The Number of Responding CD4 T Cells and the Dose of Antigen Conjointly Determine the Th1/Th2 Phenotype by Modulating B7/CD28 Interactions

Christopher D. Rudulier, K. Kai McKinstry, Ghassan A. Al-Yassin, David R. Kroeger and Peter A. Bretscher

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The Number of Responding CD4 T Cells and the Dose of Antigen Conjointly Determine the Th1/Th2 Phenotype by Modulating B7/CD28 Interactions

Christopher D. Rudulier, K. Kai McKinstry,1 Ghassan A. Al-Yassin, David R. Kroeger,2 and Peter A. Bretscher

Our previous in vivo studies show that both the amount of Ag and the number of available naive CD4 T cells affect the Th1/Th2 phenotype of the effector CD4 T cells generated. We examined how the number of OVA-specific CD4 TCR transgenic T cells affects the Th1/Th2 phenotype of anti-SRBC CD4 T cells generated in vivo upon immunization with different amounts of OVA-SRBC. Our observations show that a greater number of Ag-dependent CD4 T cell interactions are required to generate Th2 than Th1 cells. We established an in vitro system that recapitulates our main in vivo findings to more readily analyze the underlying mechanism. The in vitro generation of Th2 cells depends, as in vivo, upon both the number of responding CD4 T cells and the amount of Ag. We demonstrate, using agonistic/antagonistic Abs to various costimulatory molecules or their receptors, that the greater number of CD4 T cell interactions, required to generate Th2 over Th1 cells, does not involve CD40, OX40, or ICOS costimulation, but does involve B7/CD28 interactions. A comparison of the level of expression of B7 molecules by APC and CD4 T cells, under different conditions resulting in the substantial generation of Th1 and Th2 cells, leads us to propose that the critical CD28/B7 interactions, required to generate Th2 cells, may directly occur between CD4 T cells engaged with the same B cell acting as an APC. The Journal of Immunology, 2014, 192: 5140–5150.

Several different subsets of CD4 T cells have been described, each with a distinct functional phenotype. Among these different subsets, Th1 and Th2 cells have been shown to mediate resistance and susceptibility to a number of pathogens and some cancers (1–6). Currently, licensed vaccination protocols are generally poor at generating Th1 imprints that guarantee a predominant Th1 response upon a subsequent exposure to the Ag. Thus, an accurate and complete understanding of how Th1 versus Th2 cells are generated is paramount to developing efficacious immunotherapeutic approaches aimed at pathogens and cancers in which resistance is associated with a predominant Th1 response.

Many different factors are known to affect the Th1/Th2 differentiation of naive CD4 T cells. The cytokine environment in which CD4 T cells are activated is a potent determinant of their Th1/Th2 phenotype. In general, IL-12 produced by dendritic cells (DCs) is thought to be of primary importance for the generation of Th1 cells. The production of IL-4 is critical for the substantial generation of Th2 cells. Studies have identified basophils (7, 8) and NKT cells (9) as the critical source of IL-4, whereas other studies demonstrate that naive CD4 T cells themselves produce sufficient levels of IL-4 for the development of Th2 cells (10–12). Yet other studies have implicated various costimulatory molecule receptors, such as OX-40 (13, 14), ICOS (15), and CD28 (16–19), in the differential development of Th2 over Th1 cells. Although costimulation is typically considered to be delivered to a T cell by an APC, functional interactions between T cells via costimulatory molecules and their receptors have been described (20); this is especially true for CD28 and the CD80/86 costimulatory molecules (21–24). Ag dose is also known to affect the Th1/Th2 phenotype of the ensuing response. Most in vivo observations show that lower and higher doses, respectively, favor the generation of Th1 and Th2 cells (5, 25–30), although some in vitro observations are inconsistent with this conclusion (31).

