Enhancement of Antigen-Specific Immunoglobulin G Responses by Anti-CD48

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Key Words

Cytokines · Immune response · CD48 · CD244 · Natural killer cells · B cells · NP-Ficol · Interferon- γ · T-independent antigen

Abstract

CD48 is a glycosylphosphatidylinositol-anchored protein expressed ubiquitously on many cell types. Despite the poor ability to signal on its own, CD48 can activate cells via interaction with its counter receptors CD2 and CD244 as well as influence the function of other cell surface molecules by costimulatory activities. We show, herein, that injection of anti-CD48 antibodies into mice can augment the antibody response to a T-independent antigen, NP-Ficol, that is representative of antigenic determinants expressed on the surface of various pathogens, such as *Streptococcus pneumoniae*. In C57BL/6 mice, enhancement of the response is dependent on natural killer (NK) cells as well as on the presence of CD2 and CD244, ligands for CD48, suggesting a requirement for direct interaction between NK and B cells. Interestingly, in this case, despite a similar augmentation by anti-CD48 in BALB/C mice, the response is independent of NK or T cells, suggesting that help for this response can be derived from other innate cell types. These results provide a pathway by which CD48, when appropriately activated, can influence the course of an antigen-specific antibody response via the innate system.

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Introduction

CD48 is a glycosylphosphatidylinositol-anchored protein expressed ubiquitously on many cell types. Early experiments examining its function showed that CD48-deficient mice have impaired T-cell responses upon activation [1], hypothesized to be due to alterations in costimulatory activity when cells are stimulated via the T-cell receptor [2–4]. Presumably, CD48 functions by interaction with antigen-presenting cells (APCs) via CD2, a receptor for CD48. However, interestingly, mice lacking CD2 do not exhibit similar deficiencies [5], possibly because CD48 can also interact with another receptor, CD244. Although CD2 is expressed on B as well as on T cells, CD244 expression is more restricted, being present on all natural killer (NK) cells but only on a subpopulation of CD8 T cells and in low density on other cell types.

CD48 itself has also been shown to have receptor activity. Crosslinking of CD48 on NK cells leads to aggregation of CD244 on the same cell, resulting in phosphorylation of tyrosine residues and subsequent activation of cytokine production [6]. In vitro experiments utilizing interleukin (IL)-2-propagated NK cells have indicated that stimulation of B cells by NK cells requires the presence of CD48 on B cells and leads to interferon (IFN)- γ -independent activation of downstream exons of the immunoglobulin (Ig) locus as revealed by germline transcriptions [7, 8]. However, this activation does not result

in productive DNA recombination, leading to the expression of functional transcripts unless the cells are also stimulated via the B-cell receptor [8]. On mast cells, CD48 itself may have receptor activity as well [9].

In order to assess the relevance of these *in vitro* experiments, we have examined the effect of anti-CD48 stimulation on the *in vivo* response to a TI-II antigen, NP-Ficoll, which can induce Ig production independent of cognate help from T cells but can be modulated by NK cell activity [10]. The results show that injection of anti-CD48 antibodies into C57BL/6 (B6) mice prior to antigenic stimulation results in enhanced expression of IgG1 and IgG2c, responses that are otherwise relatively low. The increase is dependent on the presence of NK cells as well as on CD2 and CD244 expression. Surprisingly, despite the induction of a similar enhancement by anti-CD48 in BALB/C mice, NK cells do not appear to be involved. These results show that CD48 can participate in a T-independent antigen-specific response and further implicates the non-specific help exerted by NK cells and/or other innate cells for specific immune responses.

Materials and Methods

Mice

C57BL/6, BALB/c, BALB.NK1.1 [11], CD2^{ko}, 2B4^{ko} (CD244) and CD2/2B4^{ko} [12] were bred and maintained under specific pathogen-free conditions at the UT Southwestern Animal Resources Center.

