Brief Definitive Report

Soluble CD40 Ligand Can Replace the Normal T Cell–derived CD40 Ligand Signal to B Cells in T Cell–dependent Activation

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Summary

We have constructed a soluble chimeric fusion protein between the mouse CD8α chain and the mouse CD40 T cell ligand. This protein binds to both human and mouse B cells. By itself it induced a modest degree of B cell proliferation, but together with antiimmunoglobulin (anti-Ig) antibody it greatly stimulated B cell proliferation, as determined by both [^{3}H]thymidine uptake and increase in cell numbers. These data are evidence that the CD40 ligand on T cells provides a signal that drives B cell proliferation. This signal is synergistic with that delivered by anti-Ig antibody.

T cells activated either physiologically by peptide-bound MHC molecules (1) or activated in vitro by antibodies to CD3 (2) induce vigorous B cell proliferation. This B cell proliferation is contact dependent, and can be mimicked at least in part by membrane preparations from activated T cells (3). Although the molecules responsible for this activation have until recently been poorly understood, the ligand for the CD40 molecule in B cells has always been considered a likely candidate. mAbs that crosslink CD40 induce B cell proliferation, especially in conjunction with other stimuli such as antibodies to Ig (4).

Recently we and others have identified a T cell ligand for the CD40 molecule that is a 35-kD protein expressed as a monomer on activated T cells (5–7). The cDNA encoding this molecule has been cloned (5). Expression of the mouse CD40 ligand (mCD40L) in human EBV-transfected B cells stimulated B cell division (5), which was interpreted as indicating that no other signal was necessary for B cell proliferation. However, it is difficult to exclude other intercellular molecular interactions besides the one of interest in systems that rely on the expression of transfected molecules.

Here we describe the development of a soluble form of mCD40L that was obtained by constructing a fusion protein from the extracellular portion of the mouse CD8α chain and the extracellular portion of mCD40L (mCD40L-mCD8α).

Materials and Methods

Construction and Expression of Soluble mCD40L-mCD8α Chimeric Protein. The primers used to amplify the extracellular portion of mouse CD8α were: 5′(GGGACGAGAAGCTGAATTCGTCGAAACTGT), and 3′(TCCTCTTCGACCTTATCCAAGGCGAAGTCCAATCCGGTCC). The primers for mouse CD40L were: 5′(GGACCGGATTGGACTTCGCCTTGGATAAGGTCGAAGAGGA), and 3′(TGTGTGAAGCTTAGGACAGCGCACTGTTCA).

PCR was used to amplify cDNA encoding the extracellular portion of mouse CD8α protein together with the extracellular portion of mouse CD40L (5). Primers encoding the 3′ sequence of the CD8 fragment and the 5′ portion of mCD40L were designed to overlap; annealing of the PCR products yielded a hybrid template. The template encoding the chimeric construct was selectively amplified using external primers specific for the 5′ region of CD8 and the 3′ region of mCD40L, each primer containing appropriate restriction sites for subcloning into the expression vector pH β APr-1-neo (8). The resulting PCR fragment of ~1.3 kb was ligated into the expression vector above, and transfected into competent *Escherichia coli*, which were selected on ampicillin-containing agar plates. Plasmids from resistant clones of *E. coli* containing the correctly inserted fragment were identified by digestion with EcoRI and HindIII. The resulting plasmid is henceforth called pH β APr-1-neo mCD40L-mCD8α. CD8α was chosen because the extracellular portion has been shown to be readily secreted (9).

Construction of Chimeric Human IgG1 Molecules. The primers used to amplify the extracellular portion of the mouse CTLA4 gene were: 5′(TTACTCTACTCCCTGAGGAGCTCAGCACATTTGCC), and 3′(TATACTTACCAGAATCCGGGCATGGTTCTGGATCA). The primers used to amplify mouse CD40 were: 5′(CGTAGAGCTCACCATGGTGTCTTTGCCTCGGCTGTGCG), and 3′(TATACTTACCCATCCGGGACTTTAAACCACAGATG).

