to migrate out of this location. This, to our knowledge previously undescribed, immunological pathway potentially plays a role in the presentation of gut microbiota-derived Ags to peripheral T cells.

Keywords: Antigen presenting cell (APC) · B-1 cell · B-1a cell · IL-10 · Peritoneal cavity

Introduction

B cells are traditionally known as precursors of Ab-producing cells. Nevertheless, it is well accepted that their non-Ab mediated functions, which include Ag presentation to CD4⁺ T cells and the secretion of cytokines, play important roles during both active immune responses and maintenance of immunological homeostasis [1–4].

Unstimulated, resting B cells are reported to preferentially expand Foxp3⁺ Treg cells [5, 6]. Likewise, specific subsets of IL-10-producing regulatory B cells are described to be involved in the maintenance or induction of IL-10-producing Tr1 Treg cells [7, 8]. However, there are few reports on possible specialized roles that other B-cell subsets might exert when acting as APCs.

Murine B-1 cells are innate-like B cells that differ from B-2 cells (follicular B cells and marginal zone B cells) as reviewed by Baumgarth [9], Suzuki et al. [10], and Sindhava et al. [11]. Briefly, B-1 cells are well known for their ability to produce natural antibodies, that is, polyreactive IgM present in preimmune mice involved in both the early defense against pathogens and in the maintenance of homeostasis. B-1 cells can furthermore be distinguished from B-2 cells by their abundant presence in the peritoneal cavity (PerC) and the differential expression of cytokines. Especially the predominant secretion of IL-10 is well known [12].

B-1 cells are found in relative large amounts in the PerC where they are characterized by a specific combination of surface markers including CD11b. Most of the B-1 cells in the PerC of BALB/c
mice are B-1a cells characterized by the expression of CD5, while a smaller population of CD5⁻ cells are termed B-1b cells. The absolute number of B-1 cells in the spleen and the PerC are thought to be comparable, however the splenic B-1 population is much harder to distinguish from B-2 cells due to the lack of CD11b expression. Other B-1-specific surface markers such as low expression of B220, intermediate expression of CD5, or expression of CD43 can also be markers for B-cell activation, anergy, or of plasma blasts [9, 13–15], which makes the identification of splenic B-1a cells challenging. The relationship between B-1a cells from the PerC and peripheral (splenic) B-1a cells is not completely clear. A large part of the splenic B-1a-cell population possesses significant differences in BCR repertoires, gene expression patterns, and IgM-secreting properties compared with that of PerC B-1a cells [16–18]. On the other hand, at least some of these differences are shown to be due to influences of the microenvironment in these organs, indicating that differences between these B-1a cell subsets might be due to a different activation status and not due to intrinsic cell or origin differences [18–20]. On top of that, it has been shown that PerC B-1a cells are able to migrate to the spleen after LPS triggering [18, 21–23] and that the spleen is an important organ for the generation or maintenance of PerC B-1a cells as shown by reduced numbers of this B-cell subset in splenectomized mice [24].

Here we compare the Ag presentation of traditional splenic B cells with PerC B-1a cells in vitro. Analysis shows that PerC B-1a cells, compared with splenic B cells, increase the percentage of IL-10-, IFN-γ-, and IL-4-producing T cells, as determined via intracellular cytokine staining.

Even though our data show that PerC B-1a cells only migrate in small numbers from the PerC toward the periphery, we demonstrate that PerC B-1a cells interact with CD4⁺ T cells from the periphery since peripheral T cells visit the PerC and may engage with PerC B-1a cells at this location. To our knowledge, this pathway has not been described before.

**Results**

**PerC B cells increase IL-10-producing CD4⁺ T cells in vitro**

The PerC contains a unique B-cell population that contains a high percentage of B-1 cells. The exact ratios between the various B(-1) cell subsets in this organ, however, varies between mice breeds [25]. Around 60–70% of the PerC CD19⁺ B lymphocytes in BALB/c mice possess the B220low CD11b⁺ CD5⁻ CD23− CD43⁻ IgMhi IgDlow phenotype characteristic for B-1a cells (Fig. 1A or B). B cells in the spleen can be subdivided into follicular B cells and marginal zone B cells based on their expression of CD21/35 and CD23 while B-1 cells, that are included in the B220low CD5⁻ population, constitute only a minor fraction (1~5%) of the splenic B-cell population (Fig. 1C).