Our group has previously shown that the number of responding CD4 T cells can affect the development of Th1 versus Th2 cells. Using an adoptive transfer system, we demonstrated that lethally irradiated mice reconstituted with a relatively low or high number of syngeneic splenocytes, and given a constant dose of SRBC, respectively, generate predominant Th1 and Th2 responses (32). The critical cell in the higher number of splenocytes, required to support the generation of Th2 cells, was shown to be a CD4 T cell, leading us to propose that low numbers of responding CD4 T cells give rise to Th1 cells, whereas higher numbers facilitate the development of Th2 cells. In another study, we demonstrated that C57BL/6 mice generate a Th1 response to SRBC when immunized with a low number of SRBC but generate a predominant Th2 response either when immunized with the same number of SRBC coupled to hen egg lysozyme (SRBC-HEL) or when immunized with a high dose of SRBC (33). It occurred to us that, by coupling HEL to the SRBC, we may have facilitated the interaction between HEL-specific and SRBC-specific CD4 T cells, thereby increasing the total number of responding CD4 T cells and...
causing the development of SRBC-specific Th2 cells. This possibility was consistent with the further finding that HEL transgenic mice, known to be tolerant of HEL at the level of CD4 T cells, mount a Th1 SRBC-specific response to a low-dose challenge of SRBC regardless of whether HEL was coupled to the SRBC.

Despite the findings described above, relatively little is known about the cellular and molecular mechanisms by which the number of responding CD4 T cells and the dose of Ag jointly affect the development of Th1 versus Th2 cells. To investigate these mechanisms, we developed an in vitro system that faithfully reproduces the in vivo dependency of the Th1/Th2 phenotype on the number of CD4 T cells and the amount of Ag present. We employed this in vitro system to ascertain whether CD4 T cell number affects the expression of different Th1/Th2-polarizing costimulatory molecules and receptors on the surface of T cells and APC; the role of Ag dose in determining the development of Th1 and Th2 cells with different numbers of responding CD4 T cells; whether B cells and DCs could both mediate the effect of CD4 T cell number on the development of Th1 and Th2 cells; and whether CD4 T cell number affected the production of pro-Th1 and pro-Th2 cytokines from APC and T cells.

Materials and Methods

Animals

BALB/c mice were obtained from Charles River Canada. DO11.10 mice were obtained from the animal colony of the College of Medicine, University of Saskatchewan, where they were bred from breeders purchased from Jackson ImmunoResearch Laboratories. BALB/c-I-J4m220H/j mice were purchased from Jackson ImmunoResearch Laboratories. All experiments were approved by the University of Saskatchewan’s Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Adoptive transfer and immunization

BALB/c mice were lethally irradiated with 750–850 R using a 60Co source (Gammaxell 220; Atomic Energy of Canada) and were both reconstituted and, when appropriate, immunized on the day of irradiation by a single i.v. injection at the tail.

Coupling of OVA to RBCs

A 5% solution of washed SRBC or chicken RBC (CRBC) in PBS was incubated with an equal volume of a 0.005% solution of tannic acid in PBS for 20 min at 37°C, mixing every 5 min. Tannic acid–treated SRBC were immediately washed once in PBS, resuspended to a 5% solution in PBS, and then incubated with 200 μg OVA for an additional 20 min at 37°C, mixing every 5 min. After four to six further washes in PBS, suspensions used for immunization were made by dilution in PBS.

Preparation of T cells and APC

Splenic DO11.10 CD4 T cells were purified by negative selection using the CD4+ T cell isolation kit II (Miltenyi Biotec). T cell–depleted splenocytes were obtained by depleting BALB/c splenocytes of T cells using CD90.2 microbeads (Miltenyi Biotec). DCs were purified from BALB/c splenocytes using pan DC microbeads (Miltenyi Biotec). B cells were purified from BALB/c splenocytes using a B cell isolation kit (Miltenyi Biotec). All isolations/depletions were performed as per the manufacturer’s instructions.

In vitro culture

Unless otherwise indicated, CD4 DO11.10 T cells were plated with 5 × 10^4 T cell–depleted BALB/c splenocytes, 0.3 μM OVA323–339 peptide, and 1 μg/ml LPS (Escherichia coli B4: 0111) in a single well of a 96-well V-bottom tray (Corning) for 4 d. Where indicated, functional grade anti-CD28 (37.51; eBioscience), functional grade anti-CD40L (1C10; eBioscience), or functional grade anti-OX40 (OX86; eBioscience), functional grade anti-CD40L (1C10; eBioscience), or functional grade anti-ICOS (7E.17G9; eBioscience) Ab were added to the cultures at the indicated concentration.

