Article

Distinct Effect of CD40 and TNF-Signaling on the Chemokine/ Chemokine Receptor Expression and Function of the Human Monocyte-Derived Dendritic Cells

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A key and limiting step in the process of human monocyte-derived dendritic cells (mDCs) for clinical use is their *in vitro* maturation and *in vivo* migration. We previously observed that CD40 signal facilitated human mDC growth and maturation. To further explore this process, mDCs generated with GM-CSF and IL-4 were co-cultured with apoptotic tumor cells for 24 hours, followed by incubating with anti-CD40 monoclonal antibody or TNF-α for 48 hours to generate mature DCs. The chemokine/chemokine receptor expression and functions of mature DCs upon various stimuli were determined. The expression of costimulatory molecules on apoptotic tumor cell-loaded mature DCs co-cultured with either anti-CD40 antibody (anti-CD40-DCs) or TNF-α (TNF-DCs) were up-regulated compared to immature DCs, consistent with the abilities of these cytokine to drive DC maturation *in vitro*. The mRNA levels of chemokines such as stromal cell-derived factor-1α (SDF-1α), EBV-induced molecule 1 ligand chemokine (ELC), and IFN inducible protein-10 (IP-10) in anti-CD40 activated DCs were increased and the dendritic cell-specific chemokine 1 (DC-CK1) was moderately up-regulated as compared with other mature DCs. The corresponding chemokine receptors CXCR4 and CCR7 of anti-CD40-DCs were significantly expressed. The CXCR3 expression on activated T cells stimulated by anti-CD40-DCs was also increased. Moreover, the anti-CD40-DCs had a stronger ability to stimulate T cell proliferation than any other DCs. The NF-κB activity was much higher in anti-CD40-DCs than that of TNF-DCs. These results offer further evidence of the importance of the CD40 signal in developing efficient human DC vaccines for cancer immune therapy. *Cellular & Molecular Immunology*. 2008;5(2):121-131.

Key Words: dendritic cell, chemokine, chemokine receptor, CD40, CD40 ligand

Introduction

Dendritic cells (DCs) are the most important professional antigen presenting cells (APCs). Immature DCs capture antigens at the site of deposition, followed by migration to lymphoid organs，where they become mature and present the processed antigens to naïve T cells to initiate the adoptive immune response (1, 2). DCs at different differentiation stages have distinct properties such as antigen processing, expression of costimulatory molecules and chemokine receptors, as well as secreting cytokines and chemokines (3-6). Understanding how these properties of DCs are regulated is important for designing effective DC based vaccines against cancers.

Several lines of evidence suggest that immature DCs could migrate to the tumor site, capture tumor antigens, and then migrate to secondary lymphatic organs to stimulate naïve T cells (7, 8). Nevertheless, the functions of immature DCs can be inhibited by suppressive cytokines in tumor microenvironments (9, 10). As a result, vaccination with immature DCs led to immunologic tolerance to tumor-associated antigens. Therefore, currently used DC vaccination strategies are based on mature DCs that are derived from cultures stimulated by proinflammatory factors TNF-α, CD40 ligand or other factors (11). These mature DCs have potent abilities to stimulate naïve T cells despite of the inhibitory effect of tumor microenvironment (12). However, once matured, DCs may partially lose their abilities to ingest

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antigen and migrate into the secondary lymphoid organs *in vivo*, which is critical to trafficking of active T cells to tumor site to play an effective anti-tumor immunity (13). As a consequence, the outcome of DC vaccine based tumor immunotherapy is not satisfactory. Therefore, defining optimal conditions that will stimulate DC maturation while maintaining their ability for *in vivo* migration are crucial for the design of effective DC-based tumor vaccine therapies.

CD40 is expressed on the surface of several different kinds of APCs, such as B cells, monocytes and DCs. It is generally accepted that CD40/CD40L is a powerful stimulus in DC function and maturation (14-16). Previous experiments have demonstrated that maturation of DCs through CD40 signaling can promote a protective CD8 T cell mediated immunity that is independent of CD4 T cell help (17). We previously observed that CD40/CD40L signal could facilitate DC growth and maturation, antagonize the negative effect of IL-10, and reinforce DC ability to accelerate T cell activation and expansion (18). Thus, it is likely that the CD40 signal is more potent than TNF-α to effectively stimulate DC maturation and function.