In vivo Cell Type-Specific Depletions

B6 and BALB.NK1.1 mice were depleted of NK cells by intraperitoneal injection of 75 µg anti-NK1.1 antibodies on days -5 and -2 before treatment with anti-CD48. This treatment did not deplete NK T cells and kept NK cells depleted for more than 2 weeks, as determined by the retention of CD3⁺NK1.1^{lo}NKp46⁻ cells in splenocytes. NK cells in BALB/C mice were depleted with a single injection of 20 µl of anti-asialo GM1 antibodies on day -1. T or T regulatory (T_{reg}) cells were depleted with anti-CD4 (125 µg/mouse), anti-CD8 (100 µg/mouse) or anti-CD25 (1 mg/mouse) 2 days prior to the injection of anti-CD48. Effective depletion of each reagent was confirmed by FACS analysis of both peripheral blood lymphocytes (PBLs) and splenocytes. Anti-IFN-γ was injected (75 µg/mouse) 2 days prior to and 1 day after the injection of anti-CD48.

Antibodies

Hamster anti-CD48, control hamster Ig, rat anti-CD2 and control rat Ig were purchased from Biolegend. Anti-CD244-1 and CD244-2 were purchased from BD Pharmingen. Mouse anti-NK1.1, rat anti-CD4 (GK1.5) and rat anti-CD8 (YTS) were purified from hybridoma culture supernatants using Gamma Bind (Pharmacia Fine Chemicals). Rabbit anti-IFN-γ from serum was similarly purified. Anti-asialo GM1 was purchased from Wako

Chemicals. Anti-CD25 (PC61) was a kind gift from Dr. Bruce Blazer. All staining reagents, including anti-CD19, anti-CD4, anti-CD8, anti-CD3, anti-CD69, anti-B220, anti-CD86 and SA-PCP were purchased from eBioscience or Biolegend. Anti-CD25 (7D4) was purchased from BD Biosciences. Goat anti-NKp46 and donkey anti-goat Ig was purchased from R&D Systems. Horseradish peroxidase-conjugated antibodies for ELISA were purchased from Southern Biotechnology. All antibodies were titrated before use.

FACS Analysis

PBLs were collected on the FACScan flow cytometer and analyzed on CellQuest (BD Biosciences).

Anti-CD48 Treatment and Immunization

Two days prior to immunization, groups of 5 or 6 animals were treated with anti-CD48 dissolved in PBS. Titrations ranging from 25 to 200 µg/mouse yielded similar results. Therefore, most experiments utilized 50 µg/mouse. F(ab')₂ fragments of anti-CD48 (a kind gift from Dr. Michael Bennett) also yielded similar activation patterns. Serum was collected from all animals at days 0, 7 and 12 or 13 after injection with 40 µg/mouse NP-Ficoll (Solid-Phase Sciences) dissolved in PBS or, as indicates for one experiment, in 100 µl RIBI (Sigma).

Antibody Responses

Ig ELISA was performed as previously described [13]. The isotypes and the subclasses of bound Ig were detected by horseradish peroxidase-conjugated, isotype-specific anti-mouse Ig antibodies (Southern Biotechnology) and developed with the substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma). Results shown represent dilutions which were most sensitive to changes in antibody levels. ELISA results were evaluated by an automated ELISA reader (Molecular Diagnostics) at an optical density of 405 nm. Statistical significance is indicated only when *p* values were ≤0.05, obtained by 1-tailed unpaired Student's *t* testing for the probability that the two indicated groups were similar. Similar levels of significance were obtained with analysis by the Wilcoxon 2-sample test.

Results

In vivo Stimulation of NK Cells by Anti-CD48

Our previous findings showed that ligation by anti-CD48 can induce the transcription of IL-13 mRNA in IL-2-propagated NK cells and that this stimulation is correlated with a relocation of one of the two ligands for CD48 and CD244, on the same cell [6]. In order to determine if anti-CD48 can stimulate NK cells *in vivo*, PBLs were isolated on consecutive days after injection of the antibody into B6 mice. Figure 1b shows, in a representative experiment, that CD69 expression on gated NK cells (R2; fig. 1a) was significantly increased by day 2 after injection. NK cell sizes were enhanced at an even earlier time (fig. 1e). In contrast, neither peripheral blood B nor T cells exhibited detectable activation (fig. 1b). Injection of anti-

Fig. 1. a–d Activation of NK and T cells with anti-CD48. PBLs isolated from individual B6 mice prior to and on days 1 and 2 after the injection of anti-CD48 antibodies (50 μ g/mouse) were stained and assessed by FACS analysis. Expression of CD69 (**b**) and CD244 (**d**) on NK (R2 in **a**), T (R3 in **a**) or B cells (CD19⁺) on day 1 (open symbols) and on day 2 (closed symbols) are shown.

