Costimulatory Pathway of T Cell Activation

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INTRODUCTION

Data establishing that T cell activation requires both T cell receptor (TCR)-mediated signals and simultaneously delivered costimulatory signals, have accumulated over the past 20 years (June, C. H., 1994)

Antigen specific T cell activation and subsequent induction of effector function require two signals for activation : signal 1, which is provided by TCR, when it is engaged by its specific antigen, and signal 2 (costimulation), which is provided by ligation of one or more of T cell surface receptor. Engagement of the TCR alone (i.e. signal 1 without signal 2) has been reported to induce a long-lasting state of T cell anergy, rendering cells unresponsive to subsequent antigenic stimulation (Schwartz, R. H., 1990).

The best characterized costimulatory pathway is B7-CD28 interaction. CD28 is a receptor expressed on the surface on T cell, which bind with B7 (B7.1, B7.2), molecules that can be expressed on many types of antigen presenting cells such as activated B cells, macrophage, dendritic cells.

The findings which almost fueled research in this area are several experiments in which the allograft or xenograft survival can be prolonged by the blockade of B7-CD28 interaction without using any other pan-immunosuppressants (Deborah J. Lenschow, 1992, Hua Lin, 1993, Mohamed H. Sayegh, 1995)

Recently, blocking B7-CD28 and CD40-CD40 ligand pathway simultaneously have been shown to induce allograft acceptance almost indefinetely in rodents and primates (Christian P. Larsen, 1996, Allan D. Kirk, 1997)

In additon to the situation of transplatation, induction of T cell anergy by blocking the costimulatory pathway of T cell activation seems to have important potential in medicine. Induction of T cell anergy by blocking the costimulatory pathway of T cell activation have also been shown to prevent active induction of autoimmune disease (Samia J. Khoury, 1995). Expresion of B7 molecule in malignant cell by genetic engineering can induce rejection of the malignancy and gene therapy utilizing these findings is in clinical trial (Robert T. Fenton, 1995)

Since the introduction of CD28-B7, additional ligand for CD28 has been suggested and recently with the study of CD28 knock-out mice, evidences for CD28 independant costimulatory pathway have been accumulating.

Our laboratory has been working on identifying molecules that are expressed on activated B cell in search for the molecule that can affect costimulatory activation of T cell. Here is the brief introduction of the work done in our laboratory.

METHODS

1. Cells

- 5C2 cells : Ia-negative mouse B lymphoma

cell line, M12.C3, transfected with defective I-$A^k\alpha$ and I-$A^k\beta$

- C8A3 : Mouse T cell line autoreactive to I-A^k

- The cells were routinely cultured in complete medium (RPMI 1640 medium supplemented with 10% FBS, 2mM L-glutamine, 50uM beta-mercaptoethanol, 100U/ml pennicilin-streptomycin)

2. Generation of Monoclonal Antibody

- 5C2 cells are cultured with 300ug/ml dibutyryl cAMP for 16 hours

- Immunize Lewis rat s.c. in hind foodpad with 6×10^6 activated 5C2 cells

- 4 days later, popliteal lymph node cells were fused with SP/20 murine myeloma cells at the ration of 2:1

- Fused cells were resuspended in IMDM with 10% FBS, 2mM L-glutamine 0.1 mM b-ME, 10% P388D1 culture supernatent

- Culture supernatent was screened with IF for stimulated and unstumulated 5C2 cells

- Hybridoma of interest was subcloned and purified with protein G column

3. FACS analysis

1×10^6 cells in 50μl of FACS buffer (PBS containing 1% FBS and 0.02% NaN_3) were stained in a V-shaped microtiter plate with either FITC- or PE-labeled mAbs or both at 4℃ for 30min. In the case of staining mouse splenic non-T cells or LPS blasts, propidium iodide (Sigma) at 4g/ml was also added to the FACS buffer. The cells were then washed three times with FACS buffer and analyzed on a FACS scan (Becton Dickson, San Jose, CA). Routinely, 5×10^3 cells were collected for each sample and dead cells were excluded by gating out propidium iodide-stained cells during the data acquisition or analysis.

