Enhancement of T cell immune response to lymphoma cells by CD28/CD80 alternative pathway*

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Abstract The aim of this study is to determine whether myeloma and lymphoma tumor cells can function as efficient antigen presenting cells (APC) to enhance the co-stimulation of T cells. The expression and function of T cell activation-related molecules, especially CD80, CD28, CD40 and CD40 ligand (CD40L), were studied on nine human myeloma cell lines (HMCL) and two B lymphoma cell lines. In the case of myeloma cell lines, the cells generally lacked CD80 antigen and expressed a heterogeneous CD40, and the expressions of CD40 and CD80 molecules could not be induced by either CD28 stimulation or CD40 ligation. Conversely, in the two B lymphoma cell lines, tumor cells expressed both CD80 and CD40 to some extent. CD28 stimulation could obviously increase the expression of CD80, CD40 and some adhesion molecules, and therefore generate a more efficient anti-tumor cell immunity. In conclusion, CD28 stimulation combined with CD40 antibody or soluble CD40 ligand may be a promising immunotherapeutic approach to B lymphoma.

Keywords: lymphoma, myeloma, CD40-CD40 ligand, CD28/CD80 pathway, anti-tumor response.

Optimal antigen-specific T cell activation and expansion have been recently known to require at least three steps[1,2]: (1) contact of antigen presenting cells (APC)—B cells, macrophages as well as dendritic cells with T cells through adhesion molecules such as CD40-CD40L, CD54-CD11a/CD18 and CD58-CD2; (2) presentation of antigenic peptides bound to molecules of the major histocompatibility complex (MHC) on APC to the T cell receptor; and (3) T cell activation is dependent on the interaction between CD28 and their ligands such as B7-1 (CD80) or B7-2 (CD86) on APC. It has been demonstrated that CD28-CD80 interaction provides an essential co-stimulating event to induce T cell intracellular signal transduction, interleukin-2 (IL-2) secretion and promote T cell survival. A disruption of the CD28-CD80 interaction may result in induction of T cell anergy[3,4]. It was reported[5,6] that the interaction of CD40 with its ligand on T cells is a critical initiation factor for the up-regulation of CD80 family molecules on APC. Therefore, CD28-CD80 and CD40-CD40L are the most important molecules involved in the regulation of immune responses.

B lymphoma and multiple myeloma are B cell neoplasias affecting the different stages of B cell differentiation. It is puzzling why these tumor cells could not mediate T cell immunity as APC-like normal B cells. We wondered therefore whether there were abnormal expressions of CD80, CD40 and

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their ligands or possibly deficient interactions between them. Here we analyzed the expressions of these molecules on human myeloma cell and B lymphoma cell lines, and studied possible mechanisms of CD40-CD40L as well as CD28-CD80 pathways involved in cell-to-cell interaction. We found that human myeloma cell lines did not express CD80 and expressed CD86 very weakly, while CD40L stimulation could not increase the expressions of co-stimulatory molecules on either myeloma or lymphoma cell lines. Interestingly, in lymphoma cell lines CD28 stimulation did greatly increase the expression of CD40 and of some adhesion molecules, and enhanced the capacity of T cell anti-tumor immunity. This may provide a promising immunotherapeutic approach in B lymphoma.

1 Materials and methods

1.1 Antibodies and reagents

Monoclonal antibodies (MAbs) against CD40, CD80, CD11a, CD18, CD54, CD49d, CD49e, CD44, CD40L antigens, FITC-conjugated goat antibodies to mouse IgG were purchased from Immunotech S.A. (Marseille, France). MAb to CD80 was produced in our institute.

Human recombinant IL-6 (rhIL-6) was kindly provided by Professor Liu Xinyuan (Biochemical Institute in Shanghai). PHA was purchased from Sigma (St Louis, Mo, USA), and tritiated thymidine was purchased from Academy of Military Medical Sciences (Beijing).