To determine if the above-described PerC B-cell population (Fig. 1A) differentially increases CD4⁺ T-cell subsets compared with the splenic B-cell population, we first analyzed both the Foxp3 and IL-10 expression of T cells when both B-cell populations were used as APCs. To that matter, CFSE-labeled OVA-specific T cells were activated with either naive PerC or splenic B cells in the presence OVA protein.

The relative expression of Foxp3 in the T cells that had been primed by B cells from the PerC tended to be lower compared with that of T cells primed by splenic B cells (Fig. 2A or B). However, when PerC B cells were used as APC, a significantly higher percentage of T cells produced IL-10 compared with cultures in which splenic B cells had been used as APC (Fig. 2C or D). In order to determine whether this effect was due to the presence of B-cell-secreted IL-10, we selectively neutralized this cytokine using a neutralizing monoclonal Ab against IL-10. A reduction in the amount of T cells positive for intracellular IL-10 was found, but the selective enrichment of the cytokine-production cells was not completely blocked (Fig. 2E).
PerC B-1a cells differentially increase CD4+ T-cell subsets in vitro compared with splenic B cells

We sought to determine if the B-1a cells present in the PerC B-cell population were responsible for the enrichment of IL-10-producing T cells demonstrated in Figure 2. CD19+ CD11b+ CD5+ B-1a cells were FACS-sorted from the PerC B-cell population and these cells were used as APCs in co-cultures in a similar way as described above. To further characterize T-cell subsets by their cytokine profile, we analyzed IL-10, IFN-γ, IL-17, and IL-4 production by T cells. When FACS-sorted B-1a cells were used as APCs, the enrichment of IL-10-producing T cells was similar compared with that of cultures where the whole PerC B-cell population was used as APC (Fig. 3A and B). This indicates that indeed PerC B-1a cells have the potential to increase the percentage of IL-10-producing T cells. Furthermore, more IL-4-producing cells were observed, coinciding with a small increase in the percentage of IFN-γ-producing cells (Fig. 3A and B). No differences in the amount of T cells capable of expressing IL-17 were found, mainly because almost none of the T cells were producing this cytokine under these conditions.

Since B-1a cells are present in both the PerC and the spleen, we performed co-cultures using splenic B-1a cells to investigate if this B-cell subset enriches T-cell subsets in a similar way as PerC B-1a cells. The splenic CD19+ B220low CD5+ B cell population that contains splenic B-1a cells was FACS-sorted and splenic CD19+ B220hi CD5− B cells served as a control. The data indicate that the B-1a-enriched splenic B-cell fraction tended to enrich IL-10-producing
Figure 3. Compared with splenic B cells, PerC B-1a cells differentially enrich T-cell subsets. CD19+ CD5+ CD11b+ B-1a cells were FACS-sorted from the PerC and compared with splenic B cells in a co-culture that was performed in a similar way as described in the legend of Figure 2. Cytokines in the proliferated T cells were stained intracellularly after restimulation. (A) Flow cytometry plots showing gating strategies are representative of two independent experiments. (B) The percentage of cytokine-expressing T cells after co-culture with B-cell subsets from the PerC and the spleen is compared. Gating was performed as in (A). Data are shown as mean ± SD and are pooled from two independent experiments with technical duplicate or triplicate measurements. (C) The percentage of cytokine-expressing T cells after co-culture with the indicated splenic B-cell subsets is compared. Data are shown as mean ± SD and are pooled from three independent experiments with duplicate measurements. *p < 0.05; **p < 0.01; Student’s t-test.

T cells as well, although the increase was not to the same extend as seen by PerC B-1a cells. Splenic B-1a cells furthermore seemed to enrich IL-17-producing T cells (Fig. 3C).

In summary, our data show that PerC B-1a cells, compared with splenic B cells, increase the percentage of IL-10-, IFN-γ-, and IL-4-producing T cells, as determined by intracellular cytokine staining. Especially the percentage of IL-10 and IL-4 positive cells was increased (~3.2-fold and ~5.3-fold, respectively, versus ~1.6 for IFN-γ). Interestingly, when splenic B-1a cells are used, IL-17-producing T cells are enriched.

PerC B-1a cells create different cytokine environments after T cell-B cell communication

The cytokines that are secreted into the supernatant after T cell-B cell communication were measured and are compared in

Figure 4. Significant higher amounts of IL-10 were found when PerC B cells or FACS-sorted B-1a cells were compared with splenic B-cell cultures.