ELISPOT

OVA323–339–specific cytokine-producing cells were enumerated using the ELISPOT assay, as previously described (11). Peptide–specific cytokine spots were enumerated by subtracting the spots produced in wells without Ag from those produced in the presence of 0.3 μM OVA323–339 peptide. The number of spots generated in the presence of Ag was generally >50-fold greater than in the absence of added Ag. The total number of cytokine-producing cells per culture was calculated by multiplying the number of OVA323–339–specific cytokine–producing cells by the dilution factor. The Th1/Th2 phenotype of DO11.10 CD4 T cells was determined by comparing the ratio of IFN-γ–IL-4–producing cells.

CFSE labeling

A total of 1 × 10^7 freshly isolated CD4+ DO11.10 T cells was labeled with a final concentration of 5 μM CFSE in 1 ml PBS containing 5% FCS for 5 min at room temperature. The cells were then washed three times with 10 vol PBS containing 5% FCS to remove any residual dye.

Flow cytometry

Fluorochrome-coupled mAbs recognizing the DO11.10 TCR (KJ1-26; eBioscience), CD80 (16-10A1; eBioscience), CD86 (GL1; eBioscience), and CD19 (1D3; eBioscience) were employed. Data were collected using a Beckman-Coulter EpicsXL flow cytometer and analyzed using FlowJo software (Tree Star).

Statistical analysis

Significance was set at p < 0.05 and assessed by unpaired t tests or by ANOVA with post hoc analyses relying on Bonferroni’s multiple comparison test (GraphPad Prism v5.04). Data are represented as mean ± SD of the mean.

Results

The number of OVA-specific CD4 T cells affects the Th1/Th2 phenotype of the anti-SRBC effector CD4 T cells generated upon in vivo immunization with SRBC conjugated with OVA

Our group has previously shown that low and high numbers of responding CD4 T cells, activated under otherwise identical conditions, respectively, give rise to Th1 and Th2 cells. To further analyze the underlying mechanism, we reconstituted lethally irradiated BALB/c mice either with 2 × 10^7 syngeneic splenocytes alone or with additional splenocytes from DO11.10 transgenic mice, which contain a transgenic TCR specific for residues 323–339 of OVA in the context of self class II MHC molecules I-A^D. The reconstituted mice were also each given SRBC coupled to OVA (OVA-SRBC). Mice receiving the DO11.10 splenocytes were typically given 1 × 10^6 of such cells. This is approximately equivalent to the number of CD4+ T cells specific for a complex Ag in a naive mouse, which has been estimated at 1,000–10,000 (34, 35), given that TCR transgenic CD4+ T cells account for ~10% of lymphocytes in the spleens of DO11.10 mice (data not shown) and that only 10% of transferred CD4+ T cells persist in the host (36). The number of SRBC-specific IFN-γ– and IL-4–producing cells present in the spleen on day 7 was quantified using the ELISPOT assay. As shown in Fig. 1A, mice reconstituted with syngeneic splenocytes and challenged with 2 × 10^6 SRBC generated a predominant Th1 response to the SRBC, whereas mice reconstituted with both syngeneic and additional DO11.10 splenocytes generated a mixed Th1/Th2 response to SRBC. The critical cell type within the DO11.10 splenocytes, required to modulate the anti-SRBC response, was identified as a T cell, as mice reconstituted with syngeneic splenocytes and additional DO11.10 splenocytes, or purified DO11.10 T cells, both generated a Th1/Th2 anti-SRBC response when immunized with OVA-SRBC (Fig. 1B). Thus, it appears that the OVA-specific CD4 T cells can modulate a Th1 anti-SRBC response to a mixed Th1/Th2 mode induced by the SRBC-OVA challenge. This modulatory capacity of OVA-specific CD4 T cells on the generation of anti-SRBC CD4 T cells required not only the presence of OVA but that OVA be coupled to the SRBC, as no modulation of the anti-SRBC response was seen by
the OVA-specific CD4 T cells in the presence of both SRBC and OVA coupled to CRBC (OVA-CRBC) (Fig. 1C). Furthermore, both OVA-SRBC and OVA-CRBC were equally immunogenic as assessed by the generation of OVA-specific cytokine-producing cells (Fig. 1D). This suggests that only APC presenting both SRBC and OVA are able to mediate the effects of T cell number on the Th1/Th2 phenotype of the response.

