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CD4⁺ T Lymphocytes Expressing CD40 Ligand Help the IgM Antibody Response to Soluble Pneumococcal Polysaccharides via an Intermediate Cell Type

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Streptococcus pneumoniae is a major human pathogen that frequently causes pneumonia, septicemia, and meningitis, particularly in young children, the elderly, and immunocompromised patients. Protection against infections with S. pneumoniae is mediated through Abs against the capsular polysaccharides (caps-PS). We previously showed that the murine Ab response to caps-PS is dependent on CD40-CD40L interaction. In the present paper, we addressed the question of whether the CD40-CD40L-mediated modulation of the anti-caps-PS immune reaction is the result of a direct interaction between B lymphocytes and T lymphocytes or of an indirect interaction. SCID/SCID mice reconstituted with B lymphocytes from wild-type mice did not mount anti-caps-PS Abs. SCID/SCID mice reconstituted with B lymphocytes from wild-type mice and CD4⁺ T lymphocytes from wild-type mice but not CD4⁺ T lymphocytes from CD40L knockout mice stimulated the anti-caps-PS Ab response. This indicated that CD4⁺ T lymphocytes stimulated the anti-caps-PS Ab response in a CD40-dependent manner. SCID/SCID mice reconstituted with B lymphocytes from CD40 knockout mice and CD4⁺ T lymphocytes from wild-type mice generated an anti-caps-PS Ab response that could be inhibited by MR1, a blocking anti-CD40L Ab. These data indicated that CD4⁺ T lymphocytes stimulated the anti-caps-PS Ab response in an indirect way. Finally, lethally irradiated CD40 knockout mice reconstituted with bone marrow from wild-type mice mounted an anti-caps-PS Ab response that was comparable to the Ab response in wild-type mice, revealing that the required CD40 was on hemopoietic cells. In conclusion, we provide evidence that CD4⁺ T lymphocytes expressing CD40L stimulate the Ab response to soluble caps-PS by interacting with CD40-expressing non-B cells. The Journal of Immunology, 2006, 176: 529–536.

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3 Abbreviations used in this paper: caps-PS, capsular polysaccharide; TI, T independent; TNP, trinitrophenyl; MFI, mean fluorescence intensity; Ts, suppressor T lymphocyte; Ta, amplifier T lymphocyte.
indirectly modulate B lymphocyte activity via CD40L. Direct ligation of CD40 on B lymphocytes, with subsequent stimulation, is one possibility. However, an indirect mechanism whereby T lymphocytes interact with APCs cannot be excluded. Contact of T lymphocytes with macrophages and/or dendritic cells through CD40-CD40L interaction could induce the release of multiple cytokines that can further stimulate B lymphocytes. To elucidate the question of whether the CD40L-dependent stimulation of the anti-caps-PS Ab response is the result of a direct or an indirect effect was the main goal of this study.

Materials and Methods

Materials

Pneumovax, a 23-valent pneumococcal vaccine containing 25 μg of the caps-PS (Danish nomenclature) types 1–5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F, was obtained from Aventis Pasteur MSD. Pneumococcal caps-PS were obtained from American Type Culture Collection. C-polysaccharide was obtained from Statens Serum Institute (Copenhagen, Denmark). MR1, a monoclonal blocking Ab to murine CD40L, was provided by Bioceres. Polyclonal hamster IgG from tumor induced in C57BL/6 mice were obtained from 10 P's. PE-labeled anti-mouse CD4 (L3T4) and PE-labeled anti-mouse CD8a (Ly-2) were obtained from BD Biosciences. Peroxidase-conjugated goat-anti-mouse IgM and IgG were obtained from Nordic Immunological Laboratories. Goat serum and PBS were obtained from Invitrogen Life Technologies. 3,3'-5,5'-Tetramethylbenzidine was purchased from Dako Diagnostics. HCl 1 N and H2SO4 3 N solution were obtained from Merck. N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, 5 g dissolved in 500 ml of AD, was obtained from Merck. Dynabeads were obtained from Dynal Biotech. Nucleic acids purified from bone marrow were purified from Statens Serum Institute (Copenhagen, Denmark). Bone marrow transplantation

