Article

Fine-Tuned Expression of Programmed Death 1 Ligands in Mature Dendritic Cells Stimulated by CD40 Ligand Is Critical for the Induction of an Efficient Tumor Specific Immune Response

Tao Gu[1, 2], Yibei Zhu[1, 2], Cheng Chen[1], Min Li[1], Yongjing Chen[1], Gehua Yu[1], Yan Ge[1], Shiyong Zhou[1], Huan Zhou[1], Yong Huang[1], Yuhua Qiu[1] and Xueguang Zhang[1]

During maturation, murine myeloid dendritic cells (DCs) upregulated the expressions of CD11c, CD25, CD40, CD80, CD86, MHC II and programmed death 1 ligands 1 and 2 (PD-L1 and PD-L2). Differential expression patterns of PD-L1 and PD-L2 were found when DCs were triggered by CD40 ligand and TNF-α. PD-L1 expression was repressed and PD-L2 expression remained unchanged in mature CD40-ligated DCs, whereas TNF-α stimulated DCs kept high expression of PD-L1 and significantly enhanced PD-L2 expression on DCs. Proliferations of T lymphocytes stimulated by immature DCs were enhanced by blockade of the PD-1 and PD-1 ligand interaction. But inhibitive effects were found in T lymphocytes stimulated by CD40-ligated DCs. With the fine-tuned expressions of PD-L1 and PD-L2, CD40-ligated DCs could sustain a longer activation period and elicit a more efficient T lymphocyte activation. *Cellular & Molecular Immunology*. 2008;5(1):33-39.

Key Words: dendritic cell, CD40 ligation, PD-L1, PD-L2, antitumor immunity

Introduction

Dendritic cells (DCs), the most potent professional antigen presenting cells (APCs), which expressed high levels of MHC class I and II and costimulatory molecules, were efficient in phagocytosing antigens, migrating to lymphoid organs, and presenting antigens to naïve T lymphocytes. DCs played an important role in initiating innate and adaptive immune responses, especially in establishing an efficient antitumor immunity (1, 2). While immature DCs had a specialized antigen uptake and processing machinery, mature DCs exerted an extraordinary capacity to present antigens and stimulate naïve T cells. Functionally, mature DCs were over 100 times more potent than macrophages in activating naïve T cells *in vitro* (3, 4).

Apoptotic tumor cells represented an attractive alternative to provide DCs with abundant MHC class I and class II epitopes (5-8). As unfractionated antigenic "material", they could be efficiently phagocytosed by immature DCs *via* cell surface expressions of αvβ3, αvβ5 and CD36 and elicit tumor specific immune responses. However, antigen uptake was insufficient to induce full DC maturation. In order to induce DC maturation and trigger their transition from immature Ag-capturing cells to mature Ag-presenting cells, various cytokines or biological factors had to be applied. Notably, several studies demonstrated that CD40 ligation of DCs (either by cross-linking of CD40 on the surface of DCs by soluble CD40L, by ectopic expression of CD40L upon transfections or by mAb CD40 agonists) was most potent to induce antitumor immunity (3, 4, 9-14). However, the mechanisms of CD40 ligation on DC maturation remained to be elucidated.

Recently, it was well realized that the balance between positive versus negative signals on DCs was critical in the activation of T lymphocytes and the generation of an efficient protective immunity. PD-1, a member of the immunoglobulin (Ig) superfamily, was expressed on a subset of thymocytes and upregulated on T and B lymphocytes, monocytes and myeloid cells after activation. PD-1, a CD28 homolog, harbored two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in its cytoplasmic domain (15, 16). Programmed death 1 ligand 1 (PD-L1, or B7-H1) (17) and PD-L2, or B7-DC (18, 19) were members of the B7 family which shared approximately 38% amino acid identity. PD-L1 mRNA was constitutively expressed in heart, lung, kiney, liver and spleen and was found to be upregulated by IFN-γ in

[1]Medical Biotechnology Institute and Clinical Immunology Laboratory for Jiangsu Province, Soochow University, Suzhou, Jiangsu 215007, China;

[2]Tao Gu and Yi-Bei Zhu contributed equally to this work;

[3]Corresponding to: Dr. Xueguang Zhang, Medical Biotechnology Institute, Soochow University; Renmin Road 708, Suzhou, Jiangsu 215007, China. Tel: +86-512-6512-5022, E-mail: smbxuegz@public1.sz.js.cn

