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The Duration of Signaling through CD40 Directs Biological Ability of Dendritic Cells to Induce Antitumor Immunity

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Although it has been demonstrated that the functions of dendritic cells (DCs), including Ag capture, Ag presentation, and migratory activity, change dynamically with their maturation, the most appropriate conditioning of DCs for anticancer immunotherapy is still unclear. The help signal is one of the most potent stimuli for DC maturation and is provided by the interaction of CD40 expressed on DCs with CD40 ligand on CD4+ T cells. To elucidate the appropriate conditioning of DCs for anticancer immunotherapy, we examined the biological activity of DCs stimulated with immobilized anti-CD40 Ab. DCs stimulated for 3 h (3h-DCs) still showed an immature phenotype, but exhibited augmented migration toward secondary lymphoid tissues. Subcutaneous injection of 3h-DCs facilitated priming of T cells, which could mediate potent antitumor therapeutic efficacy, in draining lymph nodes and successfully induced protective immunity. In contrast, 24h-DCs showed a mature phenotype with good Ag presentation ability to induce cell killing by adoptively transferred CD8+ T cells when injected at tumor sites; however, they showed no migratory activity and were unable to induce protective immunity when injected s.c.. This is the first report that functionally distinct DCs, either for the priming phase or for the effector phase, could be obtained by conditioning with CD40 stimulation and that the duration of stimulation determines the biological outcome. The usage of DCs conditioned for the priming phase might provide significant advantages in anticancer immunotherapy.


Dendritic cells (DCs)$^2$ are believed to be the most potent APCs that are capable of picking up Ags in nonlymphoid tissues and carrying them to secondary lymphoid organs to prime T cells in response to maturation stimuli such as danger and help signals. By contrast, the presentation of Ags by DCs without activation results in the elimination of effector T cells that have a cognate TCR or induction of regulatory T cells in secondary lymphoid tissues (1–3). Thus, the presence or absence of maturation signals for immature DCs in peripheral tissues acts as a switch to induce either an adaptive immune response or tolerance (4–6). Since tumor cells show only slight antigenic differences from self-somatic cells even though they have altered Ags that T cells can recognize, it is likely that the peripheral tolerance system is activated in the absence of relevant stimulation for DCs. We reported that lymph nodes (LNs) draining a growing weakly immunogenic murine fibrosarcoma contain a few CD62L$^{low}$ effector memory-type T cells that are capable of mediating therapeutic efficacy against organ metastases when adaptively transferred (7, 8). However, suppressor T cells that can abrogate the antitumor reactivity of CD62L$^{low}$ T cells are also induced in LNs draining growing tumors (9).

The major CD4+ T cell help signal for DC maturation is provided by the interaction between CD40 expressed on DCs and CD40 ligand (L) on activated CD4+ T cells (10–12). It is demonstrated that CD40 stimulation induces the migration of DCs into secondary lymphoid tissues by up-regulating the expression of CCR7 (13, 14). Indeed, CD40 stimulation for DCs is essential in the priming phase, since mice lacking CD40L showed defective priming ability accompanied by a disrupted migration of DCs for regional LNs (15). We previously reported that a vaccination with CD40L-transduced tumor cells effectively primed T cells, which exhibited therapeutic efficacy against established organ metastases (16). However, little is known about how to obtain DCs suitable for T cell priming by ex vivo conditioning.

The primed T cells that migrate into nonlymphoid tissues must be restimulated to exhibit effector function. In tumor immunology, it was long considered enough that in the effector phase, CD8+ T cells recognize peptides bound to MHC class I Ag expressed on tumor cells. However, it was shown that CD8+ T cells require the presentation of Ag by DCs receiving the CD4+ T cell help signal to acquire the license to kill tumor cells (17–20). We previously demonstrated that CD62L$^{low}$CD4+ T cells derived from tumor-draining LNs are capable of mediating potent antitumor reactivity against tumors that express MHC class I but not class II Ag (8). A bone marrow chimera model revealed that bone marrow-derived APCs are essential in the effector phase of antitumor immune responses induced by CD4+ effector T cells (21). Genetically modified tumor cells that secrete IFN-γ and express CD80 to mimic activated APCs successfully enhanced the antitumor immune response (22). Thus, it is likely that DCs receiving help signals play an important role in not only the priming phase but also the effector phase.