Previous reports indicated DC maturation is characterized by the up-regulation of costimulatory molecules and a coordinated switch in chemokine receptor expression patterns (19-22). Dendritic cells utilize distinct sets of chemokine receptors, depending on the maturation stages. Immature dendritic cells express CCR1, CCR2, CCR4, CCR5, CXCR1, and CXCR4, while mature dendritic cells express a limited set of chemokine receptors, CXCR4 and CCR7 (19, 22, 23). Dendritic cells also produce chemokines that are responsible for attracting T cells. Activated T cells, which express high levels of CXCR3, are chemotactic to CXCR3 ligand, IP-10 (24, 25). Naïve T cells are chemotactic to DC-CK1 (26). Although results from different groups indicate CD40 and TNF-α can facilitate DC maturation and function, little is currently known about the mechanism of CD40 or TNF-α on the maturation of human DCs. To further study these mechanisms, we compared DCs matured by TNF-α and anti-CD40 by examining levels of chemokine secretion, surface expression of chemokine receptor, as well as DCs' abilities to stimulate T cells.

In this report, we used antigen-load and then added anti-CD40 antibody or TNF-α to induce the maturation of monocyte-derived DC. We determined the expression profile of chemokines and chemokine receptors of DCs on various stages of maturation with different stimulation methods. We have found that anti-CD40-stimulated DCs not only exert maturation characteristics but also have much higher T cell proliferation and migration stimulating abilities. Thus, CD40 signaling might be more effective than TNF-α in inducing effective human anti-tumor DC vaccine.

Materials and Methods

Reagents and antibodies

The control Abs and a series of rat anti-human mAbs, including CD1α, CD80, CD83, CD86, HLA-DR, CXCR3, CXCR4 and CCR7 were used as direct conjugates to FITC or PE (Immunotech Company, France). Anti-human CD40 stimulatory monoclonal antibody (5C11) was prepared in our lab (Biotechnology Institute, Soochou University, China) (27); rhGM-CSF and rhIL-4 were purchased from Amagen Company (USA). Chemoattractants SDF-1α/CXCL12 and secondary lymphoid tissue chemokine (SLC/CCL21) (ELC homologous chemokine secreted by stromal cells in secondary lymphoid tissue) were obtained from BD Company (USA). Anti-human IP-10/CXCL10 antibody and human IP-10 ELISA Kit were supplied by R&D Systems (USA). IP-10 mAb and goat anti mAb DC-CK1 were purchased from Pepro Tech (USA) and Santa Cruz-Biotechnology (USA) respectively.

Cell preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood of healthy volunteer donors (Suzhou Red Cross Blood Center, China) by standard density gradient centrifugation with Ficoll (Shanghai Second Chemistry Factory, China). Monocytes were purified by positive sorting using anti-CD14-conjugated magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The $CD14^+$ cells that were more than 95% pure as revealed by FACS analysis (BD Biosciences, USA) were seeded in 6-well flat bottom plates (Costar, Cambridge, MA) in RPMI 1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% fetal calf serum (Hyclone, USA), 0.02 mmol/L L-glutamine, 100 U/ml penicillin-streptomycin, and rhGM-CSF 100 ng/ml, rhIL-4 50 ng/ml in a humidified incubator at 37°C in 5% CO_2. The medium was renewed every 2 to 3 days. At day 7, we used DDP (Cis-Diamine-dichloroplatinum) with Raji cell line (ATCC, USA) for 2 h to achieve early period apoptosis tumor antigen. The apoptosis ratio was 34.3 ± 7.6%. The apoptotic Raji cells as tumor antigens were incubated with the dendritic cells at the ratio of 1:3. Twenty-four hours later, we added 20 ng/ml of TNF-α (Sigma, USA) or 2 μg/ml of 5C11 to the culture system and cultured for additional 48 hours to induce DC maturation (27, 28). The control group was cultured without exogenous cytokine after apoptotic Raji cells were loaded. T cells were obtained from the sheep RBC-bound PBMC fraction by hypotonic lyses. This method yielded more than 95% purified T cells, as assessed by flow cytometry stained with an anti-CD3 mAb. Then, we used different DC groups to stimulate T cells with the proportion of 1:50 respectively. Five days later, we obtained activated T cells.