Bone marrow transplantation

Bone marrow from 8-wk-old C57Bl/6 mice was flushed from femurs and tibias with PBS and passed through sterile mesh filters. CD40 knockout recipient mice were conditioned with total body irradiation administered as a single exposure (9 Gy). The efficacy of the irradiation procedure was verified by irradiation of three mice without replacement of bone marrow. All three mice died within 3 wk, which confirmed the efficacy of the total body irradiation. Irradiated recipients received an i.v. injection of 5 × 108 bone marrow cells and were monitored daily for survival. Twelve weeks after bone marrow transplantation, mice received injections i.p. with Pneumovax. Fourteen days after injection with Pneumovax, blood was drawn and anti-caps-PS Abs were measured using ELISA. Engraftment was checked by flow cytometric analysis of CD40 expression on CD19+ cells.

Results

CD4+ T lymphocytes from CD40L knockout mice fail to stimulate the anti-caps-PS Ab response

To investigate the role of the CD40-CD40L interaction in the stimulatory effect of CD4+ T lymphocytes, we examined whether CD4+ T lymphocytes obtained from CD40L knockout mice stimulated the anti-caps-PS Ab response to the same extent as CD4+ T lymphocytes obtained from wild-type mice without T lymphocytes did not mount an Ab response to caps-PS, whereas SCID/SCID reconstituted with B lymphocytes isolated from wild-type mice together with CD4+ T lymphocytes obtained from wild-type mice clearly generated anti-caps-PS Abs. Conversely, SCID/SCID mice reconstituted with B lymphocytes from wild-type mice together with CD4- T lymphocytes isolated from CD40L knockout mice did not mount an Ab response to caps-PS. These results underscore the importance of the CD40-CD40L interaction in the CD4+ T lymphocyte-mediated modulation of the anti-caps-PS immune response and are consistent with our previous finding (9) that MR1 inhibits the stimulatory effect of CD4+ T lymphocytes in the murine anti-caps-PS immune response.

To verify that B lymphocytes remained functional after transfer into SCID/SCID mice, we measured the Ab response of B cell alone reconstituted SCID/SCID mice to TNP-Ficol, which is another TI Ag. In SCID/SCID mice reconstituted with B cells (in the absence of T lymphocytes), the anti-TNP-Ficol Ab response 1 wk after immunization was 238 ± 65 MFI (mean ± SD) (n = 3). This was significantly higher than the Ab level measured in SCID/SCID mice in the absence of B lymphocytes, which was 4.4 ± 7.3 MFI (n = 7) (p = 0.016; Mann-Whitney U test). For comparison, the anti-TNP-Ficol Ab response in wild-type mice amounted to 667 ± 40.7 MFI. These data indicated that B lymphocytes remained functional after transfer into SCID/SCID mice, even in the absence of T lymphocytes.

CD4+ T lymphocytes stimulate the anti-caps-PS Ab response by B lymphocytes through the CD40-CD40L interaction in an indirect way

To examine whether the stimulatory effect of CD4+ T lymphocytes on the anti-caps-PS Ab response is the result of a direct interaction between B and T lymphocytes or of an indirect effect, we studied the effect of CD4+ T lymphocytes on the Ab response
of B lymphocytes obtained from CD40 knockout mice. If T lymphocytes directly interact with B lymphocytes through the CD40-CD40L interaction, then no effect of CD4+ T lymphocytes will be observed. If, however, CD4+ T lymphocytes exert their effect indirectly, e.g., via interaction with APCs, then CD4+ T lymphocytes will stimulate the anti-caps-PS Ab response of CD40 knockout B lymphocytes.