There have been reports that the kinetics and duration of CD40L expression on CD4+ T cells are regulated by costimulatory signals

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$^2$ Abbreviations used in this paper: DC, dendritic cell; LN, lymph node; CM, complete medium; m, murine; MIP, macrophage-inflammatory protein; SLC, secondary lymphoid tissue chemokine; BM-DC, bone marrow-derived DC; iDC, immature BM-DC; L, ligand.
and cytokines such as IL-2, IL-12, IL-15, IFN-γ, and TGF-β, although CD40 is constitutively expressed on DCs (23–26). A recent study demonstrated that CD40L expression on CD4+ T cells can be separated into an early TCR-dependent phase and a later extended phase, and that the biological outcome of CD40 signaling for B cells depends upon the duration of interaction (27). Interestingly, only Th1 CD4+ T cells exhibit a later extended phase.

To elucidate the significance of the help signal duration, bone marrow-derived DCs (BM-DCs) were stimulated with immobilized anti-CD40 Ab for 0, 3, 6, 9, 12, and 24 h. Our data demonstrate that the duration of the stimulation through CD40 is critical in directing the biological function of DCs, either for the priming phase or for the effector phase.

Materials and Methods

Mice

Female C57BL/6 (B6) mice were purchased from CLEA Laboratory (Tokyo, Japan). They were maintained in a specific pathogen-free environment and used for experiments at the age of 8–10 wk.

Tumors

MCA 205 is a fibrosarcoma of B6 origin induced by i.m. injection of 3-methylcholanthrene (28). Single-cell suspensions were prepared from solid tumors by enzymatic digestion as described previously (29). An MCA 205 tumor cell line was established and maintained in vitro.

mAbs and flow cytometry

Hydromidas producing mAbs against murine CD4 (GK1.5, L3T4), CD8 (2.43, Lyt-2), and murine CD62L (MEL.14) were obtained from the American Type Culture Collection (Manassas, VA). Anti-CD4 mAb, anti-CD8 mAb, and anti-CD62L mAb were produced as ascites fluid from sublethally irradiated (500 cGy) DBA/2 mice. PE-conjugated anti-H-2Kb (AF6-88.5), PE-conjugated anti-I-Ak (AF6-120.1), PE-conjugated anti-CD80 (16-10A), PE-conjugated anti-CD86 (GL1), FITC-conjugated anti-Thy1.2 (30-H12), PE-conjugated anti-CD11b (M1/70), FITC-conjugated anti-CD11c (HL3), and PE-conjugated anti-CD62L (MEL.14) were purchased from BD PharMingen (San Diego, CA). Analyses of cell surface phenotypes were conducted by direct immunofluorescence staining of 0.5–1 × 10^6 cells with conjugated mAbs. In each sample, 10,000 cells were analyzed using a FACScan flow microfluorometer (BD Biosciences, Sunnyvale, CA). For DC staining to avoid nonspecific binding with the FcR, Fc block (BD PharMingen) was used according to the manufacturer’s instructions. PE-conjugated subclass-matched Abs used as isotype controls (M5/114.15.2, G155-178, 2C11) were also purchased from BD PharMingen.

Fractionation of T cells

T cells in the LN cell suspension were concentrated by passing through nylon wool columns (WAKO, Osaka, Japan). To yield highly purified (90%) cells with down-regulated CD62L expression (CD62L<sup>−</sup>), LN T cells were further isolated by a panning technique using T-25 flask precoated with goat anti-rat Ig Ab (Jackson ImmunoResearch Laboratories, West Grove, PA)anti-CD62L Ab (MEL.14), and sheep anti-rat Ig Ab (AbicantiCD62L Ab-coated DynaBeads M-450 (Dynal, Oslo, Norway). T cells with high CD62L expression (CD62L<sup>high</sup>) were obtained as cells attached to flasks coated with goat anti-rat Ig Ab (anti-CD62L Ab. In some experiments, cells were further separated into CD4<sup>+</sup> and CD8<sup>+</sup> cells by depletion using magnetic beads as described previously (8).