Another group of B6 mice were injected with anti-CD2 antibodies, and CD69 expression was determined in the same way (**c**). MFI = Mean fluorescence intensity. **e** Size profile of gated NK cells at the times indicated. Results are representative of two independent experiments.

CD2 antibodies at the same concentration (50 μ g/mouse) did not induce significant increases in CD69 expression on NK cells although T cells were activated. Interestingly, the expression of CD244 on NK cells was also significantly upregulated upon in vivo stimulation of CD48 but not by anti-CD2 (fig. 1d), whereas the expression of CD2, the other ligand for CD48, was not altered (data not shown). Therefore, anti-CD48 can activate NK cells that have not been propagated in vitro.

Enhancement of Isotype Switching via CD48

To assess the functional significance of the activation of NK cells by anti-CD48, the antibodies were injected prior to antigenic challenge by the T-independent antigen NP-Ficoll. The T-independent type II response was chosen to restrict the effect to that of direct crosslinking of the B-cell antigen receptor without T-cell stimulation via major histocompatibility complex class II restricted help [for a review, see 14]. Figure 2a and b indicates in a

Fig. 2. NK cell-mediated enhancement of antigen-specific switch to downstream isotypes by anti-CD48 and dependence on NK cells. **a** B6 mice were injected with either control hamster Ig or anti-CD48 antibodies (aCD48; 50 μ g/mouse) 2 days prior to immunization with NP-Ficoll. Subclass-specific antigen-specific antibodies were determined on days 0, 7 and 12. **b** Mean responses of each group are representative of two independent experiments. **c** B6 mice (5–6 animals per group) were injected with either control mouse Ig or anti-NK1.1 antibodies (aNK1.1) on days

–5 and –2 prior to the injection of anti-CD48 antibodies. Two days later, all animals were immunized with NP-Ficoll, and serum was collected on days 7 and 12. Mean responses on day 12 are representative of two independent experiments. **d** Groups of B6 mice were injected with control Ig or anti-CD48 on day –2, or with anti-IFN- γ (aIFN- γ ; 60 μ g/mouse) on days –3 and –1 in addition to anti-CD48 prior to immunization with NP-Ficoll on day 0. Mean responses on day 12 are representative of two independent experiments.

representative experiment that this stimulation resulted in an average more than 2-fold enhancement in the IgG1 response and in a somewhat lower stimulation of the IgG2c and IgG3 responses in B6 mice. The kinetics indicate that the IgM responses occur at earlier times, but in

some animals, can diminish with time. The responses of the other classes were relatively low at day 7 but peaked at day 12 or 13. The extent of enhancement of each isotype varied between experiments and was not dependent on the concentration of anti-CD48 injected, as similar

results were obtained from 25 to 200 μg per mouse (data not shown).

We next investigated the role of NK cells in this enhancement since they have been shown to be responsible for the augmentation of the IgG2a/c responses to both TI-I and TI-II antigens upon stimulation by poly(I:C). NK cells were depleted prior to the injection of anti-CD48 and the subsequent challenge with NP-Ficoll. As shown in figure 2c, in the absence of NK cells, enhancement of both IgG1 and IgG2c, but not IgG3, responses were completely eliminated.

The Effect of Anti-CD48 Is Mediated via CD2 and/or CD244

IL-2-propagated NK cells have been shown to be able to activate the germline transcription of IgG2a required for initiation of switch recombination to the downstream isotype [7]. Contact between the two cells is required for the activation that is mediated via CD2 and/or CD244 on NK cells interacting with CD48 on B cells. In order to determine if the activation by NK cells *in vivo* also initiates switch recombination via CD2, we examined the response of CD2^{ko} mice. Figure 3a shows in a representative experiment that injection of the antibody failed to enhance any isotype-specific response to NP-Ficoll.