1.2 Cell lines

The XG-1, XG-6, XG-7 and XG-4 IL-6-dependent human myeloma cell lines (HMCL) were established in our laboratory[7], the RPMI 8226, U266 HMCL, Daudi, Raji were purchased from ATCC(Rockville, MD, USA). The XG cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol and 1 ng/mL of IL-6. The RPMI 8226, U266, Daudi and Raji cells were routinely cultured in the medium without IL-6. Human XG1-B7, XG6-B7 and XG7-B7 cells were obtained by transfection with a B7-1 retrovirus, they stably expressed a high density of B7-1. Human CD28 transfectant (DWT6.11) was obtained by transfecting full-length human CD28 cDNA in PHβ-prneo vector into murine T cell hybridoma. CD40 ligand-positive cells were obtained by transfecting full-length human CD40L cDNA in pcDNA3 vector into COS cell lines. All cell lines were free of mycoplasma contamination as assayed by an ELISA kit from Boehringer Mannheim (Germany).

1.3 Flow cytometry

Indirect immunofluorescence was performed by incubating 5×10^5 cells with 1 μg of murine mAb in 100 μL of staining buffer containing PBS with 0.25% bovine serum albumin and 0.01% sodium azide for 45 min at 4℃. Isotype-matched Ig was used as a control (murine IgG). The cells were then stained with FITC-conjugated goat antibody against mouse IgG. Flow cytometry was performed with an XL apparatus (Coulter, USA).

1.4 Allogeneic mixed lymphocyte reaction (allo-MLR)

Peripheral blood mononuclear cells (PBMNCs) were isolated by Ficoll/Hypaque density gradient

centrifugation from healthy volunteers. Allogeneic PBMNCs were co-cultured with the mitomycin-treated tumor cell lines (1×10^7 cells in 50 μg/mL mitomycin for 45 min at 37℃) and, after 60 h of co-culture, cells were pulsed with 1 μCi of tritiated thymidine per well for another 12 h, then harvested and counted. Allogeneic PBMNCs were also cultured with PHA (1 μg/mL) as the positive control. All microculture tests were carried out in triplicate, and stimulation index (SI) were calculated as follows: $SI = cpm_{(T\ cells + HMCL)}/cpm_{(T\ cells)} + cpm_{(HMCL)}$.

1.5 Cell proliferation assay

To investigate the effects of CD28 and CD40L stimulations on the proliferation of myeloma and lymphoma cells, the cells were co-cultured with mitomycin-treated CD28 or CD40L cell lines. After 24 h and 48 h co-culture, the cell number was counted under the microscope.

2 Results

2.1 Expressions of CD40, CD80 and adhesion molecules on human lymphoma and myeloma cell lines

The expressions of CD40, CD80 and adhesion molecules on 9 HMCL and 2 B lymphoma cell lines were observed. The percentages of labelled cells are given in table 1.

Table 1 Expressions of CD40, CD80 and adhesion molecules on myeloma and lymphoma cell lines

Cell line	CD80	CD40	CD11a	CD18	CD54	CD49d	CD49e	CD56	CD44
myeloma cell lines									
XG1	2.4	1.6	2.2	6.4	99.8	99.9	2.3	5.8	99.0
XG1B7	88.1	0.5	0.8	99.4	98.3	99.9	0.3	0.9	96.7
XG6	14.4	21.3	1.1	6.9	99.8	88.5	9.2	1.1	99.6
XG6B7	89.0	0.4	0	0.2	99.7	42.7	0.3	0.1	99.5
XG7	0.5	0	0	0.1	96.3	99.1	0.8	8.9	99.7
XG7B7	95.0	0.8	0.1	39.2	96.1	98.5	2.7	34.9	99.8
XG4	1.1	12.9	0.7	4.9	98.9	74.4	2.6	1.5	99.7
U266	3.0	0.1	1.4	2.0	95.8	98.7	3.1	3.5	95.9
8226	1.2	0.3	0.7	15.4	99.1	2.6	62.1	0.6	1.3
B lymphoma cell lines									
Raji	76.2	40.3	31.3	21.2	40.1	11.3	nd	nd	nd
Daudi	80.2	46.9	0.1	0	36.0	0.1	0.1	nd	nd

Note: results are the percentage of cells labelled by the different antibodies. nd: not done.

2.2 Enhanced expressions of CD40 and adhesion molecules on lymphoma cell lines triggered by CD28 molecules

The results are outlined in tables 2 and 3. After Raji and Daudi cells were co-cultured with CD28 transfected cell lines for 24 h at 37℃, the CD40 expression on them increased from 40.3% to 99.7% and 46.9% to 97.3% respectively. CD11a, CD18, CD54 and CD49d expressions were also enhanced, and reached nearly 100% on Raji cells and 66%—96% on Daudi cells. The induced expression can be blocked by adding the CD80 antibodies (20 μg/mL) to the above co-culture.