BM-DCs

DCs were generated from bone marrow cells according to the procedure described in the report by Inaba et al. (30), with some modification. In brief, bone marrow cells obtained from femurs and tibias of naive mice were placed in T-75 flasks for 2 h at 37°C in complete medium (CM) containing 10 ng/ml recombinant murine GM-CSF (a gift from KIRIN, Tokyo, Japan). CM consists of RPMI 1640 medium supplemented with 10% heat-inactivated LPS-qualified FCS, 0.1 M mannitol, and 5 × 10<sup>5</sup> M 2-ME (Sigma-Aldrich, St. Louis, MO). Nonadherent cells were harvested by gentle pipetting. Approximately 80% CD11c<sup>−</sup>CD11b<sup>+</sup> cells and 20% CD11c<sup>+</sup> CD11b<sup>−</sup> cells were obtained. For some experiments, CD11c<sup>+</sup> cells were further purified with anti-CD11c-coated immunomagnetic beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer’s instructions.

Activation of DCs

To settle DCs on a T-25 flask precoated with goat anti-rat Ig Ab and anti-CD40 Ab (323; Serotec, Oxford, U.K.) as soon as possible, they were plated in 4 ml of CM supplemented with GM-CSF (10 ng/ml). Anti-CD40 Ab-coated flasks were prepared as described below. T-25 flasks were coated with 2 ml of goat anti-rat Ig Ab solution (100 μg/ml) overnight at 4°C. The Ab solution was removed and the flasks were washed with PBS twice. The flasks were further coated with rat anti-murine CD40 Ab (100 μg/ml) for 30 min at 37°C in a 5% CO<sub>2</sub> incubator and were used immediately after washing with PBS.

Tumor-draining LN cells

B6 mice were inoculated s.c. with 2 × 10<sup>5</sup> MCA 205 tumor cells in the bilateral flanks. Inguinal LN draining tumors were harvested 7–10 days after s.c. tumor inoculation. Single-cell suspensions were prepared mechanically as described previously (29).

Adoptive immunotherapy

B6 mice were injected i.v. with 6 × 10<sup>5</sup> MCA 205 tumor cells in 1 ml of HBSS to establish pulmonary metastases (29). Three days after tumor inoculation, mice were sublethally irradiated (500 cGy) and then infused i.v. with T cells isolated from LNs draining conditioned DCs for 7 days. The BM-DCs were cocultured overnight with irradiated (5000 cGy) MCA 205 tumor cells to acquire tumor-related Ag and stimulated with immobilized anti-CD40 Ab for 3 h before s.c. inoculation. Mice were followed up and survival time was recorded. The significance of differences of survival time between groups was analyzed with the two-sided Wilcoxon rank sum test. A value of p < 0.05 was considered to be significant.

Cytokine ELISA

One hundred thousand CD4<sup>+</sup> or CD8<sup>+</sup> T cells were cocultured with 1 × 10<sup>7</sup> DCs as stimulators for 12 h in a 96-well plate containing 200 μl of CM. Supernatants were harvested and assayed for mIFN-γ content by a quantitative sandwich enzyme immunoassay using a mIFN-γ ELISA kit (Genzyme, Cambridge, MA) according to the manufacturer’s instructions.

Immunization models

B6 mice were immunized intradermally in the left flank with 2 × 10<sup>6</sup> conditioned DCs. The DCs were cocultured with irradiated MCA 205 tumor cells (5000 cGy) overnight and stimulated with immobilized anti-CD40 Ab for 0, 3, or 24 h before inoculation. Twenty-one days after immunization, these mice were inoculated s.c. along the midline of the abdomen with 3 × 10<sup>6</sup> MCA 205 tumor cells. Diameters of skin tumors were measured twice weekly with a caliper, and size was recorded as the average of two perpendicular diameters.

RT-PCR

Total RNA was isolated from immunobead-sorted CD11c<sup>+</sup> BM-DCs after stimulation with immobilized anti-CD40 Ab using Isogen (Nippon Gene, Tokyo, Japan) and used for CDNA synthesis. The CDNAs were used as templates for PCR (94°C for 2 min, 56°C for 30 s, and 72°C for 1 min), and 25 cycles were performed using primers specific for mCCR7 (forward, 5'-GCTCAACCTGGCCTGGCAGACATCC; reverse, 5'-CCACTTGGAGTGTTGATCAAGGCTCC). To ensure the quality of the procedure, RT-PCR was also performed using primers specific for β<sub>2</sub>-microglobulin.

In vitro migration assay

Recombinant mouse macrophage-inflammatory protein 3β (MIP-3β; R&D Systems, Minneapolis, MN) was diluted at 10 ng/ml in assay medium (CM without FCS), and 600-μl aliquots were placed in 24-well culture plates (Costar, Cambridge, MA). Transwell culture inserts of 0.6-μm diameter and 5-μm pore size (Costar) were placed in each well, and 5 × 10<sup>4</sup> DCs after conditioning in 100 μl of assay medium were added to the upper chamber. After incubation at 37°C in 5% CO<sub>2</sub> for 2 h, the cells that migrated to the bottom chamber were harvested and counted by light microscopy.