4. Antigen Presentation Assay

- Untreated or db-cAMP-activated 5C2 cells were irradiated (3000 rad) and used as Antigen Presenting Cell (APC)

- 2×10^4 APC were cocultured with 1×10^5 I-Ak autoreactive T cell hybrid C8A3 in 200ul pre well in 96 well plate for 16 to 18 hours

- Culture suprenatent was collected and assayed for the presence of IL-2 by measuring the incorporation of ^3H thymidine by IL-2 dependent T cell line, CTLL

5. cDNA expression cloning

- cDNA library constructed from poly (A) RNA from db-cAMP stimulated 5C2 cells

- cDNA ligated to PEF-BOS mammalian expression vector

- The library was transfected into COS cells by the DEAE-dextran method

- 48 hours after transfection, panning with 2D10 Ab and goat anti-rat IgG coated plates

- Episomal DNA recovered from panned cells and transformed into E coli, DH 10B and the plasmid DNA was reintroduced into COS cell again

- Repeat cycle of expression and panning twice again

- Narrow down to single clone and confirm with specific reactivity to 2D10 by IF

6. CD4 T cell Stimulation with and without putative costimulation

- Isolate B-cell and CD4+ T cells from mouse spleen using cell separation column (from Ancell) after RBC lysis.

- Stimulate B cell with LPS 15ug/ml for 48 hours

- Coculture 5×10^4 irradiated LPS stiumlated B cell and CD4+ cell with anti-CD3 Ab (0.5ug/ml) in 96 well plate with addition of indicated antibody.

- Pulse the well with ^{3}H-thymidine 42 hours after coculture

- Harvest the cell 6hours after pulse and count the radioactivity

RESULTS

On the basis of immunofluorescence staining and FACS analysis (picture will be presented in the meeting), we subcloned 4 kinds of antibody against activated B cell and did experiments and obtained following results.

A. Antibody 2D10

We subcloned interesting antibody against activated B cell (named as 2D10) which recognize antigen on the db-cAMP stimulated 5C2 cell line (B cell line) with FACS as Fig. 1.

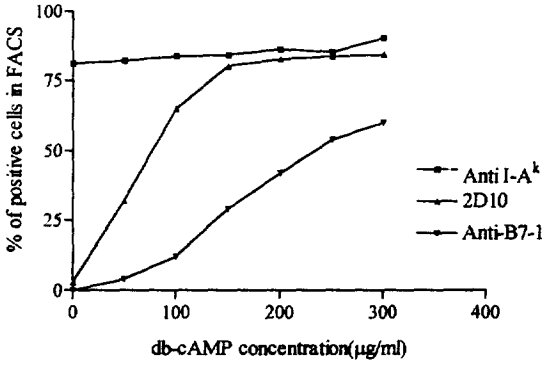


Fig. 1. Expression of molecule recognized by 2D10 on surface of db-cAMP actiated B cell(5C2).

5C2 B lymphoma cells(5×10^5 cells/well) were cultured in the presence of various concentrations of db-cAMP in 6-well culture plates for 24 h. The cells were then harvested, washed, and stained with FITC-labeled mAb anti-B7-1, 2D10, or anti-I-Ak as positive control.

This antigen recognized by this antigen was also found on the activated splenic B cell as Fig. 2.

2D10 blocked the antigen presentation of db-cAMP-activated 5C2 cells to C8A3 T hybrid in Fig. 3.