FIGURE 2. The stimulatory effect of CD4+ T lymphocytes on the Ab response to caps-PS is indirect. SCID/SCID mice were reconstituted with 1) wild-type murine spleen cells depleted of CD4+ and CD8+ T lymphocytes (leaving B lymphocytes) (A), 2) with CD40 knockout murine spleen cells depleted of CD4+ and CD8+ T lymphocytes (leaving CD40 knockout B lymphocytes) (B), 3) with wild-type murine spleen cells depleted of CD4+ and CD8+ T lymphocytes (leaving wild-type B lymphocytes) (C), 4) with CD40 knockout murine spleen cells depleted of CD8+ T lymphocytes and CD4+ T lymphocytes (leaving CD40 knockout B lymphocytes) together with wild-type murine spleen cells depleted of B lymphocytes and CD8+ T lymphocytes (leaving wild-type CD4+ T lymphocytes) (D). The B lymphocyte fraction contained >85% B lymphocytes and <1.5% CD4+ or CD8+ T lymphocytes. The CD4+ T lymphocyte fraction contained >80% CD4+ T lymphocytes and <2.5% B or CD8+ T lymphocytes. The cells were suspended in 500 μl of 0.9% NaCl and injected i.p. into SCID/SCID mice. The total amount of B lymphocytes was the same in all conditions and amounted to ± 10 × 10^6 cells. The total amount of CD4+ T lymphocytes was the same in all conditions and amounted to ± 4 × 10^6 cells. The IgM Ab response to caps-PS serotypes 3, 4, 14, and 19F was measured in duplicate 14 days after vaccination with Pneumovax. Results shown are from a representative experiment that was done three times with similar outcome.

FIGURE 1. CD4+ T lymphocytes from CD40L knockout mice fail to stimulate the anti-caps-PS Ab response. SCID/SCID mice were reconstituted with wild-type murine spleen cells depleted of CD4+ and CD8+ T lymphocytes (leaving B lymphocytes) (A), with wild-type murine spleen cells depleted of CD4+ and CD8+ T lymphocytes (leaving B lymphocytes) together with wild-type murine spleen cells depleted of B and CD8+ T lymphocytes (leaving CD4+ T lymphocytes) (B), or with wild-type murine spleen cells depleted of CD4+ and CD8+ T lymphocytes (leaving B lymphocytes) together with CD40L-/- murine spleen cells depleted of B and CD8+ T lymphocytes (leaving CD4+ T lymphocytes) (C). The B lymphocyte fraction contained >80% B lymphocytes and <1.5% CD4+ or CD8+ T lymphocytes. The CD4+ T lymphocyte fraction contained >85% B lymphocytes and <1.5% CD4+ or CD8+ T lymphocytes. The cells were suspended in 0.9% NaCl and injected i.p. into SCID/SCID mice. The total amount of B lymphocytes was the same in all conditions and amounted to ± 10 × 10^6 cells. The total amount of CD4+ T lymphocytes was the same in all conditions and amounted to ± 4 × 10^6 cells. The IgM Ab response to caps-PS serotypes 3, 4, 14, and 19F was measured after 14 days. Results shown are from a representative experiment that was done three times with similar outcome.
SCID/SCID mice were reconstituted with purified B lymphocytes obtained from the spleen of CD40 knockout mice in the presence or absence of purified CD4+ T lymphocytes obtained from wild-type mice. As a control condition, SCID/SCID mice were reconstituted with B lymphocytes of wild-type mice in the presence or absence of T lymphocytes from other wild-type mice. The SCID/SCID mice were immunized with Pneumovax and the Ab response to caps-PS serotypes 3, 4, 14, and 19F was measured after 14 days. Results are shown in Fig. 2. SCID/SCID mice reconstituted with B lymphocytes obtained from either CD40 knockout mice or wild-type mice did not mount an Ab response to caps-PS. SCID/SCID mice reconstituted with B lymphocytes from wild-type mice and CD4+ T lymphocytes from wild-type mice clearly generated an augmented Ab response when compared with SCID/SCID mice reconstituted with only B lymphocytes from wild-type mice (p < 0.05, Kruskal-Wallis) for all serotypes tested and for at least four serum dilutions, confirming the stimulatory effect of CD4+ T lymphocytes on the Ab response to caps-PS. SCID/SCID mice reconstituted with B lymphocytes from CD40 knockout mice together with CD4+ T lymphocytes from wild-type mice generated significantly higher (p < 0.05, Kruskal-Wallis) for all serotypes tested and for at least four serum dilutions) anti-caps-PS Abs than SCID/SCID mice reconstituted with B lymphocytes from CD40 knockout mice. In the presence of wild-type CD4+ T cells, the absence or presence of CD40 on the B cells did not make a statistically significant difference (p ≥ 0.5, Kruskal-Wallis). These results suggest that the stimulatory effect of CD4+ T lymphocytes on the Ab response to caps-PS was indirect.