In vivo migration assay

Immediately before s.c. injection, DCs were labeled with 5 μM CFSE (Molecular Probes, Eugene, OR) in HBSS at 37°C for 15 min and washed.
twice. Mice were injected s.c. in the left flank with $2 \times 10^6$ labeled DCs. Twenty-four hours after inoculation, inguinal LNs were removed. LN cells were stained with PE-conjugated anti-CD11c Ab and analyzed with a microfluorometer.

Secondary lymphoid tissue chemokine (SLC) binding assay

BM-DCs were exposed to 100 ng/ml rSLC (R&D Systems) for 25 min and stained with biotinylated anti-SLC Ab (Genzyme) after Fc block. DCs were double stained and analyzed with a microfluorometer after staining with PE-conjugated streptavidin (BD PharMingen) and FITC-conjugated anti-CD11c Ab.

Results

Expression of MHC class II Ag and costimulatory molecules on BM-DCs stimulated with immobilized anti-CD40 Ab

The CD40L expressed on activated CD4\(^+\) T cells is a homotrimer and the maturation signal through CD40 is thought to be generated by the cross-linking of CD40 molecules. In this study, CD40 stimulation was achieved by interaction with immobilized agonistic anti-CD40 Ab to mimic the physiological stimulation with membrane-bound CD40L. To examine the duration of stimulation required to induce phenotypic maturation, BM-DCs stimulated with immobilized anti-CD40 Ab for 3 or 24 h were stained with FITC-conjugated anti-CD11c Ab and PE-conjugated anti-H-2K\(^b\) Ab, PE-conjugated anti-I-A\(^\beta\) Ab, PE-conjugated anti-CD80 Ab, or PE-conjugated anti-CD86 Ab and were analyzed with a microfluorometer. Immature BM-DCs (iDCs) after 5 days of culture expressed low levels of MHC class II Ag, CD80, and CD86 before stimulation. BM-DCs that were stimulated for 24 h (24h-DCs) showed an increase in MHC class II, MHC class I, MHC class II, CD80, and CD86 expression, whereas BM-DCs stimulated for 3 h (3h-DCs) still showed an immature phenotype (Fig. 1). Coculture with gamma-irradiated MCA 205 tumor cells resulted in minimum change in the maturation state of DCs.

Tumor Ag presentation activity of BM-DCs was augmented by 24-h stimulation with immobilized anti-CD40 Ab

To ascertain whether phenotypic maturation was accompanied by augmented tumor Ag presentation activity, the IFN-\(\gamma\) production by CD62L\(^{low}\) memory effector T cells derived from LNs draining a growing MCA 205 tumor was measured. A significant increase of IFN-\(\gamma\) secretion by CD62L\(^{low}\)CD4\(^+\) T cells was observed when they were cocultured with 24h-DCs that had captured tumor Ag, compared with 24h-DCs without Ag loading (Fig. 2A). In contrast, captured Ag was not effectively presented with MHC class II Ag by iDCs or 3h-DCs during coculture for 12 h, since almost an identical amount of IFN-\(\gamma\) production by T cells was observed in the presence of DCs without Ag loading. A significant increase in IFN-\(\gamma\) production by CD8\(^+\) T cells was also observed when they were cocultured with 24h-DCs that had acquired tumor Ags (Fig. 2B).

In vivo migration activity of BM-DCs stimulated with immobilized anti-CD40 Ab

To test whether the capacity to migrate to secondary lymphoid tissues is acquired by BM-DCs stimulated through CD40, we examined inguinal LN cells draining CFSE-labeled DCs that were conditioned with immobilized anti-CD40 Ab. LNs were harvested 24 h after DC inoculation in the left flank. LN cells were stained with PE-conjugated anti-CD11c Ab and were analyzed with a microfluorometer. CFSE-positive cells were observed only in LN cells draining 3h-DCs (Fig. 3). All of the CFSE-positive cells were CD11c positive.