Chimeric Ig molecules expressing the extracellular portions of the mouse CTLA4 gene (10) or mouse CD40 gene (11) and the human IgG1 constant domains were created as follows. External primers encoding the 5′ portion and the 3′ portion of mouse CD40 and CTLA4 were used to amplify the extracellular portion of mouse CD40 (from mouse B cell cDNA), and mouse CTLA4 (from a plasmid containing the mouse CTLA4 cDNA, kindly provided by Dr. Pierre Golstein, Centre d'Immunologie, INSERM-CNRS de

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Marseille Luminy, France). Each primer contained appropriate restriction sites for subcloning into the human IgG1 expression vector (12), together with a 3′ splice donor site within the 3′ primer to correctly splice to the human γ1 exons.

Transfection. J558L hybridoma cells were transfected with pH β APr-1-neo mCD40L-mCD8α and selected in the presence of G418-sulphate (Gibco, Paisley, Scotland) or Xanthine (Sigma Chemical Co., St. Louis, MO) and mycophenolic acid (Calbiochem-Behring Corp., La Jolla, CA) (12). Cells were cultured at 5×10^4/ml. Selection medium was added 24 h after the transfection, and clones were screened (see Results and Discussion) when they grew up 2 wk later.

Immunoprecipitation of mCD40L-mCD8α and Purification of Chimeric Molecules. Positive transfectant clones were adjusted to 4×10^6/ml cells in methionine-free media (Gibco) supplemented with 10% dialyzed FCS with antibiotics, 2-ME, and glutamine supplements. 1 mCi of [^{35}S]methionine was added to 1 ml of cell suspension, and cells were cultured for 4 h at 37°C.

After incubation, cells were washed thoroughly in PBS (pH 7.2) and lysed for 30 min at 4°C in 0.5% NP-40 containing buffer supplemented with PMSF (Sigma Chemical Co.). The lysate was spun at 10,000 *g* for 10 min, the supernatant removed, and precleared with protein A and protein G beads (Pharmacia, Uppsala, Sweden). The supernatant was aliquoted and incubated with either mCD40-Hγ1 prebound onto protein A beads or rat anti-mouse CD8 that had previously been bound onto protein G beads. After washing, protein was eluted from the beads by boiling, and run under reducing and nonreducing conditions on 12% SDS-PAGE gels.

Antibodies and Flow Cytometry. The mAbs used in these experiments were against human CD19 (HD37, Boehringer, Mannheim, Germany), human CD3 (TR66 [13]), human CD4 (10A12), human CD8 (OKT8; American Type Culture Collection, Rockville, MD), human IgD (δTa4.1; American Type Culture Collection), mouse CD8 (53-6.72; American Type Culture Collection), and mouse κ (187.1 [14]). Fluorescent second-step conjugates were goat anti-mouse IgM-PE (Southern Biotechnology Associates, Birmingham, AL), anti-mouse CD8 FITC (Becton Dickinson & Co., Mountain View, CA), mouse anti-rat Ig FITC (Jackson ImmunoResearch Labs., Inc., West Grove, PA), sheep anti-mouse FITC (Silenus, Hawthorn, Australia), and goat anti-human IgM PE (Southern Biotechnology Associates).

Activated B cells (5×10^5 cells/well) were stained with saturating quantities of anti-CD19 mAb or chimeric constructs, followed after washing by second-step fluorescent reagents. There was no detectable crossreactivity between the reagents. Cells were analyzed on a FACScan® instrument (Becton Dickinson & Co.). 5,000 gated B or T cells were analyzed using the Lysys software.

Preparation of Human Peripheral Lymphocytes and Murine Splenic B Cells. Lymphocytes from normal healthy human adults were separated from peripheral blood using Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Lymphocytes were cultured for 2 h at 37°C with 5% CO_2/air to remove adherent cells. B lymphocytes were purified using positive selection with magnetic beads. PBL at a final concentration of 10^7/ml were incubated with saturating quantities of anti-human CD19 mAb, HD37, for 1 h at 4°C. After washing, B cells were positively selected by incubating the lymphocytes with sheep anti-mouse IgG-bound Dynabeads (Dynal, Oslo, Norway) as recommended by the manufacturer. After magnetic separation, the B cells were washed and incubated overnight at 37°C to allow the magnetic beads to dissociate from the B cells, in tissue culture medium (IMDM, supplemented with 10% FCS, 2 mM glutamine [Gibco], 100 IU/ml penicillin, 100 μg/ml streptomycin [Gibco], and 2-ME). The resulting cell preparation was >98% B cells as assessed by FACS® (data not shown).