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endothelial cells, keratinocytes, DCs (20) and several tumor cell lines. PD-L2 mRNA was constitutively expressed in the liver and at lower levels in lung and spleen, and preferentially expressed in bone marrow derived and splenic DCs (18, 19). Previous studies indicated that PD-1 and PD-1 ligands function as important negative regulators since their interaction played an important role in repressing immune responses and maintaining peripheral tolerance (16, 21, 22). However, experimental data on the upregulation of PD-L1 and PD-L2, together with the positive costimulatory molecules CD40, CD80, and CD86, during DCs maturation provided some doubt on the role of PD-L1 and PD-L2 in controlling T lymphocyte activation. Therefore, we were interested in determining whether PD-L1 and PD-L2 expressions in mouse $CD8\alpha^+$ myeloid DCs deliver stimulatory or inhibitory signals for T cells in controlling an immune response. We found that PD-L1 expression was repressed while PD-L2 expression remained unchanged in mature CD40-ligated DCs. Moreover, interruption of PD-L1 and PD-L1 ligand contacts between T cells and mature CD40-ligated DCs resulted in a suppression of T cell proliferation. All data suggested that the fine-tuned expression of PD-1 ligands on mature DCs contributed to efficient tumor specific immune responses.

Materials and Methods

Mice and cell lines

Female BALB/c mice (H-2^d) and C57BL/6 mice (H-2^b), 6-8 weeks old, were obtained from Chinese Academy of Sciences, Shanghai Institutes for Biological Science, Experimental Animal Center and were housed under specific pathogen-free conditions in the laboratory animal facility of Soochow University Medical Animal Center. Mouse tumor cell lines including myeloma SP2/0, B cell lymphoma A20 and melanoma B16 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Mouse B cell lymphoma SB4 was established in our laboratory. Murine CD40L-Chinese hamster ovary cell line (CHO) was kindly provided by Dr. Ying Wan (Third Military Medical University, Chongqing, China) and selected by 0.3 mg/ml G418. SP2/0 cells were maintained in high glucose DMEM medium supplemented with 15% heat-inactivated FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. Murine CD40L-CHO, A20 and B16 cells were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM 2-ME. All media and supplements were obtained from Gibco BRL Life Technologies.

Apoptosis induction in tumor cell lines

Apoptosis of tumor cell lines A20, B16, SB4 and SP2/0 was induced by 100 Gy ^{60}Co-gamma irradiation, followed by incubation for 10-14 h at 37°C. Early cell apoptotic rate was defined by FITC-labeled Annexin V^+/propidium iodide $(PI)^-$ staining, determined by flow cytometry (FCM) (Epics Altra, Beckman-Coulter, USA). Cell death was assessed by morphology (staining with trypan blue).

Preparation of mature DCs

After removing all muscle tissues with gauze from femurs and tibias, bones were placed in a 60 mm dish with 70% alcohol for 1 min, washed twice with HBSS and transferred into a fresh dish with RPMI 1640 medium. Bone marrow was flushed out and passed through a nylon mesh to remove small pieces of bones and debris. Erythrocytes were depleted by ammonium chloride lysis. Pro-B/T, T, B, NK lymphocytes and MHC II positive cells were killed with a cocktail of mAbs and rabbit complement for 60 min at 37°C. The mAbs were anti-CD4, anti-CD8, anti-CD19, anti-CD56, anti-Ia, and anti-B220/CD45R (Immunotech, France). Cells were then plated in 24-well plates at 8.0×10^5/ml in RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, 20 ng/ml recombinant murine GM-CSF (Immugenex, USA), and 10 ng/ml recombinant murine IL-4 (Immugenex). Medium was replaced every 2-3 d. On day 5 or 6, loosely adherent proliferating DC aggregates were dislodged and replated at 10^6/ml in fresh medium with cytokines. Apoptotic tumor cells were added into immature DCs (tumor cells : DCs = 1 : 3) and incubated overnight. Then, 10 ng/ml TNF-α, or mCD40L-CHO cells (treated with 0.05 mg/ml MMC for 45 min, mCD40L-CHO cells : DCs = 1 : 4, according to our previous results) were added to induce DC maturation for 48 h, respectively. Released, nonadherent and loosely adherent cells with the typical morphological features of DCs were used to activate T lymphocytes.