The absence of an apparent augmentation of isotype switch by anti-CD48 in CD2^{ko} mice could be due either to the inability of stimulated NK cells to activate B cells or to inadequate stimulation of NK cells. Since anti-CD48 induces the relocation of CD244 *in vitro* as well as an increase in CD244 expression, it is possible that activation via this route requires the presence of CD244. Therefore, we examined the ability of anti-CD48-stimulated CD244^{ko} mice to augment the NP-Ficoll response. Figure 3b shows that in the absence of this ligand, the enhancement was significantly diminished. A similar lack of enhancement was found when CD2/CD244^{ko} mice were stimulated with anti-CD48 (fig. 3c). In addition, figure 3d shows that in the absence of CD244, NK cells did not up-regulate CD69 expression upon stimulation by anti-CD48. These results correspond to our *in vitro* findings showing that activation of IL-2-propagated NK cells by anti-CD48 as measured by induction of IL-13 mRNA requires the presence of CD244. Thus, the lack of an effect on B-cell responses by anti-CD48 in CD2^{ko} mice is most likely due to the paucity of activated NK cells.

In view of our finding that CD244 is upregulated on NK cells upon stimulation by anti-CD48, it is possible that direct activation by anti-CD244 may increase the response to antigenic stimulation. Therefore, we injected

mice with allotype-specific anti-CD244 and, as a control, also injected antibodies specific for the BALB/C allotype into B6 mice. As shown in figure 3e, neither antibodies altered the response when compared to non-stimulated animals. Therefore, stimulation via the CD244 receptor, on its own, does not impact the response to a T-independent antigen.

Role of IFN- γ

So far, our results indicate that stimulation with anti-CD48 can activate NK cells and that the B-cell response to antigen can be enhanced, as revealed by increased switch recombination to downstream isotypes. Our *in vitro* experiments showed that activation of B cells requires direct interaction between NK and B cells; however, the extent of activation may be tempered by cytokines, especially IFN- γ , secreted by NK cells. To evaluate the role of IFN- γ , anti-IFN- γ antibodies were injected prior to stimulation of the animals by anti-CD48. Figure 2d shows that enhancement of IgG2c and IgG3 production was virtually eliminated in the absence of IFN- γ . However, IgG1 secretion was further enhanced. These results suggest that much of the enhancement by NK cells is mediated via a cytokine circuit which is mainly driven by IFN- γ which can, in turn, effectively inhibit the IgG1 response. Thus, enhancement of IgG1 could have been even greater in the absence of IFN- γ -mediated inhibition.

Activation by Anti-CD48 in BALB/C Mice

The IgG1 response is generally higher in BALB/C than in B6 mice. Therefore, we tested the activity of anti-CD48 in BALB/C mice to determine whether a greater enhancement can be achieved. Figure 4a shows that anti-CD48 can enhance both the IgG1 and IgG2a response. However, taking an average of 3 independent experiments, the extent of the enhancement was not significantly greater than the responses in B6 mice. In order to test the effect of depletion of NK cells in BALB/C mice, we utilized BALB.NK1.1 [11], in which the Nkrp1b gene has been replaced by the B6 Nkrp1d allele. Thus, depletion of NK cells can be readily achieved in these mice by injection of anti-NK1.1 antibodies and, as shown in figure 4b, NK cells remain depleted up to as long as 2 weeks. Interestingly, neither IgG1 nor the IgG2a enhancement was reduced by this treatment. Similar results were obtained when BALB/C mice were used and NK cells were depleted by anti-asialo GM1 treatment (data not shown). Evaluation of CD69 expression also showed that NK cells were not activated by anti-CD48 stimulation, although, in contrast to the effect in B6 mice, T-cell CD69 expression was significantly increased (fig. 4c).

Fig. 3. Role of CD2 and CD244 in the effect of anti-CD48 (aCD48). **a–c** CD2^{ko} (**a**), CD244^{ko} (**b**) or CD2/CD244^{ko} (**c**) mice were stimulated with anti-CD48 (50 μ g/mouse) for 2 days before stimulation with NP-Ficoll. On day 12 after antigen challenge, serum was collected and assessed for antigen-specific Ig. **d** Prior to (open symbols) and on day 2 (closed symbols) after anti-CD48 injection,

PBLs obtained from each of 4 CD244^{ko} mice was assessed for CD69 expression on NK and T cells. **e** Groups of B6 mice were injected with allotype-specific anti-CD244-1 or CD244-2 (aCD244-1 and aCD244-2, respectively; 100 μ g/mouse) 2 days prior to immunization with NP-Ficoll. Day 12 Ig responses were assayed together with control, non-stimulated animals.