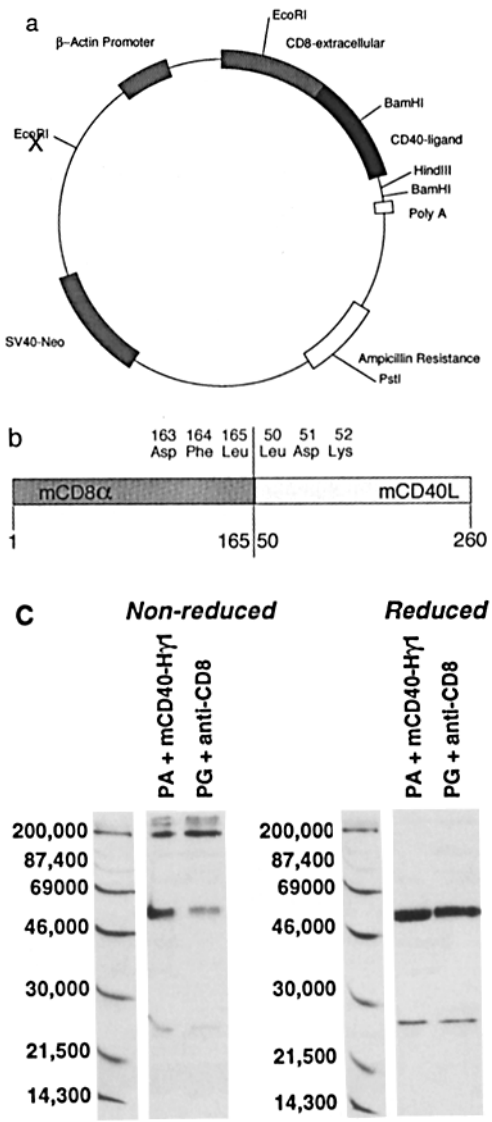

Figure 1. (*a*) Map of pH β APr-1-neo mCD40L-mCD8α plasmid. (*b*) Predicted polypeptide sequence of the mCD40L-mCD8α fusion protein showing the boundary amino acids between mouse CD8α and mouse CD40L. (*c*) Immunoprecipitates from [^{35}S]methionine-labeled J558L plasmacytoma cells that secreted mCD40L-mCD8α, run under nonreducing and reducing conditions on 12% SDS-PAGE. Lane *1*, molecular weight markers; lane *2*, the fusion protein precipitated under nonreducing conditions with mCD40L-Hγ1-bound protein A beads; lane *3*, immunoprecipitation with a rat anti-mouse CD8 mAb bound onto protein G beads; lanes *4–6*, the same treatments from a gel run under reducing conditions.

Murine B lymphocytes were prepared from 6–8-wk-old nude BALB/c female mice. Cell suspensions from minced spleens were centrifuged over Ficoll-Hypaque, washed, and then incubated for 1 h at 37°C in flat-bottomed culture dishes to remove adherent cells. The resulting preparation was >95% B cells.

Results and Discussion

Construction and Characterization of the mCD40L-mCD8α Chimeric Protein. A soluble fusion protein between mCD40L and mouse CD8α (mCD40L-mCD8α) was made as described