The number of responding OVA-specific CD4 T cells and the dose of Ag determine, in an interdependent manner, the Th1/Th2 phenotype of the in vivo anti-SRBC response

The dose of Ag is known to affect the Th1/Th2 nature of the response. However, Ag titrations are typically done in the presence of a constant number of CD4 T cells, that is, in intact mice. Thus, we wished to assess how the dose of Ag affected the nature of the response when low or high numbers of CD4 T cells were available to respond. All lethally irradiated mice were reconstituted with 2 × 10^7 unprimed syngeneic spleen cells, but the dose of OVA-SRBC was varied in either the absence or presence of the indicated number of additional DO11.10 transgenic cells. As shown in Fig. 1E, mice reconstituted with syngeneic spleenocytes and given 2 × 10^7 OVA-SRBC generate a predominant Th1 anti-SRBC response, whereas the presence of additional DO11.10 spleenocytes resulted in a mixed Th1/Th2 response. Irradiated mice similarly reconstituted with the same number of syngeneic spleen cells, but immunized with a 10-fold lower dose of OVA-SRBC, barely generated a detectable response, and the presence of the additional DO11.10 transgenic cells resulted in a substantial and predominant Th1 response. This is in line with previous findings that CD4 T cell cooperation can facilitate the activation of Th1 cells (37–39). Importantly, increasing the number of DO11.10 cells transferred with a low dose of Ag once again facilitated the development of a Th2 anti-SRBC response (Fig. 1E). Thus, both the dose
and number of responding CD4+ T cells appear to jointly determine the Th1/Th2 phenotype of the response to SRBC in vivo.

**The number of DO11.10 T cells plated per well affects the in vitro generation of Th1 versus Th2 cells**

To further investigate how the number of responding CD4 T cells affects the Th1/Th2 nature of the response, we developed a parallel in vitro system, as we anticipated that it would provide us with a greater ability to assess the nature of the underlying mechanisms. As shown in Fig. 2A, we were able to replicate our in vivo observations as low numbers (10^3) of purified CD4 DO11.10 T cells gave rise, predominantly, to Th1 cells, whereas higher numbers (3 x 10^4) gave rise predominantly to Th2 cells when cultured under otherwise identical conditions. Very similar observations were made using Rag^-/- DO11.10 T cells (Fig. 2B), indicating that our observations made with Rag^-/- DO11.10 T cells are not due to the presence of previously activated T cells. We respectively refer to cultures containing 10^3 or 3 x 10^4 DO11.10 T cells as low- and high-density cultures. Analysis of the kinetics of the responses generated in low- and high-density cultures revealed that the Th1/Th2 phenotype of the DO11.10 T cells is already determined by day 2 (Fig. 2C). We were curious whether it was simply the number of cultured DO11.10 T cells that determined the Th1/Th2 nature of the response, or whether it was the ratio of DO11.10 T cells to APC that was affecting the response. To address this question, we cultured 3 x 10^4 DO11.10 T cells with 3 x 10^5, 1 x 10^5, and 3 x 10^4 APC. As shown in Fig. 2D, as the number of APC increased, there was a clear switch from a predominant Th2 response to a predominant Th1 response. Thus, it is the ratio of responding CD4 T cells to APC in this system that affects the Th1/Th2 nature of the response and not simply the total number of responding CD4 T cells.