The DC groups were set as below: ImDCs, immature DCs cultivated as mentioned above for 7 days; Ag-DCs, apoptotic Raji cell DCs loaded for 72 h; Anti-CD40-DCs, apoptotic Raji cell DCs loaded for 24 h then cultivated with 5C11 for 48 h; TNF-DCs, apoptotic Raji cell DCs loaded for 24 h then cultivated with TNF-α for 48 h.

The T cell groups are set as below: Resting Ts, unstimulated T cells which were obtained from the sheep RBC-bound PBMC fraction by hypotonic lysis; ImDC-Ts, activated T cells stimulated by DCs from ImDCs; Ag-DC-Ts, active T cells stimulated by DCs from Ag-DCs; Anti-

CD40-DC-Ts, active T cells stimulated by DCs from anti-CD40-DCs; TNF-DC-Ts, active T cells stimulated by DCs from TNF-DCs.

Flow cytometry analysis of costimulatory molecules and chemokine receptors

Cells used were derived from at least three different donors. 2×10^6 DCs of each group mentioned above were stained with the following Abs respectively: rat anti-human CD1α, CD80, CD83, CD86, HLA-DR, CXCR4, CCR7, and 1×10^6 T cells were stained with CXCR3 mAb for 30 min on ice and washed. For direct staining, an FITC or PE-labeled isotype-matched control Ab was used as a negative control, and then analyzed by the Flow Cytometry Facility and the Beckman-Coulter's Expo32 Multiform software.

RNA extraction and real time-PCR

Total RNA from 2×10^6 DCs of different groups as mentioned above was extracted with a Trizol reagent according to the manufacturer's instructions (Invitrogen, USA). cDNA was synthesized by reverse transcriptase reaction mixture containing 0.5 μg random hexamer (Promega, USA), 200 units of Moloney murine leukemia virus reverse transcriptase (MMLV, Invitrogen, USA). This procedure followed the RNA PCR Kit from AMV Tech, Takara, Japan to detect mRNA transcription levels. A 50 μl PCR response system containing 25 μl of IQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA), 0.5 μl of gene-specific forward and reverse primers respectively (Table 1) and 5 μl cDNA was chosen. Cycling conditions included initial denaturation at 94°C for 2 min, followed by 35 circles of 94°C for 40 sec, 56°C for 50 sec, and 72°C for 1 min. The immature DC mRNA was used as a control and β-actin was included as a control for mRNA transcription level.

Chemotaxis assay

DC migration ability: a 24-well, 8 μm pore size Transwell system was used. DC Groups were set as mentioned above. 2×10^5 in 100 μl RPMI 1640 were added to the upper chamber and 600 μl RPMI 1640 with a different concentration of chemokines, as indicated, were added to the bottom chamber. To determine the number of cells that migrated non-specifically, the migration assays were performed in parallel contrast with no chemokines added to the bottom chamber. After 3 h incubation in a humidified incubator at 37°C in 5% CO_2, the cells were collected and counted with flow cytometry.

T cell migration ability to DC supernatant and tumor supernatant

In a 24-well 5 μm diameter Transwell system, DCs or tumor supernatants were centrifuged at 2,000 rpm for 5 min and any deposit was discarded to obtain the pure supernatant. Resting T cells or activated T cells (2×10^5) were stimulated by corresponding DC groups in the upper chamber and 600 μl of DCs or tumor supernatant to the lower chamber with or without a different concentration of anti-human IP-10 anti-body. After 3 h incubation, the cells in the underside well were collected and counted. The results of the chemotaxis index were calculated as follows: (the total number of the cells of interest in the bottom chamber) / (the total number of added cells) × 100%. Assays were performed in triplicates.

Table 1. Specific primers of PCR

Primer	Sequence (5'→3')	Size (bp)
CXCR4	F) GAACTTCCTATGCAAGGCAGTCCA R) CTTGTGCACAGTGTTCTCAAACTCA	531
SDF-1α/ CXCL12	F) ATGTGGCCAGAGCCAACGTCAAG R) GCCTTAGTCTAAGCTGCTACGTG	271
CCR7	F) TCCTTCTCATCAGCAAGCTGTCCT R) GCTTACTGAGCTCACAGGTGCTAC	395
ELC/ CCL19	F) CACCCTCCATGGCCCTGCTACT R) TAACTGCTGCGGCGCTTCATCT	304
DC-CK1/ CCL18	F) CAACAAAGAGCTCTGCTGCCTC R) CATAGCAGATGGGACTCTTAGAAG	317
IP-10/ CXCL10	F) TCTAAGTGGCATTCAAGGAGTACC R) AGCTTCCGGTAGTTCTTAAATGAC	205
β-actin	F) CACCCACACTGTGCCCATC R) CTAGAAGCATTGCGGTGGAC	650