T lymphocyte proliferation assay

T lymphocytes obtained from spleens of BALB/c mice (H-2^d) and C57BL/6 mice (H-2^b) by nylon wool purification were activated by DCs ligated with CD40, DCs stimulated with TNF-α, or by DCs loaded with apoptotic tumor cells, respectively. Four different tumor cell lines (including A20, B16, SP2/0 and SB4) were induced to apoptosis. Immature DCs were loaded with these four different apoptotic tumor cells, respectively. Syngeneic T lymphocytes (2×10^4/well) were incubated with corresponding DCs in 96-well culture plates according to DCs : responder T lymphocytes = 1 : 50 (according to our previous experiments, data not shown) for 56 h in RPMI 1640 with 10% FCS and 30 IU/ml IL-2. ^{3}H-thymidine (1 μCi/well) was added and the cells were harvested onto filter paper after 16-18 h. T lymphocytes stimulated with mCD40L-CHO cells (treated with 0.5 mg/ml MMC for 45 min, mCD40L-CHO cells : T lymphocytes = 1 : 50) or medium (10% FCS/RPMI 1640) only were used as controls. *In vitro* proliferation of T lymphocytes stimulated by DCs was determined by the measurement of thymidine incorporation. The stimulatory index was calculated as (experimental cpm value - background cpm value) / (blank control cpm value - background cpm value).

Cytolytic T cell assay

For cytolytic assay T lymphocytes were stimulated and grouped as above. Purified T lymphocytes at 1×10^6/ml were

co-cultured with DCs at a 50 : 1 ratio in 24-well plates in 2 ml final Vol. When T lymphocytes were stimulated for 5-6 days *in vitro*, the specific cytolytic rate of T lymphocytes induced by DCs was assessed by a 4 h standard ^{51}Cr-release assay as described previously. The assay was carried out in U-bottomed, 96-well microtiter plates. A20, B16, SB4 or SP2/0 tumor cells were used as target cells respectively. Each assay was prepared by incubation with 100 μCi/ml ^{51}Cr (Biotech, USA) for 2 h at 37°C and placed at 2 × 10^4 cells/well in triplicates. A20 or SP2/0 tumor cells were used as control target cells according to different groups of T lymphocytes (effector cells). Then, activated T lymphocytes were co-incubated with ^{51}Cr labeled target tumor cells for 4 h as E : T = 50 : 1 (according to our previous experiments, data not shown). Supernatants were harvested and counted. The specific cytolytic rate was calculated as 100 × [(experimental release - spontaneous release) / (maximal release - spontaneous release)]. Data were expressed as the mean ± SEM of three independent experiments using different apoptotic tumor cells loaded DCs.

Flow cytometric characterisation of DCs

For phenotypic analysis, myeloid DC progenitor cells (CD8α$^-$) from mouse bone marrow, DCs cultured for 3 day or 5-6 days, DCs loaded with apoptotic tumor cells, DCs stimulated by TNF-α or mCD40L-CHO cells for 24 h or 48 h and loaded with apoptotic tumor cells were stained with a panel of antibodies characteristic to DCs, including direct immunofluorescence with phycoerythrin (PE)-labeled mouse antibodies: CD8α, CD11b, CD11C, CD25, CD40, CD80, CD86 (Immunotech, France), MHC II (SouthernBiotech, USA), PD-L1, PD-L2 (eBioscience, USA) and then analyzed by FCM. Isotype-matched monoclonal antibodies were used as controls.

Determination of PD-L1 and PD-L2 mRNA synthesis by RT-PCR and real-time PCR

RNA was extracted from 5 × 10^6 DCs using Trizol (Gibco, Life Technologies) according to the manufacturer's instructions. Random primers and reverse transcriptase (MBI, USA) were used to synthesize cDNAs. Primers were designed using Primer Express Software (Primer 5.0) as follows: mouse PD-L1: 5'-CCT GCT TGC GTT AGT GGT GTA C-3' and 5'-TCA GAC TGC TGG TCA CAT TGA G-3', mouse PD-L2: 5'-ATG CTG CTC CTG CTG CCG ATA CT-3' and 5'-AGG TCC AGA TCT GGG AAG AAG AGA-3', mouse β-actin: 5'-GTA TGG AAT CCT GTG GCA TCC ATG-3' and 5'-GAC TCA TCG TAC TCC TGC TTG CTG-3'. PCR cycling conditions were 94°C for 1 min, 60°C for 1 min and 72°C for 1.5 min for 35 cycles, and 72°C for 10 min. Then, PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. Amplication of β-actin mRNA was used as a control. The levels of mRNAs were also determined by real-time PCR. 100 pmol of each primer and 1× SYBR Green master mix (PE Applied Biosystems) were added in a 50 μl PCR mixture. PCR cycling conditions were the same as above. Results were normalized to β-actin and expressed as fold increase relative to that of DCs loaded with apoptotic tumor cells which were induced by mCD40L-CHO cells for 24 h. Data were presented as the mean ± SEM of two independent experiments using different apoptotic tumor cells loaded with DCs.