**B cells, but not DCs, support the development of Th2 cells in high-density cultures**

The nature of the activating APC has been shown to affect the Th1/Th2 differentiation of naive CD4 T cells. In particular, IL-12 produced by DCs is considered by some to be required for the development of Th1 cells, whereas B cells are widely thought to exclusively facilitate the development of Th2 cells, despite the fact that B cells have also been shown to be involved in the generation of Th1 cells (40-42). Thus, we next examined the ability of DCs and B cells to support the respective development of Th1 and Th2 cells from low- and high-density cultures. As both B cells and DCs may be required for optimal responses, we employed these two APC types in various proportions. With this approach, we found that both B cells and DCs support the generation of Th1 cells in low-density cultures (Fig. 3A), whereas only B cells support the generation of Th2 cells in high-density cultures (Fig. 3B). We found that the inability of DCs to support a Th2 response was due to their production of the Th1-promoting cytokine IL-12, as adding neutralizing anti-IL-12 Ab to cultures containing a high density of T cells, DCs, and peptide resulted in the generation of Th2 cells (Fig. 3C). IL-12 was not essential for the generation of Th1 cells, as adding neutralizing anti-IL-12 Ab to low-density cultures, exclusively containing DCs as APC, did not affect the Th1 phenotype or number of effector cells generated (Fig. 3D). These findings are difficult to reconcile with the idea that DC-activated CD4 T cells, in the absence of IL-12, invariably give rise to Th2 cells by a default pathway (43) or that B cells exclusively support the development of Th2 cells. IL-12 was also dispensable for the potential significance of this finding to in vivo immune responses.

We address in the Discussion the potential significance of this finding to in vivo immune responses.

**FIGURE 2.** The ratio of T cells to APC affects the development of Th1 versus Th2 cells. (A) The indicated number of DO11.10 CD4 T cells was cultured with T cell-depleted BALB/c splenocytes as APC. (B) The indicated number of Rag^-/- DO11.10 T cells was cultured as in (A). (C) The indicated number of DO11.10 T cells was cultured as in (A). (D) A total of 3 x 10^5 DO11.10 T cells was cultured with the indicated number of T cell–depleted BALB/c splenocytes as APC. Cultures were harvested on day 4, unless otherwise indicated. Numbers above bars indicate the ratio of IFN-γ:IL-4–producing cells. The Th1/Th2 phenotype was determined by comparing the ratio of IFN-γ:IL-4–producing cells using an unpaired t test (A–C) or a one-way ANOVA with post hoc analysis relying on Bonferroni’s multiple comparison test. Data are representative of a minimum of three experiments performed with three independent culture wells per group and are represented as mean ± SD of the mean. Statistical significance was determined using an unpaired t test. **p < 0.01, ***p < 0.001, ****p < 0.0001.
the development of a Th1 response in low-density cultures containing T cell–depleted splenocytes as APC and did not increase the Th2 phenotype of high-density cultures containing T cell–depleted splenocytes as APC (Fig. 3C, 3D).

T cell–derived IL-4 is critical for the development of Th2 cells in high-density cultures

IL-4 is considered to be critical for the development of Th2 cells; however, as indicated in the Introduction, its source is controversial. We found that IL-4 is critical for the development of Th2 cells in our high-density cultures, as the addition of anti–IL-4 leads to the development of Th1 cells (Fig. 3E). The source of IL-4 in high-density cultures appears to be the T cells themselves, as $3 \times 10^4$ T cells cultured with IL-4–deficient APC still gave rise to Th2 cells (Fig. 3E).

Greater ligation of CD28 is required for the development of Th2 cells than for Th1 cells

OX-40 (13, 14), CD40 (44), ICOS (15), and CD28 (17, 45–47) have been implicated in driving the Th1/Th2 differentiation of newly activated CD4 T cells. By modulating OX40/OX40L, CD40/CD40L, and ICOS/ICOSL interactions through the addition of agonistic or antagonistic Abs, we found that these molecules were not critically involved in determining the Th1/Th2 phenotype of activated T cells in low- and high-density cultures (Fig. 4). Although the addition of anti-CD40L did not affect the phenotype of the response, it appeared to have decreased the activation efficiency of the T cells in culture, as evidenced by the reduced number of IFN-γ–producing cells without more IL-4–producing cells. In contrast, we found that the generation of Th2 cells depends on the amount of stimulation that the T cells...
received through CD28, as the addition of agonistic anti-CD28 Ab greatly increased the development of Th2 cells in low-density cultures (Fig. 5A) and slightly increased the number of IL-4–producing cells in high-density cultures (Fig. 5B). Moreover, the addition of CTLA4-Ig to high-density cultures led to the development of Th1 cells (Fig. 5C). The effect of anti-CD28 on the development of Th2 cells was independent of cellular proliferation as CD4 T cells from low-density cultures, receiving anti-CD28 or isotype-matched Ab, proliferated to the same extent, as assessed by the dilution of CFSE (Fig. 5D). Thus, the consequence of CD28 stimulation on the ratio of Th1 to Th2 cells is most likely due to a direct effect on the stimulated T cell, rather than to indirect effects arising from greater proliferation and thus increased numbers of CD4 T cells. This observation and inference is concordant with the finding that ligation of CD28 can stimulate IL-4 production by CD4 T cells without affecting their level of cell division (48).