In vitro migration activity and CCR7 expression of BM-DCs stimulated with immobilized anti-CD40 Ab

It is reported that the migration of DCs to secondary lymphoid tissues mostly depends on interaction between SLC secreted by secondary lymphoid organs and CCR7 expressed on DCs. The migratory activity of conditioned DCs responding to MIP-3\(\beta\), which is an alternative specific ligand for CCR7, was evaluated by an in vitro migration assay. Only 3h-DCs showed a good ability to migrate, whereas iDCs or 24h-DCs with the fully matured phenotype demonstrated limited activity (Fig. 4A). To clarify whether CCR7 accounts for the induced migratory activity of DCs, a RT-PCR for mRNA expression of CCR7 and a SLC binding assay.
were performed. RT-PCR revealed that 3-h stimulation with immobilized anti-CD40 Ab enhanced the expression of mRNA encoding CCR7 (Fig. 4B). In contrast, low levels of mRNA encoding CCR7 were detected in iDCs and 24h-DCs. In Fig. 4C, thick lines indicate SLC binding activity of gated CD11c+/H11001 cells and thin lines indicate control fluorescence intensity without SLC exposure. 3h-DCs exhibited augmented binding activity for SLC compared with iDCs. SLC binding activity was even abrogated when they were stimulated for 24 h.

**Priming of T cells is facilitated in LNs draining 3-h conditioned BM-DCs**

To evaluate the priming of T cells in LNs draining s.c. inoculated DCs, we examined the number and phenotype of LN cells. Kinetic analysis revealed that the number of cells in LNs draining DCs reached a peak 7 days after s.c. injection of DCs (Fig. 5, left panel). In the right panel of Fig. 5, each value, which was obtained from independent experiments with more than three mice in each group, represents the mean number of cells in LNs draining DCs for 7 days. Open squares represent averages of four experiments. LN cell numbers increased as soon as 3 days after the inoculation when 3h-DCs were injected s.c. and peaked 7 days after the inoculation in all groups (Fig. 5, left). The peak number of LN cells draining 3h-DCs was significantly more than that of LN cells draining iDCs or 24h-DCs (Fig. 5, right). The number of LN cells draining 24h-DCs was not significantly different from that of LN cells draining iDCs. We next analyzed phenotypes of LN cells draining conditioned DCs for 7 days. LN cells were stained with FITC-conjugated anti-Thy1.2 Ab/PE-conjugated anti-CD62L Ab. CD62Llow T cells in secondary lymphoid organs are considered primed by APCs. We previously reported that antitumor reactivity is exclusively mediated by CD62Llow T cells in tumor-draining LNs (7). In resting LNs, ~10–15% of T cells are CD62Llow T cells (data not shown). Phenotypic analyses showed that the proportion of CD62Llow T cells markedly increased to 31.4% in LNs draining 3h-DCs, whereas LNs draining iDCs and 24h-DCs contained 20.4 and 17.8% CD62Llow T cells, respectively (Fig. 6). Subcutaneous injection of 3h-DCs did not increase the proportion of CD62Llow T cells in nondraining LNs on the opposite side.

**T cells isolated from LNs draining 3-h DCs showed potent antitumor reactivity when adoptively transferred**

Although LNs draining a growing tumor are an excellent source of T cells that are primed with tumor-associated Ag, T cells derived from tumor-draining LNs need to be activated in vitro to exhibit antitumor reactivity (29, 31). However, we reported that LN T cells draining s.c. inoculated apoptotic tumor cells with CD40L-transduced cells could mediate potent antitumor therapeutic efficacy without any further activation when infused i.v. (16). To test whether CD62Llow T cells in LNs draining 3h-DCs were able to mediate antitumor reactivity, we infused 2 × 10⁸ CD62Llow or CD62Lhigh T cells freshly isolated from LNs draining 3h-DCs, which had acquired tumor Ag before stimulation, into mice bearing 3-day established pulmonary metastases. All of the mice that were infused i.v. with CD62Llow T cells derived from LNs draining 3h-DCs were cured (Fig. 7A). In contrast, the survival of the group that received CD62Lhigh T cells was not significantly different from that of the untreated group.
Single s.c. injection of BM-DCs stimulated with anti-CD40 Ab for 3 h successfully induced protective immunity in naive mice.

We next addressed the efficacy of conditioned DCs to induce protective immunity. Mice immunized with a single s.c. injection of conditioned DCs were challenged with 3*10^6 MCA 205 tumor cells 21 days after immunization. All of the mice immunized with 3h-DCs exhibited strong protective immunity (Fig. 7B). In contrast, skin tumor growth curves were identical among naive mice and mice immunized with iDCs or 24h-DCs.