Furthermore, we compared the effects of the different ratios between CD28 positive cells and Raji cells, and studied the time-dependent change. As indicated in table 4, in the mean fluorescence den-

sity, the 2:1 ratio group is less effective than that of 1:1 ratio group at 24 h. The expressions of CD40, CD11a, CD18, CD49d and CD54 reached to the maximum at 24 h, but after 48 h co-culture the expressions began to decrease, and some membrane molecules approached nearly the normal level.

Table 2 Enhanced expressions of adhesion and costimulatory molecules on Raji after stimulated by CD28 transfected cell lines

Group	Negative control	CD80	CD40	CD11a	CD18	CD49d	CD54
A	6.1%	76.2%	40.3%	31.3%	21.2%	11.3%	40.1%
	(1.95)	(10.9)	(5.47)	(2.22)	(1.88)	(1.81)	(8.97)
B	1.8%	99.8%	99.7%	99.7%	99.7%	99.6%	93.6%
	(2.93)	(123.9)	(29.6)	(45.4)	(9.92)	(4.98)	(11.6)
C	1.2%	0.4%	0%	0%	0%	0%	0.4%
	(1.27)	(1.38)	(0)	(0)	(1.27)	(0)	(1.30)

The results are the percentage of cells labelled by the different antibodies. The numbers in brackets indicate the mean density of the fluorescence profiles. A, Raji cells; B, Raji cells stimulated by CD28 transfected cell lines; C, Raji cells stimulated by CD28 transfected cell lines plus CD80 antibody.

Table 3 Enhanced expressions of adhesion and costimulatory molecules on Daudi after stimulated by CD28 transfected cell lines

Group	Negative control	CD80	CD40	CD11a	CD18	CD49d	CD54
A	1.0%	1.0%	46.9%	0.1%	0%	0.1%	36.0%
	(1.16)	(1.18)	(1.41)	(1.25)	(0)	(1.12)	(1.46)
B	1.8%	96.8%	97.3%	69.1%	66.6%	94.7%	96.3%
	(1.42)	(12.0)	(28.5)	(2.88)	(2.70)	(8.75)	(21.4)
C	1.6%	0.2%	0.3%	1.4%	1.6%	2.0%	0.5%
	(1.69)	(1.31)	(2.20)	(1.36)	(1.36)	(5.80)	(6.28)

The results are the percentage of cells labelled by the different antibodies. The numbers in brackets indicate the mean density of the fluorescence profiles. A, Daudi cells; B, Daudi cells stimulated by CD28 transfected cell lines; C, Raji cells stimulated by CD28 transfected cell lines plus CD80 antibody.

At the same time, we observed the cluster growth of Raji and Daudi cells stimulated by the CD28 transfected cell lines under the microscope, as shown in fig. 1. However, we found that this stimulation did not influence the growth of either myeloma cells or lymphoma cells.

Table 4 Inducible expression of adhesion and costimulatory molecules on Raji dependent on the cell number of CD28 transfected cell lines and the time of co-culture[a)]

Group	Negative control	CD80	CD40	CD11a	CD18	CD49d	CD54
A	6.1%	76.2%	40.3%	31.3%	21.2%	11.3%	40.1%
	(1.95)	(10.90)	(5.47)	(2.22)	(1.88)	(1.81)	(8.97)
B	1.8%	99.8%	99.7%	99.7%	99.7%	99.6%	93.6%
	(2.93)	(123.9)	(29.6)	(45.4)	(9.92)	(4.98)	(11.6)
C	8.5%	99.8%	85.6%	98.1%	99%	29.3%	74.1%
	(2.08)	(47.90)	(7.57)	(15.20)	(7.89)	(1.58)	(7.37)
D	1.6%	90%	37.8%	32.7%	38.6%	20.8%	47.4%
	(4.35)	(19.00)	(7.25)	(2.78)	(5.41)	(2.64)	(11.80)

a) The results are the percentage of cells labelled by the different antibodies. The numbers in brackets indicate the mean density of the fluorescence profiles. A, Raji cells; B, Raji cells stimulated by CD28 transfected cell lines (Raji cells: CD28 positive cells = 1:1, 24 h); C, Raji cells stimulated by CD28 transfected cell lines (Raji cells: CD28 positive cells = 2:1, 24 h); D, Raji cells stimulated by CD28 transfected cell lines (Raji cells:CD28 positive cells = 2:1, 48 h).