Compared with splenic B cells, IL-6 levels were significantly higher in PerC B (and PerC B-1a) cell cultures. IL-2 levels were significantly higher when whole PerC B cells were used, whereas they were only slightly enriched when FACS-sorted B-1a cultures were compared with splenic B cell cultures. IFN-γ and IL-17 levels were roughly equal in all co-cultures performed.

PerC B cells or splenic B cells in the presence of OVA protein (without T cells) produced sixfold lower levels of IL-10 while IFN-γ, IL-17a, and IL-2 became undetectable. IL-6, on the other hand, was still present (Fig. 4). This finding indicates that most of the cytokines detected in the supernatant of the co-cultures resulted from communication between T and B cells.

In summary, we detected increased levels of IL-6 and IL-10 when FACS-sorted PerC B-1a cells presented Ag to Ag-specific T cells whereas Ag presentation by splenic B cells resulted in a
cytokine environment that is characterized by the virtual absence of these cytokines.

**PerC- B-1a cells are not exclusively restricted to the PerC**

The above presented data show that PerC B-1a cells can differentially activate CD4\(^+\) T cells. However, physiologically, the PerC contains relatively few CD4\(^+\) T cells ([26] and data not shown). We therefore addressed whether PerC B-1a cells can migrate toward the periphery to communicate with peripheral T cells. For this purpose, we FACS-sorted PerC B-1a cells, labeled them with the red fluorescent dye PKH-26, and adoptively transferred them i.p. to acceptor mice that were s.c. immunized the next day. Four days later, the mice were analyzed for the presence of the transferred PKH-26\(^+\) cells. Flow cytometric analysis in combination with viable cells counts of the draining lymph nodes, as well peripheral lymph nodes, the spleen, and the blood showed that most of the transferred B cells remained in the PerC (Fig. 5B). However, a small population of transferred cells was consistently detected in the periphery (Fig. 5B).

Migration of PerC transferred B-1a cells toward the periphery was associated with the loss of CD11b (Fig. 5C), which is in agreement with LPS-induced B-1a migration experiments performed by others [18, 22]. The expression of CD5 was stable after migration (data not shown).

In conclusion we found that B-1a cells preferentially remain in the PerC, but that they are not exclusively restricted to this location, since small amounts of PerC B-1a cells were shown to migrate to the periphery under our experimental conditions.

**PerC B-1a cells present Ag to CD4\(^+\) T cells from the periphery in vivo**

The finding that PerC B-1a cells migrated toward the periphery in small numbers only, questioned the likelihood that these cells migrate to the periphery to subsequently present Ag to T cells. Therefore, the capacity of PerC B-1a cells to present Ag to peripheral T cells in vivo was further studied. Isolated PerC B-1a cells were ex vivo pulsed with OVA\(_{323-339}\) peptide and transferred to the PerC of acceptor animals via the i.p. route. CFSE-labeled OVA-specific (KJ1–26\(^+\)) CD4\(^+\) T cells were transferred to the periphery via the i.v. route on the same day. The animals were sacrificed 4 days later and analyzed for the presence of proliferating transferred T cells in various organs. Proliferating KJ1–26\(^+\) T cells were detected mainly in the PerC, in very small amounts in the mediastinal LNs, but not in the spleen or in other peripheral LNs (Fig. 6A). This finding strongly suggests that i.v.-transferred CD4\(^+\) T cells from the periphery entered the PerC to engage with Ag-presenting PerC B-1a cells. In a follow-up experiment we transferred T cells via the i.p. route to the PerC as well, to allow further analysis of the in vivo activated T cells that were otherwise present in small amounts. I.p.-transferred T cells that were in vivo activated by PerC B-1a cells were not restricted to the PerC but instead were located in the spleen (Fig. 6B, upper panel), mediastinal lymph nodes (Fig. 6B, lower panel), and other peripheral lymph nodes including the brachial, axillar, mesenteric, and lumbar lymph nodes (data not shown). Using this model, we were not able to detect differences in T-cell cytokine profiles after ex vivo stimulation (Fig. 6C) in a similar way as we showed earlier in Figure 3. Nevertheless, PerC B-1a-activated T cells found in the spleen proliferated more vigorously compared to the ones activated by splenic control B cells (Fig. 6D).

**Discussion**

Ag presentation by B cells has long been disregarded, but several papers unambiguously demonstrated the important contribution of Ag presentation by B cells for effective immune responses [2, 4, 27].