Blockade of PD-1 and PD-ligand interaction

T lymphocytes obtained from spleens of BALB/c mice (H-2^d) and C57BL/6 mice (H-2^b) by nylon wool purification were stimulated by CD40-ligated DCs, or by immature DCs cultured 5-6 days. Then 10 μg/ml anti-PD-L1 mAb (clone 10B5, hamster IgG), 10 μg/ml anti-PD-L2 mAb (clone YL-1, rat IgG1), and both were added to the mixture of T lymphocytes and mature CD40-ligated DCs or 5-6 day immature DCs (IM-DC), respectively. As control cells T lymphocytes only (T) and T lymphocytes stimulated by DCs (DC-T) with isotype-matched mAbs were used. Isotype-matched mAbs (Rat IgG1, R3-34; hamster IgG, A19.3) were obtained from BD-PharMingen and used as negative controls. All mAbs had an endotoxin level of 2 endotoxin U/mg. About 2 × 10^4/well syngeneic T lymphocytes were incubated in 96-well culture plates. Corresponding DCs were added according to DCs : responder T lymphocytes = 1 : 50 (according to our previous experiments, data not shown) for 56 h. ^{3}H-thymidine (1 μCi/well) was added and the cells were harvested onto filter paper after 16-18 h co-culture. *In vitro* proliferation of T lymphocytes stimulated by DCs was determined by the measurement of thymidine incorporation. The stimulatory index was calculated as (experimental cpm value - background cpm value) / (blank control cpm value - background cpm value). The mAbs against anti-PD-L1 and anti-PD-L2 were kindly provided by Prof. Lieping Chen (Department of Immunology, Mayo Clinic, Rochester, Minnesota, USA). Mature CD40-ligated DCs or 5-6th day immature DCs were incubated with anti-PD-L1 mAb, anti-PD-L2 mAb, or both for 2 h, respectively. Unbound antibodies were removed by washing prior to addition of T cells. This was done to ensure that effects of antibodies were due to blockade of PD-L1 or PD-L2 on DCs instead of on T cells, since PD-L1 and PD-L2 were also expressed on activated T cells.

Results

Phenotypic characteristics of DCs

During maturation, myeloid DCs (CD8α$^-$) upregulated the expressions of CD11c, CD25, CD40, CD80, CD86, MHC II. According to the expressions of CD25, CD40, CD11c, CD80, CD86 and MHC II, DCs loaded with apoptotic tumor cells were not fully mature (data not shown). However, these cell surface molecules were further upregulated when those DCs were stimulated by TNF-α or by mCD40L-CHO cells for 24 h or 48 h, respectively. This resulted in a strong upregulation of CD25 and CD40 receptors while the expressions of DC-specific surface molecules CD11c, CD80, CD86 and MHC II were also found to be increased, albeit in a less pronounced manner (data not shown). No difference in the

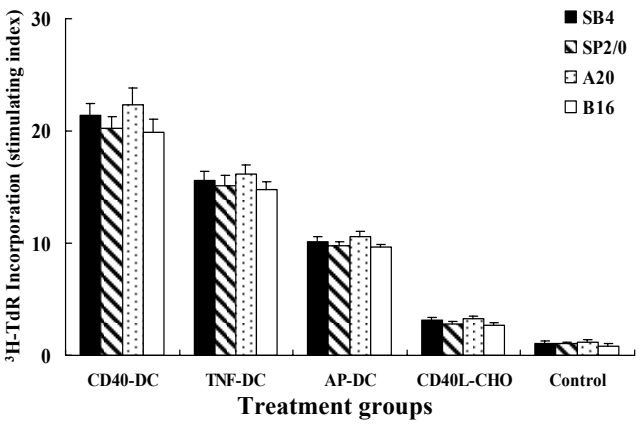


Figure 1. Proliferation of T lymphocytes stimulated by DCs which were triggered by various treatments. T lymphocytes obtained from murine spleens by nylon wool purification were stimulated by DCs stimulated by CD40 ligation (CD40-DC) or TNF-α (TNF-DC), by DCs loaded with apoptotic tumor cells (AP-DC), or by mCD40L-CHO cells (CD40L-CHO) (treated with 0.5 mg/ml for 45 min, mCD40L-CHO cells : T lymphocytes = 1 : 50) and 10% FCS/RPMI 1640 (Control). Syngeneic T lymphocytes (2×10^4/well) were incubated with DCs in 96-well culture plates according to DCs : responder T lymphocytes = 1 : 50 (according to our previous experiments, data not shown) for 56 h. Then, tritiated thymidine (1 μCi/well) was added and the cells were harvested on filter paper after 16-18 h co-culture. Results were determined by the measurement of thymidine incorporation. Data were expressed as the mean ± SEM of three independent experiments using different apoptotic tumor cells loaded DCs.