Fully mature DCs induced the therapeutic efficacy of adoptively infused Ag-primed CD8+ T cells when injected at skin tumor sites.

CD8+ T cells freshly isolated from LNs draining growing tumors exhibited little therapeutic efficacy when adoptively transferred un-restimulated ex vivo (29, 32). To test whether DCs are capable of restimulating tumor Ag-primed CD8+ T cells to exhibit antitumor reactivity in vivo, skin tumor-bearing mice were infused i.v. with CD8+ T cells derived from tumor-draining LNs and then injected s.c. with conditioned DCs at skin tumor sites. In brief, skin tumors were established by s.c. injection of 1.5*10^6 MCA 205 tumor cells on the midline of mice. Three days later, 1*10^6 purified CD62L low CD8+ T cells derived from LNs draining 10-day MCA 205 tumors were infused i.v. Conditioned DCs were inoculated into adjacent skin tumors s.c. 5 days after the CD8+ T cell infusion (Fig. 8A) or on the same day (Fig. 8B), and the diameter of skin tumors was serially measured twice a week. All of the skin tumor growth curves were almost identical before DC inoculation even though mice in certain groups received CD8+ T cell infusion. A transient but significant suppression of skin tumor growth was observed in mice immunized with conditioned DCs (Fig. 8B).

**FIGURE 4.** A, Six hundred microliters of assay medium containing recombinant MIP-3β at 100 ng/ml was placed in a 24-well culture plate. The assay medium consists of CM without FCS. Transwell culture inserts of 6.5-mm diameter and 5-μm pore size were placed in each well, and 5*10^5 DCs stimulated with immobilized anti-CD40 Ab for the periods indicated in 100 μl of assay medium were added to the upper chamber. After incubation at 37°C in 5% CO2 for 2 h, the cells that had migrated to the bottom chamber were harvested and counted by light microscopy. Statistical analyses were performed with Student’s t test. *p < 0.01. B, CCR7 mRNA expression in DCs stimulated with immobilized anti-CD40 Ab for the periods indicated. Total RNA was isolated from DCs and analyzed by RT-PCR for CCR7. β2-Microglobulin gene expression is shown to confirm that equal amounts of RNA were used in each RT-PCR. Results shown are representative of three separate experiments. C, Thick lines indicate SLC binding activity of gated CD11c+ cells and thin lines indicate control fluorescence intensity without SLC exposure. 3h-DCs exhibited augmented binding activity for SLC compared with iDCs.

**FIGURE 5.** Kinetics of cellularity in LNs draining DCs. Two million DCs stimulated with immobilized anti-CD40 Ab for the periods indicated were injected s.c. into the left flank of mice. Inguinal LNs draining DCs were harvested from three mice serially 0, 4, 7, 11, and 14 days after s.c. inoculation. Symbols indicate the mean number of cells of LNs in the left panel. In the right panel, each symbol indicates the cell number in LNs draining s.c. inoculated DCs for 7 days. Open squares and bars indicate the average and SE for four independent experiments. Statistical analyses were performed with Student’s t test. *p < 0.01.
growth was observed only in the group that received CD8⁺ T cell infusion followed by s.c. inoculation of 24h-DCs. (Fig. 8).

Adoptive transfer of tumor Ag-primed CD4⁺ T cells increased numbers of mature DCs in tumor tissues and regional LNs

To clarify whether the presence of CD4⁺ T cells influences the maturation of DCs infiltrating tumor tissues and migrating in LNs, 5 × 10⁶ MCA 205 tumor cells mixed with 5 × 10⁶ iDCs were inoculated s.c. in the left flank after sublethal (500 cGy) whole body irradiation. Five days later, 1 × 10⁶ CD62LlowCD4⁺ T cells derived either from LNs draining MCA 205 tumors or splenocytes of naive mice were infused i.v. Skin tumors and inguinal LNs were harvested 48 h after the CD4⁺ T cell infusion. Single-cell suspensions derived from skin tumor tissues and inguinal LNs were stained with FITC-conjugated anti-CD11c Ab/PE-conjugated anti-CD86 Ab and examined with a microfluorometer. The histograms in Fig. 9 show CD86 expression on gated CD11c⁺ cells obtained from digested tumor. The subpopulation with up-regulated CD86 expression was observed only when CD62LlowCD4⁺ T cells derived from tumor-draining LNs were infused. The dot plots in Fig. 9 demonstrate that CD86⁺CD11c⁺ cells increased in number in LNs of mice receiving CD62LlowCD4⁺ T cells derived from tumor-draining LNs.