Unlike the two B lymphoma cell lines, there was no detectable change of CD40 expression in a series of myeloma cell lines stimulated by the CD28-transfected cell lines (results not shown).

2.3 Inductions of CD80, CD95 and CD54 on HMCL and lymphoma cell lines by the CD40L transfectant

In order to enhance the antigen-presenting capacity of myeloma and lymphoma cells, we looked into the effect of CD40 activation on the expression of co-stimulatory molecules. As outlined in table 5, the CD40L cells did not up-regulate CD80 expression on XG2, 8226, Raji and Daudi cell lines. However, the density of CD54 was up-regulated on XG2, 8226 myeloma cells after CD40 activation; CD54 expression was not modulated on Daudi cells and was decreased on Raji cells by co-culture with CD40L transfected cells.

Table 5 Inductions of CD80 and CD54 on HMCL and lymphoma cell lines by the CD40L transfectant[a)]

Cell line	Before stimulated by CD40L transfectant		After stimulated by CD40L transfectant	
	CD80	CD54	CD80	CD54
XG2	0.5%	86.3%	0.8%	94.8%
8226	0.8%	27.7%	4.8%	58.4%
Raji	44.1%	76.3%	49.7%	45.3%
Daudi	80.2%	87.3%	72.1%	86.5%

a) The results are the percentage of cells labelled by the different antibodies.

2.4 Enhanced allo-MLR capacity of B lymphoma cells by CD28/CD80 interaction

After exposed to CD28 transfected cell lines, Raji and Daudi lines were used as stimulator cells in primary allogeneic MLR, a significant T cell proliferation was observed (figs. 1 and 2). The SI, as determined in allogeneic MLR, was enhanced from 1.73 to 2.79 for Raji and from 3.26 to 6.29 for Daudi when the ratio of stimulator to responder was 1:10.

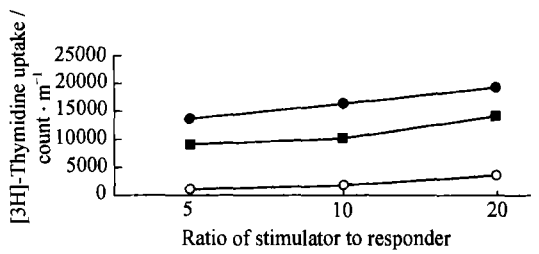

Fig. 1. Allogeneic MLR of Raji before and after exposured to CD28 transfected cell line. Raji mixed with $CD28^+$ cells (●) were more potent allostimulators than Raji plus $CD28^+$ cells blocked by CD80 antibodies (■) and PBMCs (○).

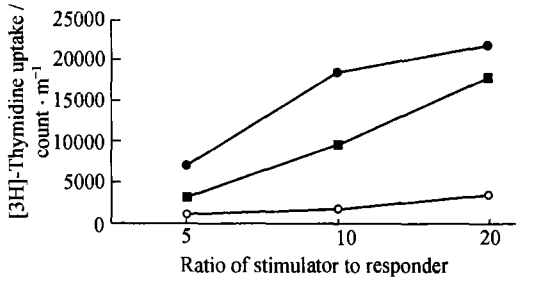

Fig. 2. Allogeneic MLR of Daudi before and after exposured to CD28 transfected cell line. Daudi mixed with $CD28^+$ cells (●) were more potent allostimulators than Daudi plus $CD28^+$ cells blocked by CD80 antibodies (■) and PBMCs (○).

3 Discussion

The aim of this study was to determine whether myeloma and lymphoma tumor cells could function as antigen-presenting cells (APC) and generate specific anti-tumor T cells by increasing their ex-

pressions of membrane molecules. As demonstrated by our results[8—10], myeloma cells expressed most of the adhesion molecules and showed variable expression of CD40, but generally no detectable CD80. The lack of CD80 on myeloma cell lines may explain the poor ability of HMCL to induce $CD8^+$ allogeneic T cell activation, IL-2 production and proliferation. As to the B lymphoma cell lines, they expressed some adhesion molecules as did the myeloma cells. Unlike the myeloma lines, however, they also expressed CD80 and CD40.