Both anti-B7-1 and 2D10 antibody, at a range of 0.04 to 10ug/ml, showed dose-dependent inhibitory effects on Ag presentation by db-cAMP-activated 5C2 cells and IL-2 secretion by T cell hybrid. At the concentration range of 0.1 to 10ug/ml, two mAbs addititively inhibited IL-2 secretion up to more than 95% in a dose-dependent manner.

cDNA insert was isolated from a db-cAMP activated 5C2 B lymphoma cDNA library by expression cloning in COS cells. The clone was composed of 1114 nucleotides with the longest open reading frame beginning with an ATG

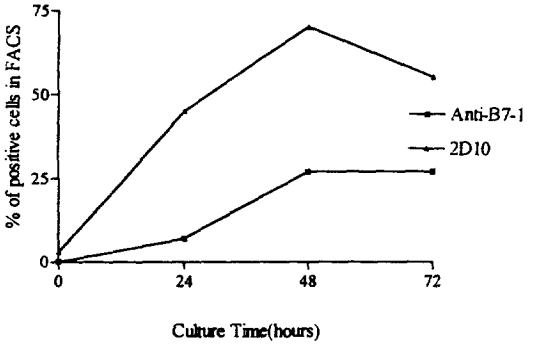


Fig. 2. Time course of the expression of molecule recognized by 2D 10 on the surface of splenic B cell stimulated by LPS.

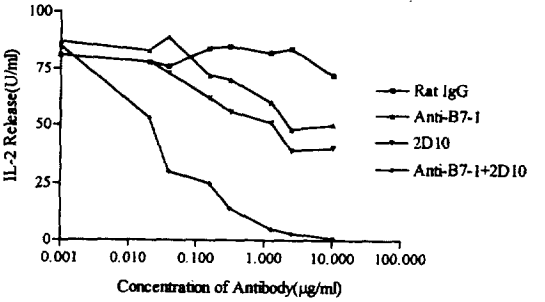


Fig. 3. Blocking effects of anti-B7 and 2D 10 mAbs on the Ag presentation of db-cAMP-activated 5C2 cells to C8A3 hybrids.

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Ag of2D10   VL.LISDAVSVETQAYFNGTAKLPCPFTKAQNISLSELVVFWQDQQKLVLYEHYLGT
mB7-1       RLSQVSSDVDEQLSKSVKDKVLLPCRYNSPHE.DESEDRIYWQKHDKVVL..SVIA
hB7-1       GLSHFCSGV.IHVTKEVKEVATLSCGHNVSVE.ELAQTRIYWQKEKKMVL..TMMS

Ag of2D10   EKLDSVNAKYLGRTSFD.RNNWTLRLHNVQIKDMGSYDCFIQKKHPTGSIILQQTLT
mB7-1       GKIK.VWPEYKNRTIYDNT.TYSLIILGLVLSDRGTYSCVVQKKERGTYEVKHLALV
hB7-1       GDMN.IWPEYKNRTIFDITNNLSIVILALRPSDEGTYECVVLKYEKDAFKREHLAEV

Ag of2D10   ELSVIANFSEPEIKLAQNVTGNSGINLTCTSKQGHPKPKKMYFLITNSTNEYGDNMQ
mB7-1       KLSIKADFSTPNITESGNPSADTK.RITCFASGGFPKPR..FSWLENGRELPGINTT
hB7-1       TLSVKADFPTPSISDFEIPTSNIR.RITCSTSGGFPEPH.LSWLENGEELNAINTT

Ag of2D10   ISQDNVTELFSISNSLSLSFPDGVWHMTVVCVLETESMKISSKPLNF...TQEPSIP
mB7-1       ISQDPESELYTISSQLDF...NTTRNHTIKCLIKYGDAHVSEDFTWEKPPEDPPDS
hB7-1       VSQDPETELYAVSSKLDF...NMTTNHSFMCLIKYGHLRV.NQTFNWNTTKQEHFPP

Ag of2D10   QT
mB7-1       KN
hB7-1       DN
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Fig. 4. Comparison of the predicted extracellular domain of molecule recognized by 2D10. 28% identity with human and mouse B7-1.