Fig. 4. Activation of NK and T cells by anti-CD48 (aCD48) in BALB/C mice. **a** Groups of 5–6 BALB.NK1.1 mice were injected with control antibody or with anti-CD48 2 days before immunization with NP-Ficoll. Another group was injected with anti-NK1.1 (aNK1.1) on days –5 and –2 prior to the injection of anti-CD48. Isotype-specific, antigen-specific Ig in serum was determined on day 7 (data not shown) and on day 13. **b** The extent of NK cell depletion was determined by FACS analysis of PBLs 14 days after injection. **c** CD69 levels on NK and T cells were determined on day 0 (open symbols) and day 2 (closed symbols) after anti-CD48 injection. Data presented in **a** and **c** are representative of two independent experiments. **d** BALB.NK1.1 mice were injected with anti-CD4 (aCD4; 125 μ g/mouse) 2 days prior to immunization with NP-Ficoll in either PBS or RIBI. Serum Ig levels were determined on day 12. **e** The extent of depletion by anti-CD4 and anti-CD8 (100 μ g/mouse) was determined by FACS analysis

of PBLs 2 days after injection. **f** Groups of BALB.NK1.1 were treated as in **a**, but instead of anti-NK1.1, one group was injected with a combination of anti-CD4 (aCD4) and anti-CD8 (aCD8) 2 days prior to the injection of anti-CD48 (aCD48). Serum Ig levels were determined on day 7 (data not shown) and on day 12 after antigen injection. Data are representative of two independent experiments. **g** Groups of BALB.NK1.1 mice were injected with either control antibody or anti-CD25 (aCD25; 1 mg/mouse) 2 days before immunization with NP-Ficoll. Mean responses on day 12 are representative of two independent experiments. **h** CD25⁺ cell depletion was assessed by FACS analysis of PBLs using anti-CD25 directed against an alternate determinant. **i** Groups of BALB/C mice were treated as in **a**, but instead of anti-NK1.1, one group was injected with anti-CD25 (aCD25) 2 days prior to the injection of anti-CD48 (aCD48) and 4 days prior to immunization with NP-Ficoll.

Fig. 5. Scenario for the most likely interaction between NK and B cells induced by anti-CD48 (aCD48). Whereas some B cells can be stimulated by a TI-II antigen to produce a basal level of Ig, the extent of the response is low. Upon stimulation by anti-CD48, NK cells are activated, resulting in an alteration of CD244 which is required before the CD2 expressed on NK cells can become effective stimulators (NKa) of B cells via CD48. Activated NK cells can then stimulate B cells that had not been activated by antigen alone. These partially activated B cells (Ba) can proceed to Ig secretion upon encounter with antigen. Whereas IFN- γ is required for the increase in IgG2c secretion, another cytokine or, more likely, the direct activation by NK cells is sufficient for the augmentation of IgG1 secretion. This enhancement is usually somewhat inhibited in the presence of IFN- γ production by NK cells but can be revealed by removal of IFN- γ .

Therefore, it was important to evaluate the role of T cells in the enhancement by anti-CD48. Since the involvement of T cells in the response to TI antigens is controversial [15, 16], we first confirmed the possible contribution of T cells in the response of BALB/C mice to NP-Ficoll. CD4 T cells were depleted prior to the injection of antigen. Figure 4d shows that anti-CD4 depletion did not affect the basal response. However, when the antigen was injected in the presence of an adjuvant (RIBI), which significantly augmented the response, prior depletion of CD4⁺ cells can reduce the increase. This effect is likely due to CD4⁺ T-cell-derived cytokines induced by adjuvant that can augment the response. The basal response was not affected by anti-CD8 depletion (data not shown), although effective depletion by injection of the two antibodies was confirmed (fig. 4e). To determine the role of T cells in the enhancement by anti-CD48, animals were injected with a combination of anti-CD4 and anti-CD8 prior to stimulation by anti-CD48. Figure 4f shows in a representative experiment that in contrast to the effect of

CD4⁺ T-cell depletion on the enhancement by adjuvant, enhancement by anti-CD48 was not affected.