However, few papers describe the Ag-presenting capacity of specialized B-cell subsets. In the current study, we describe how B-1a cells from the PerC enrich IL-10-, IL-4-, and IFN-\(\gamma\)-producing T cells when acting as APC in vitro. In a similar study, Zhong et al. [28] compared the APC abilities of PerC B-1a and splenic B cells as well and focused on Foxp3\(^+\) Treg differentiation. They found that cultures in which PerC B-1a cells were used as APCs contained fewer Foxp3\(^+\) Treg cells than cultures where splenic B-2 cells were used. This was also seen in our experiments (Fig. 2A or B).

Interestingly, the same paper describes that B-1a preferentially promoted Th1 and Th17 cell differentiation (measured via intracellular cytokine staining of IFN-\(\gamma\) and IL-17, respectively) [28]. Although we did find an increase in the number of IFN-\(\gamma\)-producing T cells in our experimental setting, we found this increase relatively modest compared with the increase in the
number of IL-10- and IL-4-producing cells (ratio PerC B-1a to splenic B-cell cultures ~1.6-fold for IFN-γ, ~3.2-fold for IL-10, and ~5.3-fold for IL-4; see Fig. 3B). No differences in the relative number of T cells expressing IL-17 were detected.

Interestingly, IL-17-producing T cells did seem to be enriched when splenic CD5 B220low B cells were used in co-cultures. It is difficult to assign this finding specifically to splenic B-1a cells due to the fact that the CD5 B220low fraction possibly contained other cells than B-1 cells. B-1 cells are notoriously hard to identify in any organ other than the body cavities due to the lack of CD11b expression and the presence of large amounts of other cells with similar surface markers [9]. CD5 is a marker for anergic B cells as well [29], and activated B cells can downregulate their expression of B220 [13, 14]. The splenic CD5 B220hi B-cell fraction consists mainly of follicular B cells, but it should be noted that it contains smaller amounts of multiple other B-cell subsets, including marginal zone B cells. Although it is technically challenging to obtain sufficient numbers of cells for analysis, it would be of interest to see a side-by-side comparison of the multiple splenic and PerC B-cell subsets in their capacities as APCs in a future study.

Analysis of the supernatants from our co-cultures showed that communication between PerC B(-1a) cells and T cells resulted in a cytokine environment with increased levels of IL-10, IL-6, and IL-2 while the levels of IL-17a and IFN-γ were not significantly altered. The latter observation supports the notion that no apparent differentiation of Th17 cells took place in co-cultures where PerC B(-1a) cells were used as APCs. This finding suggests that some of the cytokines in the PerC B-cell subset were possibly derived from other sources, such as CD5 B-1b cells or PerC B-2 B cells.

Discrepancies between cytokine levels detected in the supernatant and the cytokines that were detected intracellularly can occur due to the fact that the T cells were restimulated with PMA/ionomycin in order to facilitate intracellular detection. The supernatant furthermore contains cytokines that are produced not only by the T cells, but by the B cells as well.

Data from our migration and in vivo experiments with pulsed B cells demonstrated that B-1a cells from the PerC communicate with T cells from the periphery. This finding is important since the apparent physical separation of the B-1 cells in the PerC and the bulk of the T cells in the periphery might suggest otherwise. Migration from PerC B-1a cells toward the periphery would facilitate PerC B-1a–T cell contact, but our data show that this does not happen in abundant quantities. Data from parabiosis experiments show that B-1a cells are continuously circulating from the PerC through the omentum, toward the blood [30]. While other reports indicate that in situ labeled PerC B-1a cells showed only minimal migration toward peripheral lymphoid tissues other than the pleural cavity [31]. In general, both these reports are in agreement with our observations, since, in our model, most PerC B-1a remained inside the PerC and only relatively small amounts ended up in the periphery. After TLR triggering [21–23] or due to influenza virus infection [32] B-1a cells do migrate toward the periphery (spleen or lungs and local lymph nodes, respectively) in abundant amounts.