Immunocytochemical analysis and ELISA

To investigate DC chemokine production by immunocytochemical staining, we transferred DCs from 6-well plates to 24-well plates with a small round glass slice adhered to the bottom of the well. After DCs had adhered to the bottom, RPMI 1640 was carefully removed, followed by three washes with PBS. Cells were fixed with acetone for 2-3 min at room temperature, washed with PBS three times, followed by immunocytochemistry according to the manufacturer's instructions (Maxim Company, Fuzhou, China). The cells were stained with rabbit anti IP-10 mAb or goat anti DC-CK1 mAb for the first antibody, isotype-matched control IgG was used as a control. Cells were then incubated with biotin-conjugated anti-rabbit or anti-goat Ig Ab as the second antibodies. The immune complexes were detected by using an ABC kit and a DAB Substrate Kit form Vector Laboratories according to the manufacturer's instructions. Cells were counterstained with hematoxylin and mounted. Each immunostaining protocol was performed on cell samples from at least three different donors.

To detect IP-10 protein secretion, DCs were cultured with apoptotic Raji cells for 72 h (Ag-DCs), or cultured with apoptotic Raji cells for 24 h followed by cultivation with 5C11 (anti-CD40-DCs) or TNF-α (TNF-DCs) for 48 h. Supernatants were collected after centrifugation at 1,500× g to remove particles. The aliquots were then used to detect IP-10 concentration by ELISA with the use of a human IP-10 assay kit obtained from R&D Systems (Minneapolis, MN) according to the manufacturer's instructions.

Mixed lymphocyte reaction (MLR)