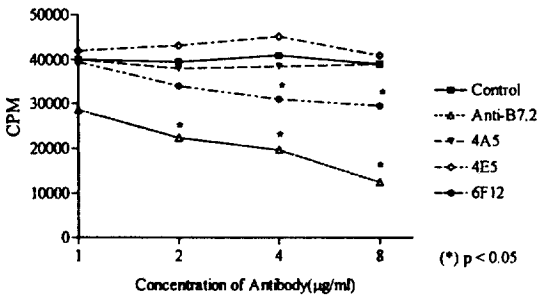


Fig. 5. Blocking effects of 4A5, 4E5, 6F12 on the antigen presentation of db-cAMP-activated 5C2 cells to C8A3 T hybrid. Irradiated db-cAMP-activated(300 μg/ml, 24hr) 5C2 cells at 2.5×10^4 cells/well were cocultured with 1×10^5 C8A3 cells in 96-well culture plates in the presence of varios concentration of indicated mAbs for 22 hours. The IL-2 released by C8A3 cells in triplicate culture supernatents was determined by the CTLL proliferation assay. Proliferation of CTLL was determined by pulsing with ^3H thymidine 36hours after culture and radioactivity determined with beta counter.

initiation condon. It encoded 303 amino acids (Fig. 4) with 28% identity with the amino acid sequence encoded by human and mouse B7-1 gene and was found to be identical with B7-2.

B. Antibody 6F12, 4E5, 4A5

Since the introduction of CD28 knock-out mice, there has been several resport that there exists CD28 independant costimulatory pathway. So we did several experiment to characterize another interesting monoclonal antibody.

Monoclonal antibody 6F12 stained strongly on both resting and db-cAMP stimulated B cell line (5C2) and 4E5 and 4A5 stained moderately on activated 5C2 cell, a little weaker on resting 5C2 cell(picture will be shown in the meeting)

The function of the molecule recognized by each mAbs was assessed in an in vitro Ag presentation assy. As in Fig 5, anti-B7.2 decreased the IL-2 production in dose dependent manner. Monoclonal antibody 6F12 decreased IL-2 production also, but effects of 4E5, 4A5 were marginal.

As was shown in several experiments of CD28 independent costimulatory T cell activation, T cell proliferation assay was more sensitive than IL-2 production in identifying molecule affecting costimulation.

So we did T cell proliferation experiment

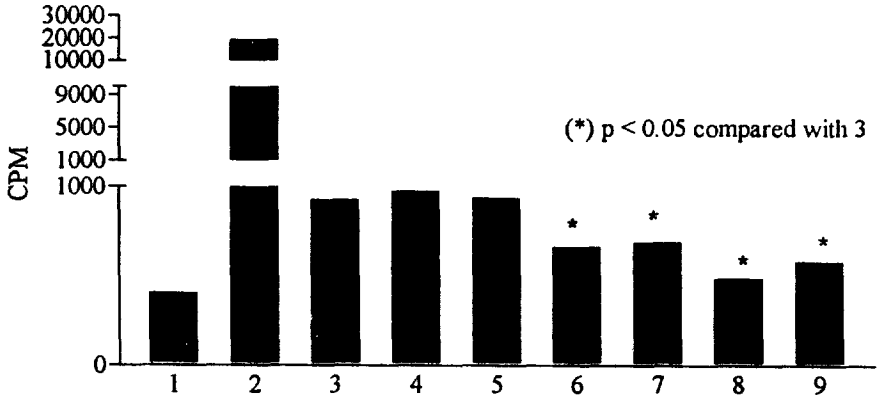


Fig. 6. Blocking effects of mAb 4A5, 4E5, 6F12 on T cell proliferation.
Splenic CD4 T cell and B cell was isolated from Balb/c spleen. LPS activated B cell and CD4 T cells(each 1×10^5 cells) were cocultured with 0.5 μg/ml anti-CD3 and indicated amouunt of each mAbs. Concentratic anti-B7. 1,2 antibody was 5 μg/ml.
1. CD4 T + α CD3
2. CD4 T +Activated B + α CD3
3. CD4 T +Activated B + α CD3 +Anti-B7
4. CD4 T +Activated B + α CD3 +Anti-B7 +4A5 5ug/ml
5. CD4 T +Activated B + α CD3 +Anti-B7 +4A5 2ug/ml
6. CD4 T +Activated B + α CD3 +Anti-B7 +4A5 5ug/ml
7. CD4 T +Activated B + α CD3 +Anti-B7 +4A5 2ug/ml
8. CD4 T +Activated B + α CD3 +Anti-B7 +6F12 5ug/ml
9. CD4 T +Activated B + α CD3 +Anti-B7 +6F12 2ug/ml