To exclude the possibility that a direct B lymphocyte-T lymphocyte interaction, other than via CD40-CD40L, was responsible for the stimulatory effect of CD4+ T lymphocytes, we studied the effect of blocking the CD40-CD40L interaction on the stimulatory effect of CD4+ T lymphocytes on the anti-caps-PS Ab response by B lymphocytes lacking CD40. SCID/SCID mice were reconstituted with B lymphocytes obtained from CD40 knockout mice and CD4+ T lymphocytes obtained from wild-type mice. The animals were then treated with MR1, a monoclonal anti-CD40L blocking Ab, or hamster IgG as an isotype control and immunized with Pneumovax. Administration of MR1 abolished the stimulatory effect of CD4+ T lymphocytes on the anti-caps-PS IgM Ab response by CD40 knockout B lymphocytes for all four serotypes measured (p < 0.05, Kruskal-Wallis) (Fig. 3). These data show that CD4+ T lymphocytes stimulate the anti-caps-PS Ab response via an effect of CD40L on non-B cells.

To demonstrate that CD40L has to be occupied to enhance the anti-caps-PS Ab response, we transferred CD40L+ T cells into CD40 knockout mice. CD40L+ T cells failed to induce an IgM (Fig. 4) and IgG (data not shown) anti-caps-PS immune response (serotypes 3, 4, 14, 19F) into CD40 knockout mice, suggesting that CD40L has to interact with CD40 to activate the anti-caps-PS immune response.

**Lack of CD40 expression on endothelium does not play a role in the anti-caps-PS Ab response**

In the above-described experiments, we showed that CD4+ T lymphocytes indirectly stimulate the anti-caps-PS Ab response via the CD40-CD40L interaction. This indicates that CD40L on T lymphocytes activates CD40 on non-B cells.

Next, we investigated whether the required CD40 is on hemopoietic cells or on nonhemopoietic cells, such as endothelium. Therefore, the anti-caps-PS Ab response was assessed in CD40 knockout mice that had been lethally irradiated and reconstituted...
with bone marrow from wild-type mice. Engraftment of bone marrow was controlled by measurement of the CD40 expression on CD19\(^+\) cells. In the transplanted mice, CD40 expression on CD19\(^+\) cells was 78, 69, 67, and 74%. In wild-type mice, CD40 expression on CD19\(^+\) cells was 67.5, 62, 61, and 63%. Previous studies using the same transplantation protocol as in this study have shown that total hemopoietic engraftment and APC repopulation was achieved (13, 14). Thus, transplanted mice have normal CD40 expression on hemopoietic cells but no CD40 expression on nonhemopoietic cells, such as endothelium. Depletion of B and CD8\(^+\) T lymphocytes resulted in a cell population consisting of \(>80\%\) CD4\(^+\) T lymphocytes and \(<2.5\%\) B lymphocytes and CD8\(^+\) lymphocytes. The cells were suspended in 0.9% NaCl and injected i.p. into the CD40 knockout mice. The total amount of CD4\(^+\) T lymphocytes injected amounted to \(\pm 6-8 \times 10^6\) cells. The results shown are means \(\pm 1\) SD of four independent experiments (representing mouse to mouse variation \((n = 4)\)).