To detect stimulatory capacity of the different DC groups to

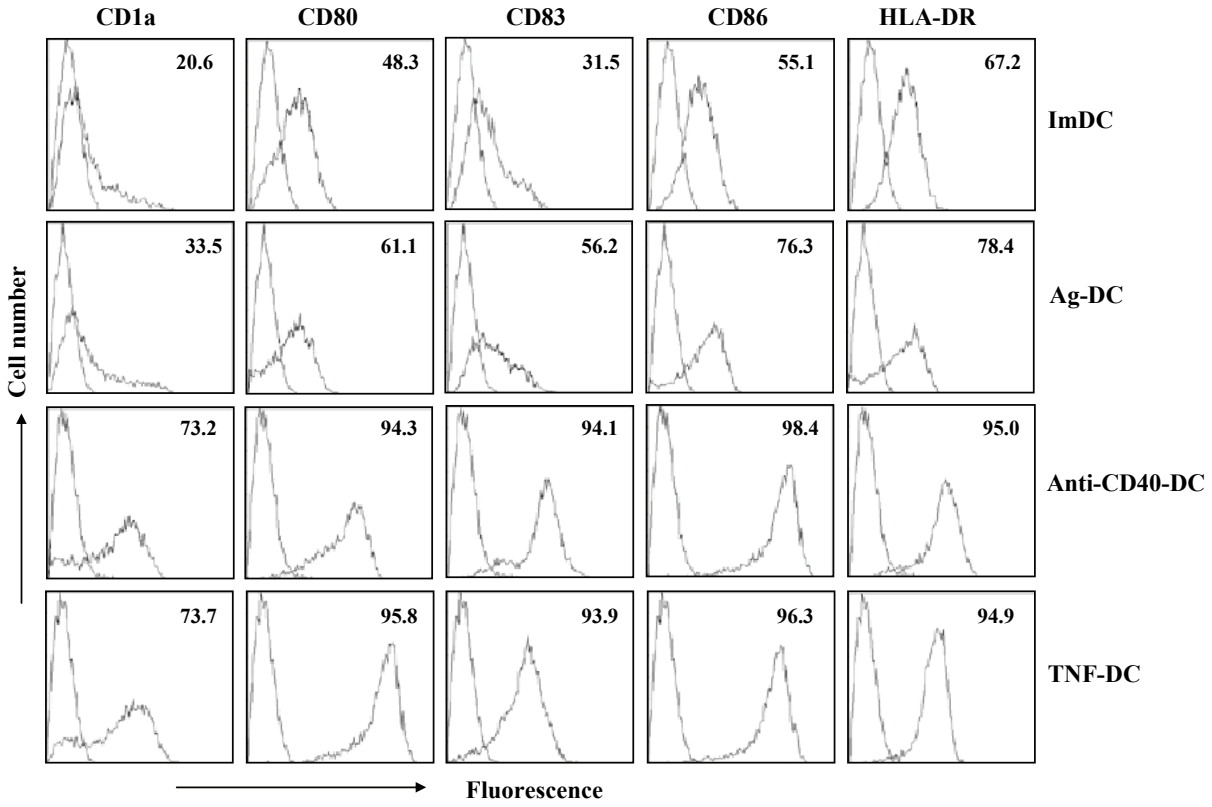

Figure 1. Expression of molecular characteristic of DC maturation analyzed by flow cytometry. DC precursors obtained from PBMCs were cultured with IL-4 and GM-CSF for 7 days as ImDCs; additional 72 h culture with apoptotic tumor cells as Ag-DCs; additional for 24 h culture with apoptotic tumor cells plus 48-hour additional culture with 5C11 as Anti-CD40-DCs; or with TNF-α as TNF-DCs. Flow cytometry was performed on DCs stained with an FITC-labeled secondary Ab and for molecules characteristic of maturation.

T cells, an allogenic MLR was performed. Briefly, purified, allogenic T cells (2×10^4/well) were cultured with 5×10^2 DCs from different groups as described above in a 96-well, flat-bottom plate in 200 μl RPMI 1640 medium supplemented with 10% FCS and 30 U/ml of IL-2 for 74 hours at 37°C in humidified air with 5% CO_2. The proliferative response of T cells was examined by pulsing the culture with ^{3}H-TdR (3.7×10^4 Bq/well) for the last 18 hours of incubation before harvesting. ^{3}H-TdR incorporation (CMP) was measured in a β counter (Pharmacia). The stimulatory index (SI) was calculated with the following formula: (cpm of test group - cpm of background) / (cpm of control - cpm of background) × 100%. Assays were performed in triplicates.

Western blotting

mDCs (1×10^7) generated as mentioned above were stimulated by 5C11 or TNF-α for additional 24 h, washed with cold PBS, and resuspended in a 500 μl sample buffer (10 mmol HEPES, pH 8.0; 10 mmol KCl; 0.1 mmol EDTA; 0.1 mmol EGTA; 1 mmol DTT; 0.5 mmol PMSF; 2.0 mg/L aprotinin). After incubation for 30 min at 4°C, 10 μl of 10% NP40 was added and the sample was vortexed for 20 sec.

Table 2. The phenotype analysis of DCs subjected to different maturation stimuli

	CD1a	CD80	CD83	CD86	HLA-DR
ImDC	21.8 ± 3.6	45.1 ± 6.4	31.9 ± 3.9	54.2 ± 4.5	68.5 ± 1.8
Ag-DC	35.3 ± 3.4	65.6 ± 5.4	55.9 ± 7.2	72.7 ± 5.5	77.3 ± 6.8
Anti-CD40-DC	71.3 ± 5.8**	94.9 ± 1.8**	94.1 ± 2.1**	97.8 ± 0.7**	94.0 ± 2.0**
TNF-DC	70.8 ± 5.4**	93.9 ± 2.2**	93.1 ± 1.1**	94.9 ± 1.9**	94.8 ± 2.0**

Expression of molecules characteristic of DC maturation on DCs subjected to different maturation stimuli as described in Figure 1. The representative results presented as mean ± SD from 5 independent experiments yielding similar results using DCs from a total of four different blood donors are shown. $^{**}p < 0.01$ *vs* ImDCs or Ag-DCs group.