Our in vivo study with peptide-pulsed B cells suggests that it is not necessary for B-1a cells to migrate out of the PerC in large amounts in order to present Ag to T cells from the periphery since peripheral T cells visit the PerC and engage with B cells in this location (Fig. 6A). Lâbadi et al. indeed showed that amongst in situ labeled PerC lymphocytes, T cells have the fastest migration rates [31]. Two recent studies indicated that the PerC T lymphocyte population is highly enriched in T cells with an Ag-experienced/memory phenotype [26,33], but not with Foxp3.
Figure 6. PerC B-1a cells present Ag to peripheral T cells, which are differentially activated. (A) FACS-sorted CD19⁺CD5⁺CD11b⁺ B-1a cells were ex vivo pulsed with OVA peptide and subsequently i.p. transferred to the peritoneal cavity of recipient mice. CFSE-labeled CD4⁺ OVA-specific (KJ1–26⁺) T cells were transferred via the i.v. route to the same animal. Shown are gated KJ1–26⁺ T cells in the indicated organs of recipient mice 96 h after transfer. Flow cytometry plot is from one experiment representative of two independent experiments. (B) B cells were transferred as in (A) whereas CFSE-labeled T cells were now transferred via the i.p. route. Shown are gated KJ1–26⁺ T cells in the spleen and mediastinal lymph nodes of recipient mice that received T cells in combination with indicated peptide-pulsed B-cell subsets. Shaded areas indicate T cells from a control animal that did not receive peptide-pulsed B cells. (C) The percentages of indicated-cytokine positive cells out of the KJ1–26⁺ T-cell population in the spleen of recipient mice (treated as in (B)), after ex vivo stimulation with PMA/ionomycin, followed by intracellular cytokine staining. Data are shown as mean ± SEM of six (PerC B-1a) to eight (splenic B cells) samples pooled from three individual experiments performed. (D) The percentage of divided KJ1–26⁺ T cells (gated as in (B)) in the spleen. Data shown are from one representative experiment out of three experiments performed. *p < 0.05, Student’s t-test.

Treg cells [26]. Earlier, it was already shown that Ag-experienced T cells present in the PerC include cells that were primed locally in the lung as well, supporting the presupposition that peripheral T cells migrate to the PerC and come into contact with PerC B cells [34]. Our experiments with i.p. transferred B and T cells furthermore demonstrate that T cells activated in the PerC do not stay in this location, but instead migrate toward the periphery (Fig. 6B).

Differences in cytokine profiles of T cells activated by B-1a cells in vitro (Fig. 2 and 3) or in vivo (Fig. 6D) can be the result of differences in microenvironments. In vitro co-cultures are efficiently simulating specific microenvironments that are important...
for the enrichment of specific T-cell subsets. We showed that enrichment of IL-10-producing T cells is dependent on a high concentration of soluble IL-10 (Fig. 2C), and we indeed demonstrated that high amounts of IL-10 were present in the supernatants of our co-cultures with PerC (B-1a) cells (Fig. 4). In vivo, splenic B cells transferred to the PerC might have been influenced by the microenvironment of the PerC, programming them into cells with B-1-like properties. The influence of the PerC microenvironment on splenic B cells has been described before [35,36]. Alternatively, ex vivo T-cell stimulation might not have been sensitive enough to detect differences in in vivo T-cell activation by the different B-cell subsets.

The finding that peripheral T cells migrate to and from the PerC, where they interact with B-1a cells, raises the question of what the contribution of this interaction on the T-cell pool might be. Evidence suggests that a large proportion of the peripheral memory T-cell pool is in fact specific for commensal microbes from the gut [37]. It is tempting to speculate that PerC B-1a cells play an unexplored role in sensing gut microbiota-derived Ags [21] or whole bacteria [38, 39], which they subsequently present to peripheral (memory) T cells. Alterations in the PerC B-1 cell population from germ-free and SPF mice support the hypothesis that PerC B-1 cells sense luminal Ags [21,40].

In summary, our data show that PerC B-1a cells communicate with CD4+ T cells from the periphery in vivo, and that PerC B-1a cells differentially enrich IL-10-, IL-4-, and IFN-γ-producing T-cell subsets in vitro. To our knowledge, this immunological pathway had not been described before. Given the fact that B-cell depletion therapies are currently being exploited for usage in a variety of T-cell-mediated autoimmune diseases [4], it is of utmost importance to understand how various B-cell subsets influence peripheral T-cell populations.

Materials and methods

Mice

BALB/c mice (8–12 weeks) were purchased from Charles River Laboratories (Maastricht, the Netherlands) and kept under standard housing conditions at the Central Animal Laboratory (GDL) of the Utrecht University. DO11.10 (OVA-TCR Tg) were bred and kept at the GDL under specific pathogen-free conditions. In one experiment, mice were immunized with 100 µL containing 1 mg dimethyl didodecyldiammonium bromide and 100 µg whole chicken OVA grade VII (OVA; Sigma-Aldrich Chemie B.V.). All animal experiments were approved by the Animal Ethics Committee (DEC) from the Utrecht University.