**Ag dose modulates the Th1/Th2 phenotype of T cells only in high-density cultures**

As shown in Fig. 5E, the dose of Ag and the number of responding CD4 T cells coordinately determine the Th1/Th2 nature of the in vivo anti-SRBC response. We show in this study that parallel events occur in our in vitro system. High-density cultures given relatively high doses of Ag supported the predominant development of IL-4–producing CD4 T cells, whereas those given lower doses of Ag predominantly gave rise to Th1 cells (Fig. 6A). In contrast, low-density cultures gave rise to Th1 cells regardless of the concentration of OVA323–339 peptide employed to activate the T cells (Fig. 6B). The effect of Ag dose in high-density cultures appears to be mediated by the level of CD28 ligation received by the DO11.10 T cells as the addition of anti-CD28 to high-density cultures, given lower doses of Ag, greatly increases the Th2 component of the response (Fig. 6C).

**Greater expression of CD86 by T cells, but not Ag-presenting B cells, is associated with the development of Th2 cells**

As shown in Fig. 6D, the number of OVA-specific CD4 T cells was significantly lower in cultures that contained B cells from high-density cultures, which express greater levels of CD80 and CD86 on their surface, as compared to cultures that contained B cells from low-density cultures. This finding indicates that greater expression of CD86 by T cells, but not Ag-presenting B cells, is associated with the development of Th2 cells. The finding that the level of CD28 stimulation controls the development of Th2 cells in our in vitro system initially suggested to us that greater levels of CD80 and CD86 might be expressed on APC from cultures that support the generation of Th2 cells. However, this does not seem to be the case. First, despite the ability of low- and high-density cultures, containing 0.3 μM OVA323–339, to respectively support the generation of Th1 and Th2 cells, B cells from high-density cultures do not express greater levels of CD80 or CD86 on their surface, at any point during

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**FIGURE 4.** Assessing the impact of OX40/OX40L, CD40/CD40L, and ICOS/ICOSL interactions on the Th1/Th2 differentiation of DO11.10 T cells. Low (A–C) and high (D–F)-density cultures, containing T cell-depleted BALB/c splenocytes as APC, were given either agonistic anti-OX40 Ab, antagonistic anti-CD40L Ab, or antagonistic anti-ICOS Ab. Cultures were harvested on day 4, and the number of IFN-γ– and IL-4–producing cells was quantified by ELISPOT. Data are representative of three experiments performed with three independent culture wells per group and are represented as mean ± SD of the mean.
culture, than B cells from equivalent low-density cultures (Fig. 7A). Second, although the dose of Ag affects the Th1/Th2 differentiation of DO11.10 T cells in high-density cultures, the expression of CD80 and CD86 by B cells in these cultures is identical (Fig. 7B). We restricted our analysis of APC to B cells because, numerically, they are the major APC type in T cell–depleted splenocytes and because we have established that only B cells can mediate the effect of T cell number on the development of Th2 cells in our system (Fig. 3B). Activated T cells are known to express CD80 and CD86. Given that there are 30-fold more DO11.10 T cells in high-density cultures than in low-density cultures, we considered it possible that the more robust CD28 signal provided to T cells in high-density cultures, given moderate to high doses of OVA323–339, might be due to T cell–T cell interactions. Indeed, we find that activated DO11.10 T cells from low- and high-density cultures express both moderate amounts of CD86 and low levels of CD80 (Fig. 7C), particularly at day 1 of culture, which is when the Th1/Th2 phenotype of the cultured cells is determined (Fig. 2C). The hypothesis that T cell–T cell interactions via CD28-CD86 contribute to the development of Th2 cells is supported by our finding that DO11.10 T cells from high-density cultures, given low doses of Ag, which develop into Th1 cells (Fig. 6A), express much less CD86 than DO11.10 T cells from high-density cultures given higher doses of OVA323–339, which facilitates the development of Th2 cells (Fig. 7D).