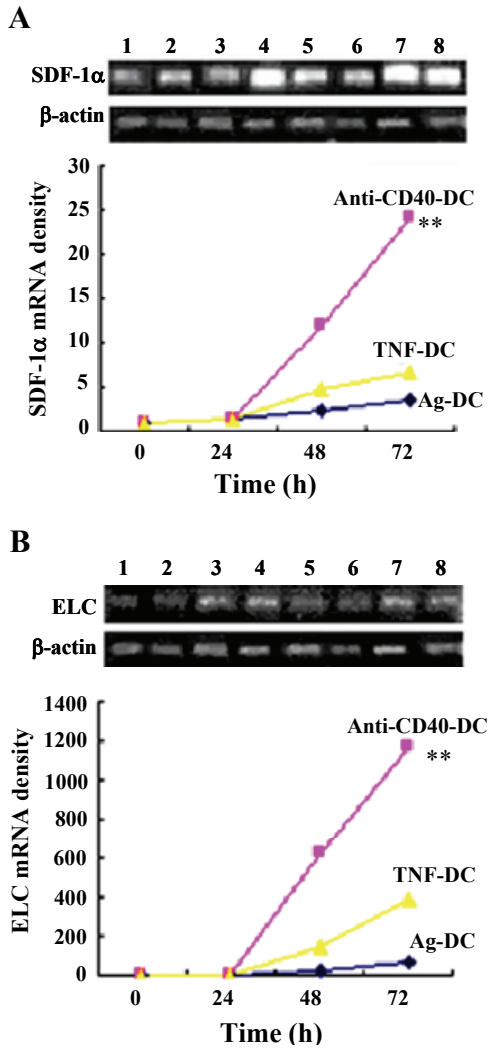

Figure 2. Enhanced SDF-1α (A) and ELC (B) expression of anti-CD40-DCs detected by quantitative real-time PCR. DC precursors obtained from PBMCs were cultured with IL-4 and GM-CSF for 7 days, then co-cultured with apoptotic Raji cells for 72 h. At 24 h of the co-culture, the anti-CD40 antibody (5C11) or TNF-α were added. Lane 1, ImDCs (day 7); Lane 2, Ag-DCs (24 h); Lane 3, Ag-DCs (48 h); Lane 4, Anti-CD40-DCs (24 h); Lane 5, TNF-DCs (24 h); Lane 6, Ag-DCs (72 h); Lane 7, Anti-CD40-DCs (48 h); Lane 8, TNF-DCs (48 h). The results were repeated 5 times by using DCs from different blood donors. $^{**}p < 0.01$ *vs* other mature DC groups.

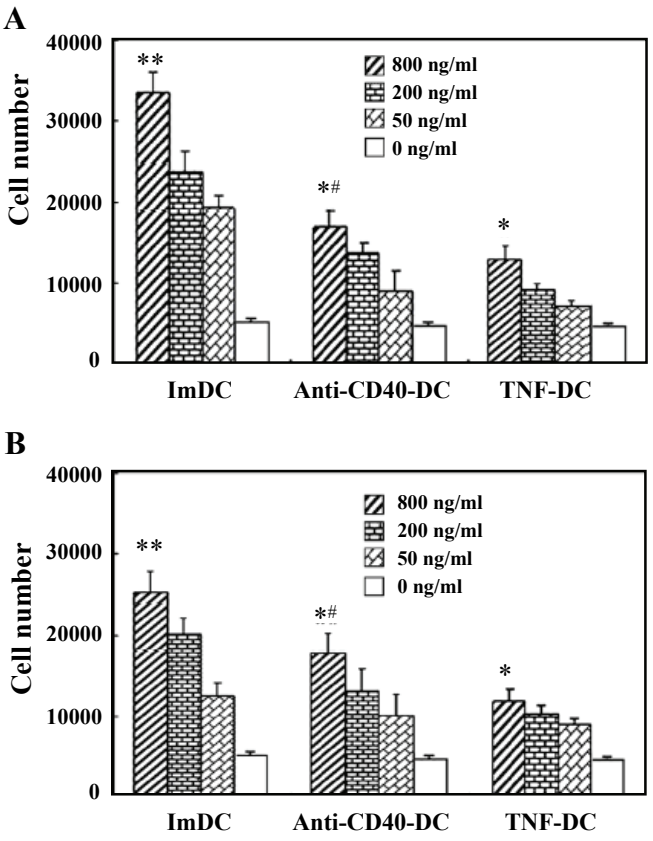

Figure 3. Anti-CD40-DCs were apt to migrate to high-levels of chemokine. Chemotactic activity of DCs are shown for different concentrations of SDF-1α (A) and SLC (B). Six hundred μl RPMI 1640 with the indicated concentration of SDF-1α or SLC were added to the lower well, DCs were added to the upper well. After 3 hours of incubation, the cells in the lower wells were collected and counted. $^{*}p < 0.05$, $^{**}p < 0.01$ *vs* other concentration in self group. $^{\#}p < 0.05$ *vs* the same concentration of Anti-CD40-DCs group.