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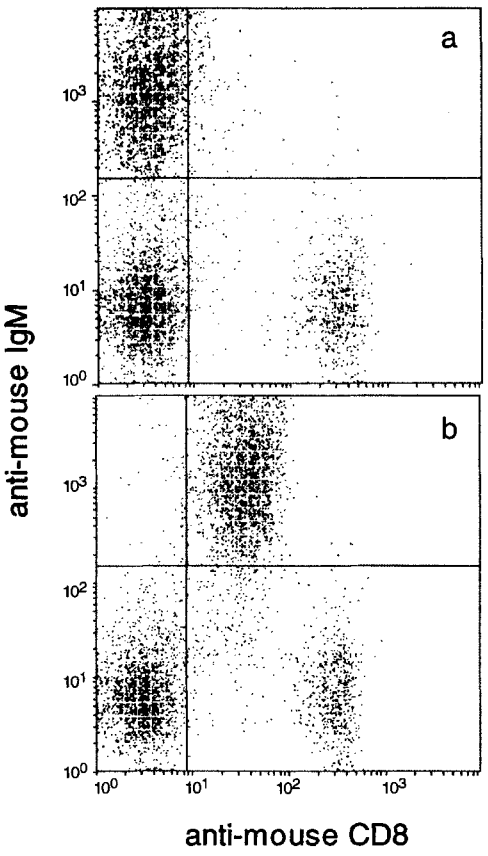

Figure 2. FACS® analysis of mouse splenic lymphocytes from a normal euthymic mouse. (*a*) Cells were stained with supernatant from J558L cells that did not secrete mCD40L-mCD8α, followed by goat anti-mouse IgM PE and rat anti-mouse CD8 FITC. (*b*) Cells were stained as in *a* except the first step included supernatant from the mCD40L-mCD8α–secreting J558L clone.

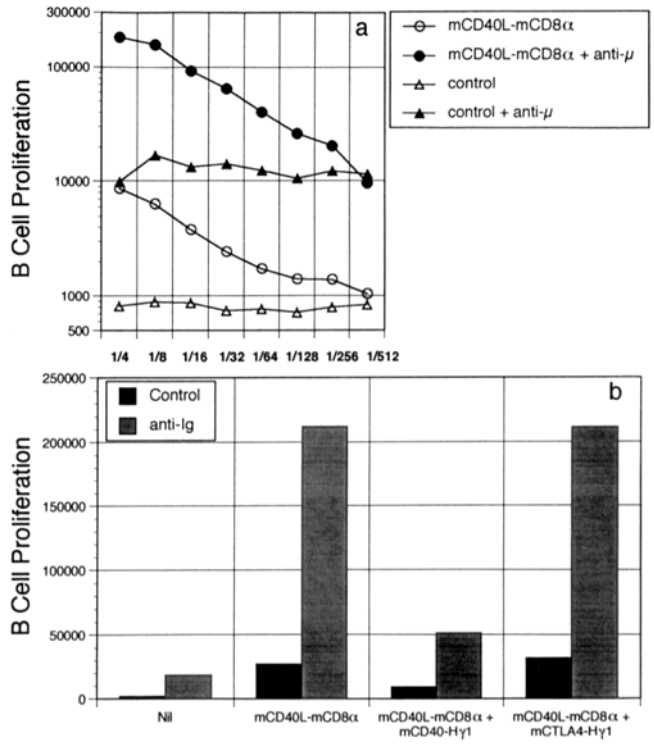

Figure 3. Effect of mCD40L-mCD8α polypeptide on mouse B cell proliferation. (*a*) Effect of mCD40L-mCD8α or a control supernatant alone or in conjunction with anti-Ig-bound Sepharose beads on murine B cell proliferation. 10^5 B cells were seeded per well, with or without a fixed dose of anti-Ig-bound beads, and a varying dose of mCD40L-mCD8α. (*b*) Effect of mCD40-Hγ1 and a control construct mCTLA4-Hγ1 on mCD40L-mCD8α–induced B cell proliferation, alone or in conjunction with an anti-Ig stimulus. Results are representative of three separate experiments. SEM were <10%.