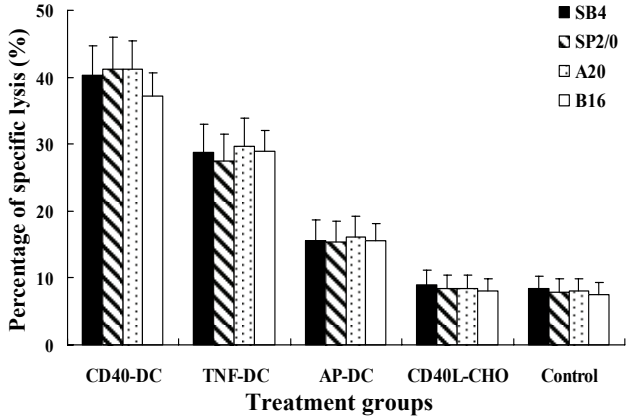


Figure 2. Tumor specific cytolytic rate of CTL activated by DCs was determined by a ^{51}Cr release assay. T lymphocyte stimulation and groups were identical to Figure 1. When T lymphocytes were stimulated for 5-6 days *in vitro*, their cytolytic ability was assessed. A20, B16, SB4 or SP2/0 tumor cells were used as target cells corresponding to different groups and were prepared by incubation with 100 μCi/ml ^{51}Cr (Biotech, USA) for 2 h at 37°C and placed at 2×10^4 cells/well in triplicate. SB4 or SP2/0 tumor cells were used as control target cells according to corresponding T lymphocytes (effector cells). Then, activated T lymphocytes were co-incubated with ^{51}Cr labeled target tumor cells for 4 h as E : T = 50 : 1 (according to our previous experiments, data not shown). Four different tumor cell lines (including A20, B16, SP2/0 and SB4) were induced to apoptosis. Immature DCs were loaded with these four different apoptotic tumor cells, respectively. Data are expressed as mean ± SEM of three independent experiments using different apoptotic tumor cells for the loading of DCs.

expression of these surface molecules was detected between DCs ligated with CD40L or stimulated with TNF-α ($p > 0.05$).

CD40 ligation DCs induced more efficient tumor specific immunity

T lymphocytes obtained from spleens of BALB/c mice (H-2^d) and C57BL/6 mice (H-2^b) were purified by nylon wool passage (> 80% CD3 positive, analyzed by FCM, data not shown). After co-culturing T lymphocytes with DCs for 24 h, we found that T lymphocytes increased in size and gradually the activation morphology appeared. Forty-eight hours later, T lymphocytes appeared to be active and in cell cycle. As determined by ^{3}H-TdR incorporation, this was more evident in the population of DCs ligated with CD40L than in DCs stimulated by TNF-α ($p < 0.05$) (Figure 1). In addition, the results of the ^{51}Cr-release assay showed that the specific cytolytic rate of T lymphocytes induced by CD40 ligation DCs (E : T = 50 : 1) was much higher than that of all other groups ($p < 0.05$) (Figure 2).

Different expression patterns of PD-L1 and PD-L2 during DC maturation

FCM results demonstrated that low levels of both PD-L1 and PD-L2 were expressed in myeloid DCs from bone marrow progenitor cells (CD8α$^-$). While induced with GM-CSF and IL-4, and PD-L2, in particular, PD-L1 expression was gradually upregulated. As shown in Figure 3A, PD-L1 expression increased to 83% ± 5.4% in 5-6th day immature DCs, while PD-L2 increased comparatively lower, with the rate of 48% ± 3.6%. In contrast, upon co-culture of DCs with apoptotic tumor cells for 20 h, expressions of PD-L1 and PD-L2 were found to be slightly upregulated only (Figure 3A).

Down-regulating PD-L1 and PD-L2 expression in DCs ligated with CD40

Differential expression patterns were found when DCs loaded with apoptotic tumor cells were stimulated by mCD40L-CHO cells or TNF-α. PD-L1 expression was remarkably reduced to 55% ± 2.9% when DCs loaded with apoptotic tumor cells were stimulated by mCD40L-CHO cells for 24 h. They maintained this moderate level for 48 h (Figure 3B). However, when stimulated by TNF-α for 24 h or 48 h, PD-L1 expression was further increased up to 94.5% ± 2.7%, 98.5% ± 1.1%, and significantly higher than that in CD40L-ligated DCs ($p < 0.05$). PD-L2 expression was slightly down-regulated and maintained at about 40%-50% in spite of CD40 ligation for 24 h, or 48 h (Figure 3) while upregulated to 85.1% ± 4.1%, 87.2 ± 4.3% when stimulated by TNF-α for 24 h or 48 h (Figure 3B), significantly higher than that of CD40-ligated DCs ($p < 0.05$). When DCs loaded