Supernatants containing cytoplasmic protein were collected after centrifugation. Nuclear proteins were obtained after resuspension of the pellet in 50 μl of sample buffer (20 mmol HEPES, pH 8; 0.5 mol KCl; 1 mmol EDTA; 1 mmol EGTA; 1 mmol DTT; 1 mmol PMSF; 2.0 mg/L aprotinin; 20% glycerol). Fifteen μg of total protein from each sample was separated by 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Antibodies against NF-κB p65, p50, and β-actin (Santa Cruz, USA) were used to investigate nuclear and cytoplasmic extracts. Alkaline phosphates developed to NBT/BCIP (Watson Biotech, Shanghai, China) reagent was used for detection according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean value ± SD. All statistical tests were performed in the Statistical Package for the Social Sciences Version 8.0 (SPSS, Chicago, IL). In most assays, ANOVA was used for comparison between groups. p values < 0.05 were considered statistically significant.

Results

Both CD40 and TNF-α signaling can induce DC maturation

To assess the influence of CD40 signaling on DC maturation and to compare its effects with TNF-α, we investigated the expression of some characteristic molecules by flow cytometry. After culturing in the presence of anti-CD40 and

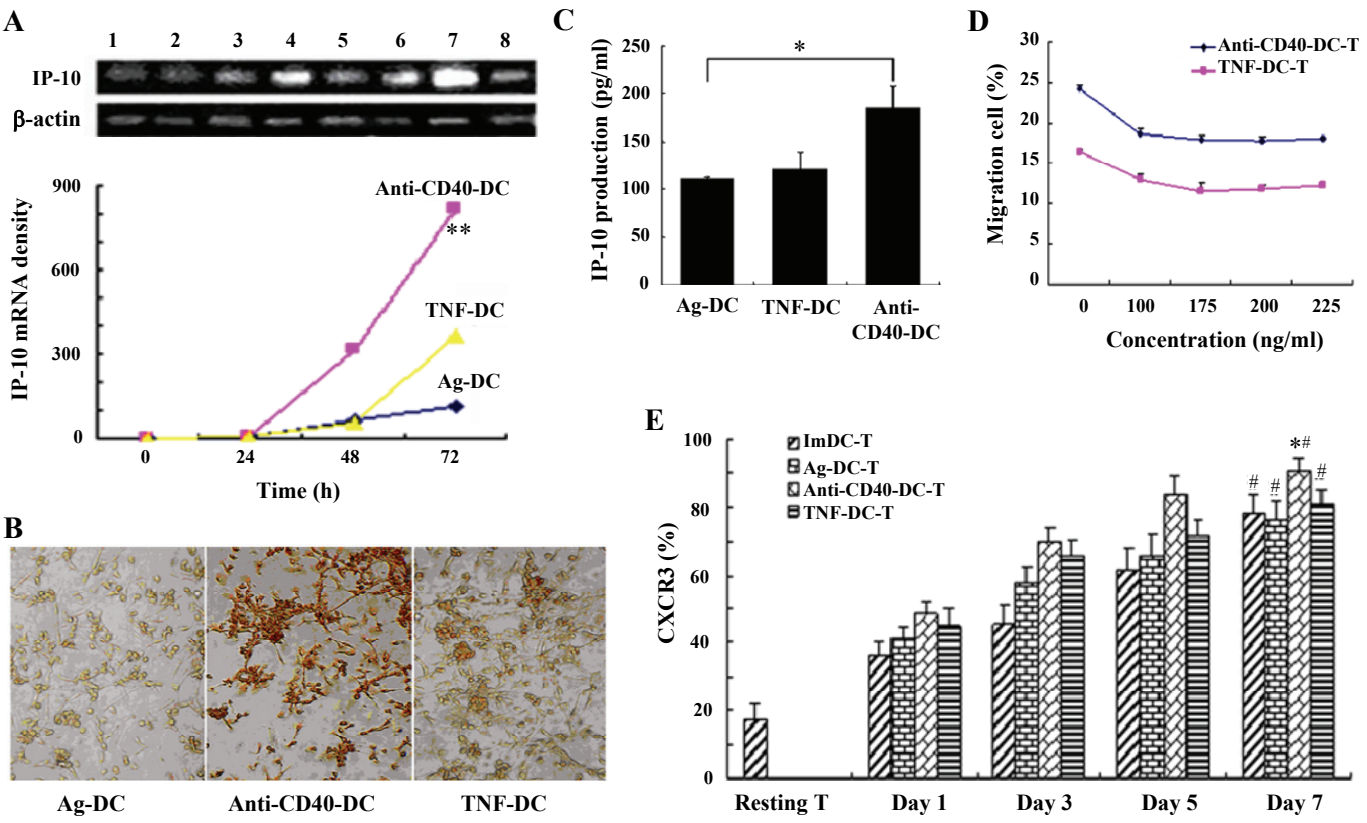

Figure 4. Anti-CD40-DCs secreted more IP-10 and enhanced the expression of CXCR3 on active T cells. (A) Quantitative real-time PCR analysis of IP-10. Anti-CD40-DCs secreted the highest level of IP-10. DC precursors obtained from PBMCs were cultured with IL-4 and GM-CSF for 7 days, then co-cultured with apoptotic Raji cells for 72 h. At 24 h of the co-culture, the anti-CD40 antibody or TNF-α were added. Lane 1, ImDCs (day 7); Lane 2, Ag-DCs (24 h); Lane 3, Ag-DCs (48 h); Lane 4, Anti-CD40-DCs (24 h); Lane 5, TNF-DCs (24 h); Lane 6, Ag-DCs (72 h); Lane 7, Anti-CD40-DCs (48 h); Lane 8, TNF-DCs (48 h). $^{**}p < 0.01$ *vs* other groups. (B) Immunocytochemical analysis of DC IP-10 protein level. Isotype control mAb staining of the corresponding group of cells revealed no immunoreactivities (data not shown). Original magnification, 200×. (C) IP-10 secretion of