in Materials and Methods. PCR was used to amplify the extracellular portion of CD40L from amino acid residues 50 (Leucine)–260 of the published cDNA sequence (5). This product was fused to the extracellular portion of a mouse cDNA encoding the CD8α polypeptide from amino acid residues 1–165 (Leucine) (15), and the resulting PCR DNA product was subcloned into the expression vector pH β APr-1 (Fig. 1 *A*). The sequence of the predicted fusion protein is shown in Fig. 1 *b*. The resulting plasmid (pH β APr-1-neo mCD40L-mCD8α) was transfected by protoplast fusion into J588L, and G418-resistant clones were obtained. Transfectant clones that grew up were tested for secretion of the mCD40L-mCD8α protein by an ELISA method; mouse CD40 linked to human IgG1 (mCD40-Hγ1) was immobilized on plates to capture chimeric protein from supernatants; and an antibody to mouse CD8 was used to identify the other 5′ portion of the chimeric molecule. A strongly positive clone (6.4) thus obtained was metabolically labeled with [^{35}S]methionine. The labeled protein was precipitated either by rat antibodies to mouse CD8 bound onto protein G beads, or by mCD40-Hγ1 bound onto protein A beads. The results of this experiment are shown in Fig. 1 *c*. A single band of ~50 kD is seen by both precipitation methods under reducing conditions; nonreducing conditions showed the existence of monomers, trimers, and higher molecular mass species, as has been reported for constructs encoding the extracellular portion of CD8α (9).

mCD40L-mCD8α Binds to Human and Mouse B Cells. To test whether the chimeric protein mCD40L-mCD8α would bind to B cell CD40, mouse splenic lymphocytes from a normal euthymic mouse were incubated with supernatant from 6.4 or a supernatant from control J558L cells, followed by incubation with the second-step reagents anti-mouse CD8 FITC or PE-conjugated goat anti-mouse IgM. Cells treated with the mCD40L-mCD8α showed CD8 positivity of the B cell population, indicating that mCD40L-mCD8α bound to B cells. This was not mediated by the CD8 part of the chimeric protein binding, as MHC class I–positive CD4-positive cells did not stain (Fig. 2 *b*). The control showed no binding to B cells (Fig. 2 *a*). There were distinct IgM-positive B cell and CD8-positive T cell populations as expected. Human B cell lines also bind mCD40L-mCD8α (data not shown), as would be predicted by the fact that the mCD40L was originally cloned using soluble human CD40 (5).

mCD40L-mCD8α Stimulates Mouse and Human B Cell Proliferation, Particularly in Conjunction with Anti-Ig. In humans, mAbs to CD40 have been shown to augment B cell prolifer-

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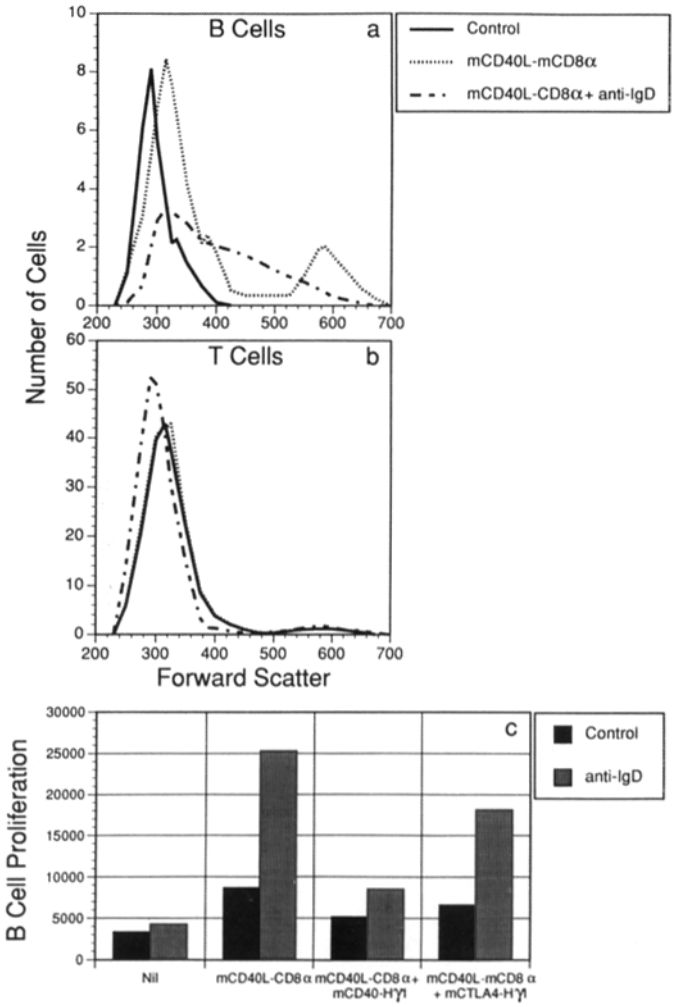