Because the depletion of T cells appeared to increase, but not to a significant degree, the enhancement by anti-CD48, a possible role of regulatory T cells is suggested. Therefore, we treated the animals with anti-CD25 antibodies (PC61) prior to immunization with NP-Ficoll. First, figure 4g shows that this depletion did not affect the basal response to the antigen despite the virtually complete elimination of CD25⁺ cells (fig. 4f). Second, to test if T_{reg} cells are affected by anti-CD48, animals were depleted in a similar manner prior to stimulation by anti-CD48 and antigenic challenge. The absence of T_{reg} cells in this case did not affect the enhancement of the response (fig. 4i). We also found that anti-CD48 did not alter the level of CD25 expression on CD4 T cells (data not shown).

Discussion

We have shown in this report that injection of anti-CD48 into B6 mice can result in activation of NK cells as manifested by an increase in size scatter as well as in CD69 expression. Interestingly, CD244 expression was also enhanced upon injection of anti-CD48. The change in CD244 expression may be related to our previous finding of colocalization of CD244 upon ligation of activated NK cells. As far as can be determined, the injection did not result in alterations of activation markers in either B or T cells, confirming previous findings that purified B cells were not activated by anti-CD48 in vitro [17]. Nonetheless, upon immunization with a T-independent antigen, the IgG responses were significantly enhanced. Importantly, the enhancement of both IgG1 and IgG2c can be eliminated by depletion of NK cells, and therefore, it is likely to be a functional consequence of the activation of NK cells.

The kinetics of the IgM responses (fig. 1a), although more rapid in onset, were not always maintained over time; therefore, any effect of anti-CD48 cannot be readily determined. However, it is clear that responses of all of the downstream isotypes were enhanced. Although the level of the increase was not extensive, it is in the order of that which can be achieved if mice were treated with poly(I:C) or adjuvant prior to antigenic stimulation [10, 13], effects which are also dependent on NK cells. Interestingly, the enhanced IgG3 response was not reduced upon depletion of NK cells. Notably, in a model of tumor cell-activated NK cell function which increased IgG re-

sponses as well, depletion of NK cells did not affect the IgG3 levels [18], although the enhancement was dependent on IFN- γ . Therefore, anti-CD48 may activate other cell types that can influence the switch to IgG3. It is not surprising that the enhancement of antigen-specific IgG2c and IgG3 was dependent on IFN- γ , most likely produced by NK cells (fig. 2d). In contrast, removal of IFN- γ prior to injection of anti-CD48 resulted in a further increase in the enhancement of the IgG1 response. We have previously shown that IL-2-propagated NK cells can induce the switch to IgG1 *in vitro*; therefore, NK cells activated by anti-CD48 must also be able to initiate the IgG1 response, but the level is dampened by the simultaneous production of IFN- γ unless the cytokine is removed beforehand.

A number of reports have clearly shown that purified NK cells can directly activate isolated B cells *in vitro* [19–21; for a review, see 22], whereas other reports have shown the involvement of NK cells in B-cell activation *in vivo* [13, 18, 23–25]; however, whether direct interaction between the two cell types is involved has not been established. We believe that enhancement by anti-CD48 provides the best evidence for the direct interaction between NK and B cells to be an essential component because the augmentation of Ig responses *in vivo* by anti-CD48 requires the presence of CD2 as well as of CD244. The requirement for CD2 correlates with *in vitro* findings that the induction of γ 2a/c germline transcription by IL-2-propagated, and therefore partially activated, NK cells occurs via a direct interaction between CD2 on NK cells and CD48 serving as a receptor on B cells [9]. In this case, it was possible to show, by transwell experiments, that the interaction between the two cell types required contact. Furthermore, enhancement was also partially compromised in the absence of CD244, suggesting that colocalization with CD48 or an increased expression of CD244 is required for sufficient activation of NK cells. Interestingly, an increase has also been shown for NK cells from poly(I:C)-stimulated animals [26]. Despite the increase in expression of CD244 upon injection of anti-CD48, stimulation of mice with anti-CD244 did not result in augmentation of B-cell responses. Thus, it appears that colocalization of CD48 with CD244 required for NK cell activation does not occur with stimulation by anti-CD244 alone. This conclusion is consistent with the structural analysis of CD244 linked to CD48 on NK cells which showed distinct differences from that of the receptors on their own [27]. These results are also consistent with recent findings that the involvement of CD244 in T-cell-mediated stimulation of B-cell responses does not require