In order to upregulate CD80 expression, we used CD40 activation known to enhance CD80 expression on several B cell neoplasias as well as on normal B and dendritic cells. We found that CD80 could not be induced significantly on either the myeloma cell lines or the two B lymphoma cell lines. Except for CD54, no adhesion or co-stimulatory molecules could be induced on HMCL by CD40 activation. These findings suggest a selective defect regarding CD80 induction by CD40 activation on myeloma and lymphoma cell lines, as was reported previously in the case of acute myeloid leukemia cells[11]. Furthermore, when we observed the CD28 stimulation in myeloma and B lymphoma cell lines, an interesting result was obtained in the two B lymphoma cell lines. The interaction of CD28-CD80 could obviously increase the expressions of CD40 and of certain adhesion molecules on the two B lymphoma cell lines. The cells expressing high levels of CD40 and of certain adhesion molecules enhanced T cell stimulatory capacity. However, we did not get the same results in the six myeloma cell lines. Therefore, in myeloma cell lines the expressions of CD40 and CD80 could not be induced by either CD28 or CD40L stimulation; this deficiency may thus explain in part why myeloma tumor cells cannot activate an effective anti-tumor reaction *in vivo*. There may be other means of increasing the expression of co-stimulatory molecules on the tumor cells. Some researchers have demonstrated that the myeloma cells can become functional APC by transfecting the CD80 gene into tumor cells and thus effectively induce an antigen specific CTL *in vitro*. As to the two B lymphoma cell lines, although they expressed CD80 and CD40 to some extent, we assume that the expressions of CD40 and CD80 on lymphoma cell lines are still insufficient to make them effective APC. Interestingly, in this work we found that CD80 stimulation in lymphoma cell lines does increase the expressions of CD40 and of certain adhesion molecules, the molecules related to the immunogenicity of tumor cells. Importantly, in normal B lymphocytes, either CD40L/CD40 or CD28/CD80 interaction can induce the increasing expressions of CD80 or CD40, respectively. Thus the two pathways exist in the normal B lymphocytes. However, in B lymphoma cells, the CD28/CD80 pathway exists, but there is a deficiency of the CD40L/CD40 pathway. The myeloma cells lack both pathways (summarized in fig. 3).

The CD40 molecule belongs to the tumor necrosis factor/nerve growth factor receptor family[5,6]. CD40 has been found in many carcinomas such as those of the bladder, ovary and liver as well as in melanomas, and this molecule is now thought to play a general role in immunoregulation. Both anti-CD40 antibody and soluble CD40 ligand (sCD40L) provide negative signals to a variety of neoplastic B-lymphocytes and inhibit tu-

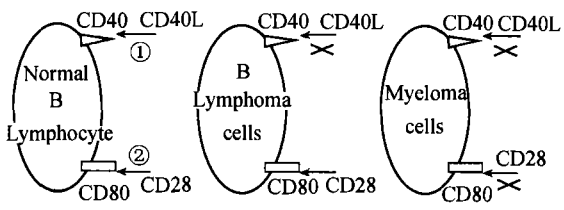

Fig. 3. CD28/B7 and CD40/CD40L pathways in normal B cells, myeloma and lymphoma cells.

mor cell growth[12]. Thus, anti-CD40 antibody and sCD40L have been considered in a new immunotherapeutic approach to these diseases[5,6]. As the present results show, CD28 stimulation can increase drastically the expressions of CD80, CD40 and of some adhesion molecules. We propose a new immunotherapeutic approach to human B-cell lymphoma. Firstly, the expressions of CD40 and some adhesion molecules on tumor cells can be upregulated through CD28-CD80 interaction. Secondly, such "upregulated" tumor cells may then activate the anti-tumoral T cells either as such or on the basis of strong CD40 expression after CD40 activation, using either anti-CD40 monoclonal antibody or CD40 ligation, employing sCD40L. The latter CD40 signal pathway might then generate more effective anti-tumor immunity. This may provide a novel concept of immunotherapeutic intervention.

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