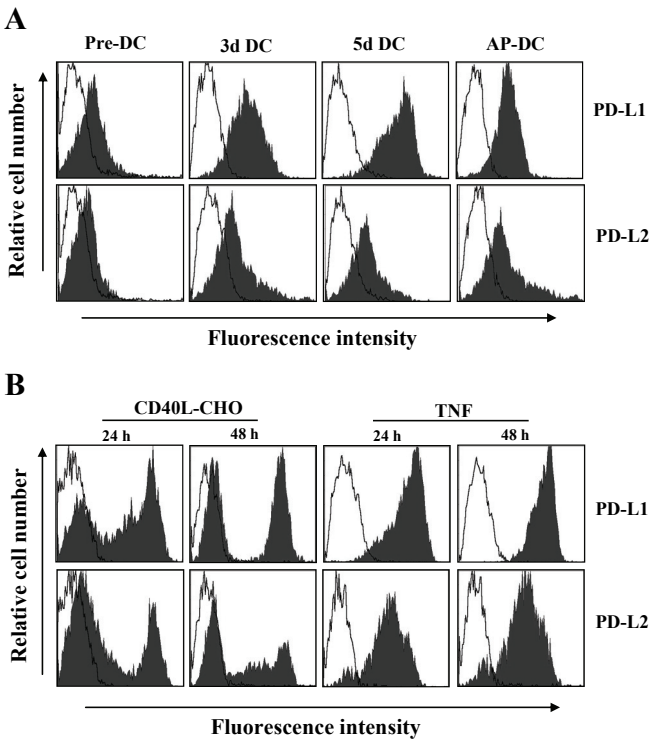
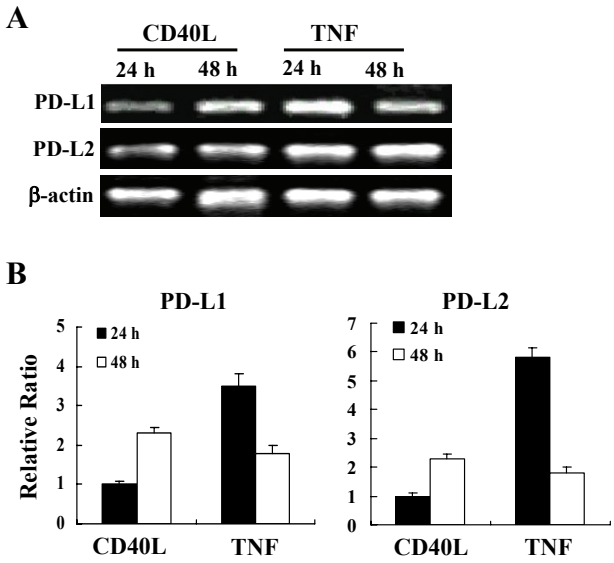


Figure 3. Expression of PD-L1 and PD-L2 during DC maturation analyzed by FCM. (A) PD-L1 and PD-L2 expression was determined for DC progenitor cells (pre-DC), DCs cultured for 3 days (3 d DC), DCs cultured for 5-6 days (5 d DC), DCs loaded for 20 h with apoptotic tumor cells (AP-DC). (B) PD-L1 and PD-L2 expression was determined for DCs loaded with apoptotic tumor cells and stimulated by mCD40L-CHO cells (treated with 0.05 mg/ml MMC for 45 min, mCD40L-CHO cells : DCs = 1 : 4) for 24 h or 48 h, DCs loaded with apoptotic tumor cells and stimulated by TNF-α (10 ng/ml) for 24 h or 48 h.



Firure 4. PD-L1 and PD-L2 mRNA levels in DCs ligated with CD40 or DCs stimulated with TNF-α detected by RT-PCR and real-time PCR. (A) PD-L1 and PD-L2 mRNA levels determined by RT-PCR for DCs loaded with apoptotic tumor cells which were induced by mCD40L-CHO cells for 24 h, for DCs loaded with apoptotic tumor cells which were induced by mCD40L-CHO cells for 48 h, for DCs loaded with apoptotic tumor cells which were stimulated by 10 ng/ml TNF-α for 24 h, and for DCs loaded with apoptotic tumor cells stimulated by 10 ng/ml TNF-α for 48 h. Amplication of β-actin mRNA was used as a control. (B) and (C) PD-L1 and PD-L2 mRNA levels determined by real-time PCR, respectively. Samples were the same as A. Results were normalized to β-actin and expressed as fold increase relative to CD40L-DCs at 24 h. Data are expressed as mean ± SEM of two independent experiments using different DCs loaded with apoptotic tumor cells.

with apoptotic tumor cells were stimulated by mCD40L-CHO cells, DCs appeared to be divided into two subsets. PD-L1 expression in one subset DCs was entirely inhibited, while in the other subset it was upregulated (Figure 3B). A similar phenomenon of PD-L2 expression was also found and shown in Figure 3B.