**Discussion**

In this study, we have detailed observations that demonstrate that CD4 T cell interactions determine the Th1/Th2 phenotype of the effector CD4 T cells generated. Both in vitro and in vivo, in the presence of sufficient Ag, low numbers of responding CD4 T cells give rise to Th1 cells, whereas higher numbers give rise to Th2 cells. Ag dose also plays a role in that low and high doses of Ag, in the presence of a relatively high number of CD4 T cells, respectively, lead to the generation of Th1 and Th2 cells. We show in the in vitro system that the development of Th2 cells at the expense of Th1 cells was critically dependent upon more robust CD28-mediated signaling. Thus, the generation of Th2 cells could be preferentially blocked by CTLA4-Ig, and agonistic anti-CD28 Ab can modulate the predominant generation of Th1 cells to the predominant generation of Th2 cells. The conclusions drawn from this in vitro analysis allow us to understand a number of in vivo observations, as we now discuss.

The ability of CD28 to drive the development of Th2 cells in vivo is well established. For example, in CD28-deficient (CD28−/−) mice, the basal level of Ig is ∼20% of normal mice. Interestingly, the majority of the circulating Ab in CD28−/− mice is of the IgG2a isotype, indicative of a predominant Th1 response, suggesting a differential role for CD28 in generating Th2 responses (16). Indeed, although CD28−/− mice mount delayed-type hypersensitivity responses against lymphocytic choriomeningitis virus that are identical in magnitude to that of wild-type mice, they have significantly reduced levels of neutralizing Ab in response to infection with vesicular stomatitis virus (16). Additional evidence for the role of CD28 in Th2 responses comes from BALB/c mice infected with *Leishmania major*. The administration of CTLA4-Ig decreases the parasite load at the site of injection.
The quantitative viewpoint, which has been presented in this work, for how the Th1/Th2 phenotype of activated CD4 T cells is determined allows one to understand previously inexplicable findings and provides a framework for the novel treatment of certain chronic diseases. For example, BALB/c mice are denoted as susceptible to L. major, as on a standard challenge with 10^5 parasites; they generate a predominant Th2 response, which is associated with progressive disease. However, there are two ways of infecting these susceptible mice such that they make a sustained Th1 response and resist the parasite. One means is by infecting with 10^6 parasites and partially depleting CD4 T cells close to the time of infection (49), and the other is by infecting susceptible mice with 10^2 parasites rather than with the standard challenge of 10^5 (28). Our proposed mechanism provides an explanation for such means of influencing the Th1/Th2 phenotype of primary and ongoing immune responses (50, 51), as decreasing the number of CD4 T cells or lowering the amount of Ag will decrease the Ag-mediated CD4 T cell interactions needed to generate Th2 cells, and so modulate the response toward a Th1 mode. In contrast, understanding the efficacy of these two maneuvers is problematic in terms of those models in which an activated APC instructs a single CD4 T cell as to the Th1/Th2 phenotype of its progeny.

There are two potential mechanisms by which higher numbers of T cells could receive greater CD28 stimulation. The B7 molecules bound by CD28 could be on the APC or other CD4 T cells; in the latter case, two CD4 T cells acting around one APC could interact. Our observations do not rigorously distinguish between these possibilities, but certain observations are more readily explained by the second possibility of direct CD4 T cell interactions. For example, the increased stimulation of T cells through CD28 in high-density cultures could not be readily explained at the APC level, as B cells, the APC type able to mediate the development of Th2 cells in our high-density cultures (Fig. 3B), did not express greater levels of B7 on their surface than those in low-density cultures. This finding led us to consider the alternative possibility that T cells are interacting directly through B7/CD28 interactions. The plausibility of this possibility is based on the facts that activated CD4 T cells express B7 molecules on their surface and that there are many more CD4 T cells under conditions favoring the generation of Th2 over Th1 cells. Moreover, the greater expression of CD86 molecules on CD4 T cells, and not the APC, in high-density cultures containing higher amounts of peptide, than in similar cultures containing lower amounts of peptide, is consistent with the idea that CD4 T cells are interacting directly.