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ACGGGACGTACCACAGAAACGGAGCCGACACGCGCGATACCCCGACGAACAACTGTCGCCAG
TGCCCTGCATGGTGTCTTTGCCTCGGCTGTGCGCGCTATGGGGCTGCTTGTTGACAGCGGTC

GTAGATCCCGTCACACAATGCACGTCACTGTTTGTCATGGAGGTGCTACCGGTCACGACACT
CATCTAGGGCAGTGTGTTACGTGCAGTGACAAACAGTACCTCCACGATGGCCAGTGCTGTGA

AAACACGGTCGGTCCTTCGGCTGACT.....................................
TTTGTGCCAGCCAGGAAGCCGACTGA.....................................

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..............CATAACATCTTTAATAAATTAGGCGGGACCCAAAGATGGACACACAA
..............GTATTGTAGAAATTATTTAATCCGCCCTGGGTTTCTACCTGTGTGTT

TGGTAAATCAAGAACTTATTTCTGTGTGAGTTGGAAATATAAATGTTTTTTTTTTTTTTTTT
ACCATTTAGTTCTTGAATAAAGACACACTCAACCTTTATATTTACAAAAAAAAAAAAAAAAA
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Fig. 7. cDNA sequence for 6F12 was found to be identical to CD40.

ACACGGACGTCGACTGAGCGAGGTCTCGACTACCCTTAGGACGGCAAGGATGACTAGGGGTA
TGTGCCTGCAGCTGACTCGCTCCAGAGCTG**ATG**GGAATCCTGCCGTTCCTACTGATCCCCAT

CCTCTCGTTGACCTGACAGGTACACAAGAGTGCCTGAAACACGGTATACGATGACACCTGTC
GGAGAGCAACTGGACTGTCCATGTGTTCTCACGGACTTTGTGCCATATGCTACTGTGGACAG

GGCACGATTTAGAACGACGACCCTGAG.....
CCGTGCTAAATCTTGCTGCTGGGACTC.....

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.....

.....AGAAATATTCCTTGAAGTT
.....TCTTTATAAGGAACTTCAA

AAAAAAAAAATAAAAAGAGATTTCCAGTGTACAATGTAAACTCAAGAATAAACTTACAAGAA
TTTTTTTTTTATTTTTCTATAAAGGTCACATGTTACATTTGAGTTCTTATTTGAATGTTCTT

CCTGGTACACATTGTCCTGACGATAGACTTATTTTCTGATCCACAACTTTTTTTTTTTTTTT
GGACCATGTGTAACAGGACTGCTATCTGAATAAAATACTAGGTGTTGAAAAAAAAAAAAAAA

Fig. 8. cDNA sequence for 4E5 was found to be identical to Fc gamma Receptor.

with each mAbs and obtained the results like Fig. 6. As shown in Fig. 6, mAb 6F12, 4E5 decreased the T cell proliferation a little more in addition to the blocking effects of anti-B7 antibodies.

cDNA insert corresponding to mAb 6F12, 4E5 was isolated from a db-cAMP activated 5C2 B lymphoma cDNA library by expression cloning in COS cells. And the cDNA was sequenced partially as in Fig. 7, 8 and was matched to be identical to CD40 and fc gamma receptor.

DISCUSSION

During the past decade, the study of T-cell costimulation has emerged as one of the most exciting areas of immunology. The reason why this area draws attention of physcians as well as immunologists, seems to me that even though this area deals with most fundamental issue of immunology, it can have great potential of application in medicine such as transplantation, autoimmune disease and tumor immunology.

Several ligand-receptor pairs function as costimulators. Among these, the best characterized is the B7:CD28/CTLA4 costimulatory pathway. It consists of two costimulatory ligands on antigen-presenting cell, B7-1, and B7-2, each of which binds to two receptors on T cells, called CD28 and CTLA-4.