These findings were confirmed by RT-PCR (Figure 4A) and real-time PCR (Figures 4B). Whereas PD-L1 mRNA levels increased 3.5 ± 0.3 fold in DCs stimulated by TNF-α for 24 h, compared to mCD40L-CHO cell stimulation ($p < 0.05$). PD-L1 mRNA levels of DCs induced by mCD40L-CHO cells for 48 h was slightly upregulated, compared to DCs stimulated with TNF-α for 48 h ($p > 0.05$). Similar results were also found for PD-L2 mRNA levels.

Blocking the interaction between PD-L and PD-1 affects the proliferation of T lymphocytes

Adding 10 μg/ml anti-PD-L1 mAb or 10 μg/ml anti-PD-L2 mAb, or both Abs to a mixture of T lymphocytes and mature CD40-ligated DCs slightly inhibited the proliferation of T lymphocytes (Figure 5). Anti-PD-L2 mAb seemed to have a more powerful ability than anti-PD-L1 mAb in inhibiting proliferation of T lymphocytes stimulated by CD40-ligated DCs, adding both Abs resulted in the strongest inhibition ($p < 0.05$). Blockade of PD-1 and PD-ligand interactions between T lymphocytes and mature CD40-ligated DCs did not enhance the proliferation of T lymphocytes.

However, blockade of the PD-1 and PD-ligand interactions between T lymphocytes and 5-6th day immature DCs could promote the proliferation of T lymphocytes (Figure 5). Anti-PD-L1 mAb seemed to have a more powerful ability than anti-PD-L2 mAb in upregulating proliferation of T lymphocytes stimulated by immature DCs. The proliferation of T lymphocytes stimulated by immature DCs was enhanced most effectively by adding anti-PD-L1 mAb and anti-PD-L2 mAb together ($p < 0.05$). Similar results were obtained by using pre-incubated DCs with mAb of anti-PD-L1 or/and anti-PD-L2 (date not shown).

Discussion

Our results indicated that tumor specific immunity relied on DCs activation through CD40-CD40L interactions. DCs

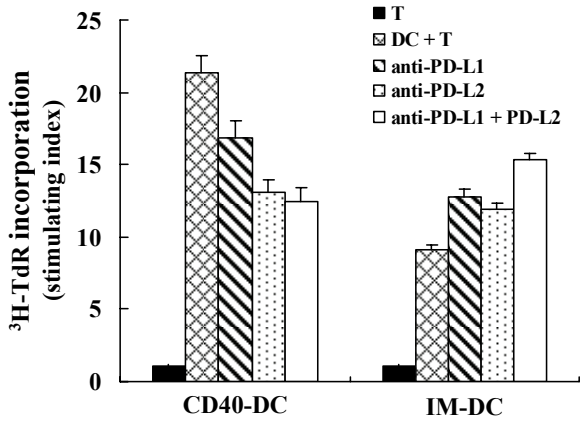


Figure 5. Blocking the interaction between PD-1 and PD-L affects proliferation of T lymphocytes stimulated by DCs. Anti-PD-L1 mAb (anti-PD-L1, 10 μg/ml), anti-PD-L2 mAb (anti-PD-L2, 10 μg/ml), or both Abs (anti-PD-L1+L2) were added to a mixture of T lymphocytes and mature DCs ligated with CD40 (CD40-DC) or of T cells and immature DCs cultured for 5-6 d (IM-DC), respectively. T lymphocytes only (T) or T lymphocytes stimulated by DCs (DC-T) were incubated as proliferation controls. Syngeneic T cells (2×10^4/well) were incubated in 96-well culture plates. DCs were added according to DCs : responder T lymphocytes = 1 : 50 (according to our previous experiments, data not shown) for 56 h, followed by the addition of tritiated thymidine (1 μCi/well). The cells were harvested onto filter paper after 16-18 h co-culture and results were determined by the measurement of thymidine incorporation. Data are shown as mean ± SEM of three independent experiments using different apoptotic tumor cells loaded with DCs.

loaded with apoptotic tumor cells and induced by CD40L transfectants were more potent to stimulate tumor specific immunity than DCs stimulated with TNF-α *in vivo* (data not shown) and *in vitro* ($p < 0.05$) (Figure 1 and Figure 2). However, the molecular mechanisms controlling the potent anti-tumor effect of CD40-ligated DCs were still poorly understood. Our results showed that CD11c, CD25, CD40, CD80, CD86, and MHC II molecules were all increased during DC maturation and appeared to be important positive molecules to induce T lymphocyte activation. But no difference was found between CD40-ligated DCs and TNF-α stimulation ($p > 0.05$).