**FIGURE 4.** Effect of wild-type CD4\(^+\) T cells on the anti-caps-PS immune response in CD40-deficient mice. CD40 knockout mice (■) and CD40 knockout mice transferred with wild-type CD4\(^+\) T cells (□) were vaccinated with Pneumovax. The immune response to caps-PS serotypes 3, 4, 14, and 19F was measured after 2 wk. The results show the IgM Ab response. The CD4\(^+\) cell fraction was obtained from wild-type murine spleen cells after depletion of B lymphocytes and CD8\(^+\) T lymphocytes. Depletion of B and CD8\(^+\) T lymphocytes resulted in a cell population consisting of \(>80\%\) CD4\(^+\) T lymphocytes and \(<2.5\%\) B lymphocytes and CD8\(^+\) lymphocytes. The cells were suspended in 0.9% NaCl and injected i.p. into the CD40 knockout mice. The total amount of CD4\(^+\) T lymphocytes injected amounted to \(\pm 6-8 \times 10^6\) cells. The results shown are means \(\pm 1\) SD of four independent experiments (representing mouse to mouse variation \((n = 4)\)).

The role of the CD40-CD40L interaction in the immune response to TNP-Ficoll

Administration of a blocking anti-CD40L Ab (MR1; 500 \(\mu\)g) exerted no effect on the anti-TNP-Ficoll Ab response. The Ab response amounted to (mean \(\pm\) SD) 399 \(\pm\) 87 MFI \((n = 4)\) and 421 \(\pm\) 175 MFI \((n = 4)\) in the absence and presence of MR1, respectively. In a separate experiment, we found that the Ab response to TNP-Ficoll was similar in wild-type mice (677 \(\pm\) 41 MFI \((n = 8)\) and CD40 knockout mice (665 \(\pm\) 100 MFI) \((n = 9)\).

**Discussion**

*S. pneumoniae* is an organism that frequently causes serious infections (1). It has already been known for a long time that protection against infections with *S. pneumoniae* is mediated by Abs to the caps-PS. Vaccination with pneumococcal caps-PS is widely used to protect people against infection with *S. pneumoniae* (15).
Besides, vaccination with caps-PS is also used to identify individuals with a selective immune defect in the immune response to polysaccharide Ags (16). Such individuals have an increased risk for infections with encapsulated bacteria.

The regulation of the Ab response to polysaccharide Ags is poorly understood. In the present paper, we demonstrate that the CD40-CD40L interaction plays a key role in the regulation of the IgM Ab response to soluble pneumococcal polysaccharides. Through adoptive transfer of B lymphocytes from wild-type mice with CD4+ T lymphocytes from either CD40L knockout mice or wild-type mice, we showed that CD4+ T lymphocytes stimulated the IgM anti-caps-PS Ab response in a CD40L-dependent manner. This excluded the possibility that the CD40L engaged in the anti-caps-PS immune reaction was from a non-T cell type, such as eosinophils, basophils, platelets, or dendritic cells. Through adoptive transfer of B lymphocytes from CD40 knockout mice with or without wild-type CD4+ T lymphocytes, and with or without MR1, a blocking anti-CD40L mAb, we showed that CD4+ T lymphocytes stimulated the anti-caps-PS immune response via an intermediate cell type. Wild-type CD4+ T lymphocytes did not enhance the anti-caps-PS immune response in CD40-deficient mice. This indicated that CD40L has to interact with CD40 to activate the anti-caps-PS immune response and excluded the possibility that there might be a second receptor for CD40L on B cells. Taken together, our data indicate that the IgM anti-caps-PS Ab response is dependent on the interaction between CD40L on T lymphocytes and CD40 on non-B cell APCs. Such interaction could induce the release of multiple soluble factors (e.g., cytokines) that can further stimulate B lymphocytes.

Transfer experiments in which mice lymphocytes were transplanted to SCID/SCID mice resulted in very weak Ig Ab responses and did not allow us to reproducibly study the IgG immune response to caps-PS. Therefore, even though CD40 on B cells is not required for the IgM response, it is possible that a direct interaction between T cell CD40L and B cell CD40 might play a role in isotype switching.