Discussion

It has been demonstrated that mature DCs possess superior Ag presentation ability to induce CTL activity and cytokine production. However, the appropriate conditioning of DCs to induce the most potent anticancer immune reaction in vivo is still unclear (18, 19, 33–42). One of the problems to be elucidated is how to obtain DCs that are capable of migrating into secondary lymphoid organs to prime T cells when injected.
In this study, we used immobilized agonistic anti-CD40 Ab as a stimulator for DCs to mimic the CD4+ T cell help signal to elucidate the appropriate conditioning of DCs for anticancer immunotherapy. Since immobilization enables Ab to cross-link with CD40 to provide a maturation signal immediately after DCs reach the bottom of flasks, we could perform precise kinetic analyses of the biological activity of DCs. The stimulation of DCs by immobilized anti-CD40 Ab seems almost identical to that by membrane-bound CD40L, since the kinetics and magnitude of phenotypic maturation and induction of migratory ability were almost the same as for DCs cocultured with CD40L-transduced tumor cells (16).

Twenty-four-hour stimulation through CD40 resulted in increased numbers of DCs with up-regulated expression of MHC class II Ag, CD80, and CD86 (Fig. 1) accompanied by the augmented presentation of MHC class I- and II-restricted Ags derived from engulfed apoptotic tumor cells (Fig. 2). These results are compatible with the report that the presentation of processed Ag preserved in endosomes required a maturation signal provided by CD4+ T cells (43, 44). By contrast, almost all of the DCs stimulated

FIGURE 8. Skin tumor growth of mice infused with CD62LlowCD8+ T cells derived from tumor-draining LNs and s.c. injection of conditioned DCs. First, 1.5 x 10⁶ MCA 205 tumor cells were injected s.c. along the midline to establish skin tumors. Three days later, mice were adoptively infused i.v. with 1 x 10⁶ CD62LlowCD8+ T cells isolated from tumor-draining LN cells after sublethal whole body irradiation (500 cGy). Two million iDCs or 24h-DCs that had acquired tumor Ag were inoculated into the adjacent skin tumor 5 days after CD8+ T cell infusion (A) or on the same day (B). Diameters of skin tumors were measured twice weekly with a caliper, and size was recorded as the average of two perpendicular diameters. Statistical analyses were performed with Student's t test. *, p < 0.05 compared with the untreated group. **, p < 0.01 compared with the untreated group.

FIGURE 9. Flow cytometric analyses of LN cells and CD11c+ cells in tumor tissues. Five million MCA 205 tumor cells mixed with 5 x 10⁶ immature DCs were inoculated s.c. in the left flank of mice. Five days later, 1 x 10⁶ CD62LlowCD4+ T cells derived from LNs draining MCA 205 tumor cells or naive splenocytes were infused i.v. Forty-eight hours after the T cell infusion, skin tumor tissues and left inguinal LNs were harvested. A single-cell suspension was stained with FITC-conjugated anti-CD11c Ab and PE-conjugated anti-CD86 Ab. The histograms show CD86 expression on gated CD11c+ cells obtained from tumor tissues. The dot plots show CD11c and CD86 expression on LN cells. Numbers indicate the percentage of CD86+ CD11c+ cells in LNs.
for 3 h showed an immature phenotype and did not present acquired Ag immediately after stimulation; however, they exhibited good migratory capacity for secondary lymphoid organs (Fig. 3).