The expressions of PD-L1 and PD-L2, especially PD-L1, were gradually upregulated when bone marrow progenitor DCs ($CD8\alpha^-$) were induced with GM-CSF and IL-4 *in vitro* (Figure 3). These were consistent with previous reports that PD-L1 and PD-L2 expressions on $CD8\alpha^-$ myeloid DCs were induced by GM-CSF stimulation (20). GM-CSF and/or IL-4 induced and maintained PD-L1 and PD-L2 expressions, especially the upregulation of PD-L1.

Obvious contrasts in PD-L1 expression were found when DCs loaded with apoptotic tumor cells were stimulated by mCD40L-CHO cells or TNF-α (Figure 3B). PD-L1 expression on DCs was found to be reduced to 55% ± 2.9% when they were stimulated by mCD40L-CHO cells for 24 h and for 48 h. However, PD-L1 expression was further increased, up to nearly 100%, when DCs were stimulated by TNF-α for 24 h or 48 h. In contrast, PD-L2 expression was slightly decreased and maintained at 42.1% ± 3.2% of moderate level, in spite of stimulation for 24 h or 48 h with mCD40L-CHO cells. PD-L2 expression was substantially increased to 85% ± 4.1% when stimulated by TNF-α for 24 h or 48 h (Figure 3B), i.e., significantly higher than by CD40-ligated DCs ($p < 0.05$). The differential expressions of PD-L1 and PD-L2 in CD40-ligated DCs or TNF-α-stimulated DCs indicated that CD40 ligation provided a potent signal to inhibit PD-L1 expression, while TNF-α stimulation promoted surface expressions of two negative costimulatory molecules, especially of PD-L2. Thus, the different capacities of CD40-ligated DCs and TNF-α-stimulated DCs to induce tumor-specific immunity might be due to the differential expression levels of PD-L1 or PD-L2. These findings were confirmed by RT-PCR and real-time PCR assays for PD-L1 and PD-L2 mRNA levels (Figure 4).

We also found that blockade of PD-1 and PD-ligand interactions between T lymphocytes and mature CD40-ligated DCs did not increase, but slightly inhibited the proliferation of T lymphocytes. All these results showed that CD40-ligated DCs maintaining moderate expression levels of PD-L1 and PD-L2 could sustain a long activation period to induce a potent tumor-specific immunity, especially for the activation of $CD8^+$ CTL. The high positive and moderate negative molecules expressed on mature CD40-ligated DCs provided the most appropriate activation and necessary feed-back signals.

Two subsets were found in mature CD40-ligated DCs. PD-L1 expression in one DC subset was totally inhibited, while high PD-L1 expression was found in the other subset. PD-L2 expression in mature CD40-ligated DCs was similar (Figure 3). These findings might be relate to the heterogeneity of DCs which seemed to exist until DCs captured antigens and became fully mature. The subset of DCs which was sensitive to CD40L stimulation and decreased PD-L1 and PD-L2 expressions should be the most important APC to elicit the potent tumor-specific immune response.

Blockade of PD-1 and PD-ligand interactions between T lymphocytes and immature DCs cultured for 5-6 days could promote the proliferation of T lymphocytes. Anti-PD-L1 mAb was superior to anti-PD-L2 mAb in upregulating proliferation of T lymphocytes stimulated by immature DCs (Figure 5). The proliferation of T lymphocytes stimulated by immature DCs was enhanced most effectively by adding anti-PD-L1 mAb and anti-PD-L2 mAb together ($p < 0.05$). These results showed an additive effect. Recent studies on the behaviour of human DCs showed that enhancement of T cell activation was most pronounced with weak APCs, such as iDCs and IL-10-pretreated mDCs, and blood monocyte-derived myeloid DCs from cancer patients and less pronounced with strong APCs, such as mDCs. These data were consistent with the hypothesis that iDC affected the balance of stimulatory versus inhibitory molecules favoring inhibition, and indicated that PD-L1 and PD-L2 contributed to the poor stimulatory capacity of iDC (23-25).

In conclusion, CD40-ligated DCs expressed not only high

levels of positive costimulatory molecules, but also moderate level of negative costimulatory molecules, such as PD-L1 and PD-L2. Excessive expressions of PD-L1 and PD-L2 were not beneficial to ultimate DC maturation and might impair the activation of T lymphocytes, but moderate expressions of PD-L1 and PD-L2 in CD40-ligated DCs could provide an appropriate balance, deliver the necessary feed back signals to induce DCs fully mature and elicit the most efficient tumor specific immune response.

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