The findings described in this report and in our earlier paper (9) are the first to implicate a role for endogenous CD40-CD40L interactions in the Ab response to purified polysaccharides. Many previous reports have stated that TI-2 responses are independent of endogenous CD40-CD40L interactions (5–8). These studies typically used TNP-Ficoll and DNP-Ficoll, except for the report of Hwang et al. (8), who studied capsular polysaccharide serotype 6. It may be that the CD40-CD40L interaction is important for certain TI-2 Ags but not for others. For example, our data indicate that the CD40-CD40L interaction is not implicated in the immune response to TNP-Ficoll. We confirmed the observation reported by Foy et al. (5) that administration of a blocking anti-CD40L Ab (MR1) did not affect the anti-TNP-Ficoll Ab response. This is consistent with the finding of Xu et al. (17) that the Ab response to TNP-Ficoll was not reduced in CD40L knockout mice, but inconsistent with the observation of Van den Eertwegh et al. (18) that TNP-Ficoll induced Th cells to express CD40L. Finally, we confirmed the observation by Castigli et al. (19) that the Ab response in CD40 knockout mice was comparable to the response in wild-type mice.
The Ab response to polysaccharide Ags in general and pneumococcal caps-PS in particular is classically believed to be “independent” of T cells and the CD40-CD40L interaction (11, 15). This is in contrast to the Ab response to protein Ags, which is dependent on T lymphocytes and the CD40-CD40L interaction.

Although T lymphocytes are not required for the Ab response to caps-PS, they have regulatory effects. Caps-PS are therefore classified as TI type 2 Ags. The regulatory properties of T lymphocytes have been described by the extensive work from Baker and his group and others (20–22) in the 70s and 80s. They found that the Ab response to purified pneumococcal polysaccharide type III (SSS-III) in athymic \( \text{nu/nu} \) mice was comparable to the Ab response in conventional thymus-bearing mice. Moreover, they reported that treatment with a T lymphocyte-depleting agent resulted in a 20- to 40-fold increase in the Ab magnitude in thymus-bearing mice but not in athymic \( \text{nu/nu} \) mice. Based on these experiments and additional experiments, Baker et al. (20) proposed that the Ab response to SSS-III is controlled by two types of regulatory T lymphocytes: suppressor T lymphocytes (Ts) and amplifier T lymphocytes (Ta). They stated that “usually the effects produced by Ts and Ta are counterbalanced: this is why the Ab response to SSS-III is similar in magnitude in thymus-bearing and athymic mice and why Ts and Ta have escaped detection for so many years.”

The existence of stimulatory and inhibitory T cells has been confirmed by other groups (23, 24) and, more recently, we further substantiated that CD4\(^+\) T lymphocytes exert stimulatory, and CD8\(^+\) T lymphocytes exert inhibitory, effects, and that these effects depend on the CD40L-CD40 interaction (9, 10). In the present manuscript, we demonstrate that CD4\(^+\) T cells expressing CD40L help the IgM Ab response to soluble pneumococcal caps-PS via an intermediate cell type.

In adoptive transfer experiments, we found that murine B cells produced significantly less Abs to soluble caps-PS in the absence of CD4\(^+\) T lymphocytes than in the presence of CD4\(^+\) T lymphocytes. Similar findings were reported by Bondada et al. (25) who found that purified B cells were unable to produce Abs against TNP-Ficoll, a TI-2 Ag. This is at odds with previously published data that indicate that the Ab response in athymic nude mice is essentially unchanged relative to euthymic mice (20, 21), indicating that the Ab response to caps-PS can occur independent of T cells and the CD40-CD40L interaction. Garg et al. (28) showed that, in contrast to in vitro culture of spleen cells, in vitro culture of lymph node cells did not respond to caps-PS, and that addition of APCs isolated from spleen cells enabled the lymph node to respond to caps-PS. It was further put forward that defects in APC function might play a critical role in the failure of neonates to respond to caps-PS (29).

In conclusion, we provided evidence that T lymphocytes expressing CD40L modulate the immune response to soluble caps-PS by interacting with non-B cell APCs expressing CD40.

Disclosures

The authors have no financial conflict of interest.

References