It is now believed that the secondary lymphoid organs are the only places where naïve T cells are primed by DCs to acquire effector functions. Thus, the ability of DCs to initiate adaptive immune responses depends upon migratory activity. The migration of DCs to secondary lymphoid tissues is regulated by the interaction between SLC, a chemokine secreted in the T cell zone of secondary lymphoid tissues, and CCR7 expressed on DCs. Disrupted migration with defective CCR7 expression results in an inability to mount an effective immunological response against pathogens (45). In vitro migration assays showed that the migratory activity of DCs responding to MIP-3β peaked when the cells were stimulated for 3 h (Fig. 4A). 3h-DCs exhibited strong binding activity for SLC accompanied by up-regulated mRNA expression for CCR7 (Fig. 4, B and C). In contrast, 24h-DCs showed rather diminished binding activity with a down-regulated mRNA expression. Thus, it is likely that the expression of CCR7 accounts for the augmented migration of 3h-DCs and reduced migration of 24h-DCs. Although it was reported that CD62L, which recognizes high endothelial venules, plays an important role in the migration of DCs to secondary lymphoid organs (46), no expression of CD62L was detected on DCs stimulated for 0, 3, or 24 h with immobilized anti-CD40 Ab (data not shown). Subcutaneous inoculation of 3h-DCs induced a marked increase in the total number of cells and the percentage of Ag-primed T cells, which were depicted as T cells with down-regulated CD62L expression, in the draining LNs (Figs. 5 and 6). 6h-DCs and 9h-DCs induced a slight but significant increase; however, 12h-DCs produced a minimum increase in draining LN cells, as did 24h-DCs (data not shown). Isolated CD62L<sup>low</sup> T cells derived from LNs draining 3h-DCs could mediate potent antigen-specific T cell deletion (Fig. 7A). To rule out the possibility that irradiated tumor cells used for loading Ag participate in the priming, CD11c<sup>+</sup> cells purified from 3h-DCs using magnetic beads were examined. The number and percentage of CD62L<sup>low</sup> T cells were almost identical to those in LNs draining unpurified 3h-DCs. The production of IFN-γ by CD62L<sup>low</sup> T cells in LNs draining purified CD11c<sup>+</sup> cells was observed to be Ag specific (data not shown). Intravenous infusion of CD8<sup>+</sup> T cells freshly isolated from LNs draining growing MCA 205 tumors has no significant therapeutic efficacy against established skin tumors. However, the inoculation of 24h-DCs into adjacent skin tumor with i.v. infusion of tumor Ag-primed CD8<sup>+</sup> T cells induced transient but significant tumor regression, whereas the inoculation of iDCs did not (Fig. 8). Thus, it seems that maturation of DCs with CD40 signaling is required to present tumor Ags to primed CD8<sup>+</sup> T cells, which migrate into tumor tissues, to induce CTL function. The limited number of infected CD8<sup>+</sup> T cells or the limited period of the effect of whole body irradiation, by which tolerogenic lymphocytes are eliminated, may be the reason why tumor growth inhibition was transient. However, the inhibition of skin tumor growth started with the 24h-DC inoculation and continued for ~10 days, even though the day of the inoculation differed between Fig. 9A and 9B. Thus, it is likely that tumor growth inhibition occurs during the period when inoculated DCs survive to present tumor Ags to CD8<sup>+</sup> T cells.

Taken together, it seems that DCs became specialized for the priming phase after a few hours of CD40 signaling. By contrast, long-term CD40 signaling produced DCs ideal for the effector phase. Furthermore, the data demonstrating protective immunity against tumor cells were obtained only when 3h-DCs were injected s.c., indicating that DCs for the priming phase are essential to initiate antitumor immunity (Fig. 7A). Previous findings raised the question of whether such a difference in the duration of interaction between CD40 on DCs and CD40L on CD4<sup>+</sup> T cells can occur under physiological conditions, since it has been reported that the duration of interaction between Ags presented on DCs and TCR on T cells is only several hours (44). However, a recent report demonstrated that Th1-type CD4<sup>+</sup> T cells have a biphasic expression of CD40L, an early TCR-dependent expression, which lasts for several hours and a later expression that extends for 72 h. Considering that the interaction between DCs and T cells is secured by a specialized structure called the immunological synapse, T cells could manipulate the biological function of DCs by changing the duration of CD40L expression. It is possible that the early short expression of CD40L induces the migratory activity of DCs to prime T cells and that the significance of the late long expression is to induce mature DCs that stay in peripheral tissues where target cells exist to restimulate primed T cells. Indeed, i.v. infusion of tumor Ag-primed CD4<sup>+</sup> T cells induced maturation of DCs infiltrating tumor tissues and increased numbers of CD8<sup>+</sup>CD11c<sup>+</sup> cells in draining LNs in an Ag-specific manner (Fig. 9).

This is the first report that functionally distinct types of DCs could be obtained from BM-DCs by conditioning with CD40 stimulation and that the duration of stimulation determined biological function. The usage of DCs that are conditioned to acquire the ability to prime T cells in secondary lymphoid organs rather than fully mature DCs alone or immature DCs might provide significant advantages in anticancer immunotherapy.

References