CD28 is 44-kD homodimeric glycoprotein of the immunoglobulin superfamily. It is constitutively expressed on most T cell and interact with B7 molecule on Antigen Presenting Cells (APC).

Role of CD28 has been demonstrated initially by the experiments in which monoclonal antibody to CD28 (anti-CD28) augment T cell res-

ponse initiated by anti-CD3 and can block the induction of anergy in T cell clones (Harding FA, 1992) Anti-CD28 causes a marked increase in the mRNA and secretion of several T cell lymphokine, including IL-2, TNF-a, GM-CSF, IFN-g.

CTLA-4 ia also a member of immunoglobulin superfamily and have a nucleotide sequence homology of about 75% with CD28. CTLA4 expression is below the threshold of detection by flow cytometry until it is transiently detectable 2 to 3 days after activation. It bind to B7 family with an affinity -20 fold higher than that of CD28. Because of its structural homology it was initially thought to serve as a functional alternative to CD28 in providing costimulatory signals to T cells. But the fact that CTLA4 plays a downregulatory role in T cell activation, was made clear by CTLA4 knock out mice. In that mice, peripheral lymphoid organs are packed with 5 to 10 times the normal number of lymphocyte, majority of them are T cell blast. In vitro T cell proliferate spontaneously and hyperresponsive to T cell antigen receptor engagement. Serum concentraions of immunoglobulin are orders of magnitude higher than normal. And it shows extensive lymphocyte infiltration of the bone marrow, lung, liver, pancrease and heart (Waterhouse P, 1995).

B7 (B7.1 and B7.2) is a ligand for CD28/CTLA4. B7 is expressed on antigen presenting cell such as macrophage, dendritic cell, activated B cell.

Unstimulated antigen presenting cell express very little or do not express B7 but it is upregulated after stimulation..

Before the discovery of B7.2, existence of additional ligands for CTLA4 has been suggested in several experiment, in which T cell response was not completely blocked by anti-B7 (Nabavi N, 1992) and also in the experiment with B7 (B7-1) knock-out mice in which T cell response remaining in B7 knock-out mice was completely blocked by CTLA4Ig (recombinant fusion protein that contains extracellar domain of CTLA4 fused to a IgG1 heavy chain) (Freeman GJ, 1993).

Since the introduction of CD28 knock-out mice, there have been several study of the immunologic deficiency of that mice in which T cell immune response and serum immunolobulin level were markedly decreased (Shahinian A, 1993). But recently, there are several reports about CD28 independant costimulatory T cell activation.

It was reported that naive CD28 deficient T cells can initiate but not sustain an in vitro antigen-specific immune response in CD28-deficient and TCR transgenic mice (Lucas PJ, 1995). When purified CD4+ T cells from wild and CD28 (−/−) mice were stimulated with soluble anti-CD3 and T depleted spleen cells or CD40L activated T depleted spleen cells, CD28 (−/−) CD4+ T stimulated with CD40L activated T depleted spleen cells showed similar proliferation as wild type and also substantial interleukin-2 production (Ding L, 1996). Also development of autoimmune disease and skin graft rejection in CD28 deficient mice have been reported (Bachmaier K, 1996, Kawai K, 1996).

These were the basis for our laboratory to search for the molecule expressed on the activated B cell. Charaterization of 3 monoclonal antibody 2D10, 6F12, 4E5 did not find any new molecule. B7-2 as costimulatory molecule is so well known and as our experiment, role of Fc gamma receptor in costimulation is not clear and need further study. But characterization of 6F12 as mAb recognizing CD40 deserve some comment since recently, there are several resport about the role of CD40 in costimulation of T cell activation, which is compatible with our findings.

As was mentioned above, Linna Ding has shown that when the CD28 (−/−) T cell was stimulated with L cells transfected with murine CD40L, it showed substantial immune response (Ding L, 1996). Guo Y has reported that the molecule expressed on B cell by CD40 L stimulation is CD44H (Guo Y, 1996), but Shinde S reported another molecule expressed on B cell by CD40L stimulation is IACM-1 (Shinde S, 1996). This area needs further study and clarification.

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