It is becoming better appreciated that, during the course of their activation, T cells form substantial homo- and heterotopic clusters around the activating APC and that direct T cell–T cell signaling occurs within these clusters (52–56). In the context of our study, we believe that critical CD28-B7 interactions may be occurring within these T cell clusters. We envisage that as the number of CD28-B7 interactions passes a threshold number, the level of CD28 stimulation experienced by the T cells may become sufficient to drive their production of IL-4, thereby inducing the development of Th2 cells (48). As we have outlined in this study, there are a number of variables that can potentially affect the degree of T cell interactions. The number of APC-presenting Ag is one such variable. When a relatively small number of APC present Ag to a relatively large number of responding T cells, the T cells are forced to interact with this limited number of APC; therefore, the degree of interaction between these T cells is at its greatest, and a Th2 response develops. However, as the number of APC-presenting Ag increases, there is a diluting-out effect that occurs, thereby reducing the degree of T cell–T cell interactions that occurs, thus leading to a Th1 response. The dose of Ag is the second such variable. As we have shown in this study, T cells exposed to a low dose of Ag express much less B7 molecules on their surface than T cells exposed to greater amounts of Ag. Therefore, APC presenting a low dose of Ag will induce the ex-
expression of less B7 by the T cells than APC presenting greater doses of Ag. We envisage, as a consequence, that T cells interacting with APC presenting a low dose of Ag will be impaired in their ability to form T cell–T cell interactions, relative to those activated by APC presenting greater amounts of Ag, and will therefore develop into Th1 cells, even if a relatively large number of CD4 T cells is responding.

We also wish to address our observations on the role of different types of APC in the envisaged CD4 T cell interactions. In most cases, Ag presentation by DCs is considered to be sufficient for the activation of naive CD4 T cells. However, there is strong evidence that B cells can work in concert with DCs to generate optimal CD4 T cell responses. For example, CD4 T cell responses generated to a number of Ags are impaired in B cell–deficient mice (57–60). In addition, by employing TCR transgenic CD4 T cells specific for an I-E–restricted peptide and different transgenic mice that express I-E only on DCs, B cells, or both, Kleindienst and Brocker (61) have demonstrated that, although DCs are sufficient to activate
the transgenic T cells, the presence of I-E on both B cells and DCs is required for maximal T cell responses. The findings of Kleidinst and Brocker are well supported by many imaging studies that demonstrate that, in lymph nodes, following their activation in the T cell zone, CD4 T cells migrate to the B cell follicle, where they interact with B cells that have migrated to the follicular edge (62) and continue to proliferate (62–65). We propose that it is during their interaction with B cells that interactions among CD4 T cells contribute to the determination of their phenotype. Clearly, this can only occur if CD4 T cells that have interacted with an IL-12–producing DC are not irreversibly Th1 polarized but can develop into, or give rise to, Th2 cells once sufficient CD28 stimulation is provided in the absence of IL-12. This may occur when large numbers of responding T cells interact around a B cell. The idea that the Th1/Th2 phenotype is plastic rather than static is supported by a growing number of reports describing the ability of both Th1 and Th2 cells to change their phenotype (66).

We think that B cells are critical APC mediating T cell–T cell interactions based on our observation that only B cells are able to support the development of Th2 cells in vitro and that SRBC must be directly coupled to OVA for DO11.10 T cells to affect the SRBC response in vivo. The requirement for SRBC to be coupled to OVA is best explained if an Ag-specific B cell is the critical APC mediating T cell–T cell interactions, as a non-Ag–specific APC, such as a DC best explained if an Ag-specific B cell is the critical APC mediating T cell–T cell interactions based on our observation that only B cells are able to obviate the T cell–T cell interactions. This may occur when large numbers of responding T cells interact around a B cell. The idea that the Th1/Th2 phenotype is plastic rather than static is supported by a growing number of reports describing the ability of both Th1 and Th2 cells to change their phenotype (66).

Disclosures

The authors have no financial conflicts of interest.

References