NK cells [28]. Thus, despite findings from *in vitro* experiments that anti-CD244 can directly activate NK cells, *in vivo*, the function of these cells may be dampened by the presence of CD48-expressing cells [29].

Upon consideration of these results, the best scenario for enhancement of the B-cell response to a T-independent antigen by anti-CD48 is depicted in figure 5. Thus, it appears that upon stimulation by anti-CD48, NK cells can be activated, resulting in the alteration of CD244 which is required before CD2 expressed on NK cells can become effective stimulators of B cells via interaction with CD48. This interaction results in the initiation of germline transcription of the γ 1 and γ 2c heavy chain loci in B cells, but crosslinking of their B-cell receptor by antigen must occur before productive Ig secretion can be augmented. Whereas IFN- γ is required for the increase in IgG2c secretion, another cytokine, or more likely the direct activation by NK cells, is sufficient for the augmentation of IgG1 secretion. This enhancement is usually somewhat inhibited in the presence of IFN- γ production by NK cells but can be revealed by the removal of IFN- γ . It should be emphasized that the requirement for antigenic stimulation precludes the non-specific activation of B cells upon encounter with activated NK cells. An alternative explanation for the apparent activation of NK cells by anti-CD48 is that the antibody blocks inhibitory effects on NK cells by CD48 expressed on neighboring cells such that they are now more activated [30]. However, if this were the case, the basal Ig responses would have been higher in the CD244^{ko} mice. Similarly, because the Ig responses were not detectably lower in the knock-out mice, another possibility, i.e. that survival of activated NK cells is reduced by the absence of CD244 [31], can be discounted.

While this scenario is reasonable for the sequence of events caused by injection of anti-CD48 into B6 mice, the process by which production of downstream isotypes in BALB/C mice is enhanced is not as easily understood. Clearly, injection of anti-CD48 can result in increases in both IgG1 and IgG2a secretion. Whereas response to TI-II antigens are major histocompatibility complex class II independent, nonetheless, T-cell participation in terms of cytokine stimulation and CD40 interactions have been documented [for a review, see 14]. However, we have shown that this increase cannot be inhibited by depletion of NK, CD4 or CD8 cells. Also, enhancement cannot be attributed to changes in the status of T_{reg} cells since depletion of T_{reg} cells did not alter the production of Igs upon activation by the TI-II antigen or by anti-CD48. We have also tested for the direct activation of B cells or

CD11b-positive cells in both peripheral blood and splenocytes shortly after antibody injection and have not detected increases in either CD86 or CD69 (data not shown). Nevertheless, a number of other mechanisms require further investigation. For example it is possible that anti-CD48 induces the production of cytokines by a subpopulation of APCs that can affect the level of Ig secretion. Such activation has previously been shown for human cells [32, 33]. Exogenously introduced IL-12 that induced activation of B-cell IgG production in BALB/C mice has been shown to be independent of both T and NK cells [34]. CD48 can also bind directly to the IL-18RA subunit which can trigger binding to IL-18RB and, in the presence of IL-18, an active signaling complex is formed [35]. Anti-CD48 has also been shown to act as an accessory molecule for the activation of human B cells via CD40-mediated signals [36], possibly provided by APCs. Any of these conditions could play a more dominant role in BALB/C than in B6 mice. Such differences in immune responses between B6 and BALB/C are not unusual, es-

pecially with regard to cytokine responses [37–39], and explanations have not always been straightforward [40, 41].

Regardless of the different mechanisms that may be involved in B6 versus BALB/C mice, the results presented herein provide evidence of a specific role for CD48 in altering the course of an immune response to a T-independent antigen, adding to the relatively limited data [42; for a review, see 43] regarding the function of this molecule.

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