Cell isolation, labeling, and cell transfers

OVA323-339-TCR Tg CD4+ T cells were obtained from the spleen of naive DO11.10 mice by negative selection with sheep-anti-rat IgG Dynalbeads (Dynal, Invitrogen) using an excess amount of anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-MHC class II (M5/114), anti-CD8 (YTS169) monoclonal Abs. Enriched T cells were routinely pure between 85 and 95%. Labeling of cells with CFSE (Molecular Probes) was performed as previously described [41].

B cells were isolated from naive BALB/c mice. Splenic B cells were isolated by positive selection using CD19-MicroBeads (MACS Miltenyi) and splenic B-cell subsets were isolated by FACS sorting (BD INFLUX). B cells were routinely pure >95% based on their expression of CD19. PerC washout cells were obtained by an i.p. injection of 10 mL of PBS supplemented with 2% FCS and 2 mM EDTA (Gibco) that was subsequently collected using a Pasteur pipette. Whole PerC B cells were isolated by positive selection using CD19 MicroBeads and PerC B-1a cells were isolated by FACS sorting (based on the expression of CD19+CD5+CD11b−). Supporting Information Fig. 1 shows the reanalysis of purified cell populations used. In some experiments, PerC B-1a cells were labeled with PKH-26 (Sigma) according to manufacturer’s instructions. Viable cell counts were determined by Trypan blue exclusion.

In experiments cells were transferred, 2 × 10^6 B cells (Fig. 5 and 6) and 1 × 10^7 OVA-TCR Tg CFSE-labeled CD4+ T cells (Fig. 6) were transferred via the i.p. and/or i.v. route. OVA323-339 peptide pulsed B cells (15 µg/mL; 2 h; 37°C; 5% CO2) were washed three times before transfer.

Flow cytometry and intracellular cytokine staining

Single-cell suspensions were washed and subsequently stained in the presence of Fc block (2.4G2) for CD11b (M1/70), CD19 (1D3), CD21/35 (7E9), CD23 (B3B4), CD4 (RM4-5), CD43 (S7), CD45R/B220 (RA3-6B), CD5 (53-7.3, biotin conjugate), DO11.10 TCR (KJ1-26), IgD (11-26c.2a), and IgM (II/41) from BD, eBioscience, or Biolegend. After washing, the samples were analyzed on a FACS Canto II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

For intracellular cytokine staining, total cells from co-cultures were stimulated for 5 h in the presence of 50 ng/mL PMA, 500 ng/mL ionomycin, 1 µg/mL LPS, and 1 µg/mL Brefeldin A (all from Sigma). Cells were then washed, stained extracellularly as indicated earlier, permeabilized using Cytofix/Cytoperm solution (BD) according to manufacturer’s instructions, and subsequently stained intracellularly for cytokines in the presence of Fc block (2.4G2) using antibodies against IFN-γ (XMG1.2), IL-10 (JESS-16E3), IL-17 (eBio17B7), and IL-4 (11B11) from BD or eBioscience.

Co-culture

Indicated B cells (3 × 10^5) were co-cultured with OVA-TCR Tg CFSE+ CD4+ T cells (1 × 10^5) in the presence of 100 µg/mL OVA in IMDM supplemented with 5 × 10^-5 M 2-mercaptoethanol,
penicillin (100 units/mL), and streptomycin (100 µg/mL) (all from Gibco) at 37°C and 5% CO₂. In some experiments, IL-10 secreted into the supernatant was neutralized using 20 µg/mL rat anti-mouse IL-10 mAb (JES-2A5).

Luminex

Cytokines secreted during the cultures were determined by Luminex. Briefly, fluoresceinated microbeads coated with capture antibodies for simultaneous detection of IFN-γ (AN18), IL-2 (JES6-1A12), IL-10 (JESS-2AS), IL-17a (TC11-18H10), and IL-6 (MP5-20F3) were added to 50 µL of culture supernatant. Cytokines were detected by biotinylated antibodies against IFN-γ (XMG1.2), IL-2 (JES6-5H4), IL-10 (SXC-1), IL-17a (TC11-8H4.1), and IL-6 (MP5-32C11) followed by PE-labeled streptavidin (all from BD Biosciences Pharmingen). Fluorescence was measured using a Luminex model 100 XYP (Luminex, Austin, TX, USA) and the cytokine concentrations were quantified using a calibration line from recombinant cytokines.

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References


Abbreviation: Perc: Peritoneal cavity

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