Figure 4. Effect of mCD40L-mCD8α, alone or in conjunction with anti-IgD, on the proliferation and size of human PBL as assessed by forward scatter. (*a*) Shows the forward scatter of gated human B cells after culture in medium only. mCD40L-mCD8α, or mCD40L-mCD8α and anti-IgD added at a final concentration of 10 μg/ml. Soluble anti-IgD did not cause B cell enlargement at this dose. (*b*) Shows the forward scatter of gated human T cells after culture in medium only, mCD40L-mCD8α, or mCD40L-mCD8α and anti-IgD added at a final concentration of 10 μg/ml. (*c*) Effect of mCD40-Hγ1 and mCTLA4-Hγ1 on mCD40L-mCD8α–induced human B cell proliferation alone or in conjunction with soluble anti-IgD. The results are representative of three different experiments. SEM were <10%.

ation initiated by anti-Ig (4). We therefore tested our chimeric molecule in functional assays. The dose-dependent effect of mCD40L-mCD8α on mouse B cell proliferation is shown in Fig. 3 *a*. mCD40L-mCD8α alone stimulated significant DNA synthesis (measured by [^{3}H]thymidine incorporation) compared with that found with a control supernatant. A comparable level of DNA synthesis was achieved by anti-Ig immobilized on Sepharose beads. mCD40L-mCD8α together with anti-Ig stimulated significantly higher levels of [^{3}H]thymidine uptake (10–20-fold higher than either stimulus alone) and this was accompanied by a large increase in B cell numbers. Such very high levels of proliferation could not be achieved by increasing the dose of either anti-Ig or mCD40L-mCD8α alone, indicating that they were synergistic. The proliferative effect of mCD40L-mCD8α on mouse B cells was specially inhibited by soluble mCD40-Hγ1 but not inhibited by an irrelevant fusion protein, mCTLA4-Hγ1 (Fig. 3 *b*). The effect of the chimeric protein was therefore to the mCD40L and not mediated by CD8 binding to MHC class I.

Human peripheral blood B cells also proliferated in response to soluble anti-IgD and mCD40L-mCD8α, and in addition, there was a significant degree of proliferation with mCD40L-mCD8α alone (Fig. 4 *c*). The addition of mCD40-Hγ1 profoundly inhibited the proliferative effect mCD40L-mCD8α, whereas mCTLA4-Hγ1 induced only partial inhibition. Analysis of forward scatter of human PBL activated with mCD40L-mCD8α alone induced a small increase in the size of human B cells. A few B cells appeared to be activated further. These cells were not aggregated but represented large B cell blasts. Soluble anti-Ig had no discernible effect. However, anti-Ig and mCD40L-mCD8α together caused virtually all B cells to enlarge (Fig. 4 *a*). T cells were unaffected (Fig. 4 *b*).

We have generated a soluble form of the type II membrane protein mCD40L by fusing the extracellular portion of mouse CD8 with the extracellular portion of mCD40L. This protein not only bound to B cells, but it also exerted specific effects on them. mCD40L-mCD8α was potently synergistic with anti-Ig in stimulating B cells to proliferate, with large clumps of dividing cells forming in cultures that had received both stimuli. A similar result has been reported for mAbs to CD40, which induce homotypic adhesion (16). The potent synergy between anti-Ig and mCD40L-mCD8α suggests that they provide distinct signals. Soluble CD40L has been useful for dissecting the distinct signals that T cells must deliver to support effective B cell activation. It will now be important to determine where and on which cells CD40L is normally expressed in vivo, and the membranes that regulate CD40L expression on resting T lymphocytes.

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