different DC groups. Supernatants from different stimuli were collected to measure IP-10 contents as described in Materials and Methods. Data were presented as Mean ± SD. A representative result from three independent experiments was shown. $^{*}p < 0.05$. (D) Blocking experiment by anti-human IP-10 antibody. Six hundred μl supernatant of Anti-CD40-DCs or TNF-DCs were added in the lower wells, incubated with anti human IP-10 antibody for 30 min, and then 3×10^5 active T cells were added in each upper well. After 3 hours, the cells in the upper and underside wells were counted. (E) The expression of CXCR3 on T cells by FACS. $^{\#}p < 0.01$ *vs* resting T cells, $^{*}p < 0.05$ *vs* other groups.

TNF-α, DC maturation markers such as CD1α, CD80, CD83, CD86, and HLA-DR were obviously up-regulated compared to immature DCs ($p < 0.05$) (Figure 1 and Table 2). These results suggest that both CD40 and TNF-α can induce DC maturation. Moreover, similar effects of anti-CD40 monoclonal antibodies on DC maturation were observed when tumor antigens from pleural fluids of lung cancer patients were used to load the monocyte-derived dendritic cells from the same patient (data not shown).

CD40L activated DCs induced higher levels of SDF-1α and ELC and express high levels of CXCR4 and CCR7

As CD40 antibody can efficiently stimulate DC maturation, we next investigated its effect on DC migration. We tested the expression of SDF-1α, ELC and their corresponding receptors CXCR4 and CCR7, which are important for regulating the migration of DCs from the periphery to secondary lymphatic organs. We found that immature DCs

Table 3. The expression of CXCR4 and CCR7 under different maturation stimuli

	CXCR4	CCR7
ImDC	37.6 ± 2.0	54.38 ± 3.3
Ag-DC	50.6 ± 5.99	64.5 ± 4.02
Anti-CD40-DC	87.68 ± 5.51*	89.4 ± 4.37*
TNF-DC	77.78 ± 5.86	81.2 ± 5.28

DC expression of CXCR4 and CCR7 was detected by FACS analysis. The DC groups and culture method were the same as in Table 2. The representative results from 5 independent experiments yielding similar results are shown. * $p < 0.01$ *vs* other DC groups.

cultured in the absence of apoptotic tumor cells produced low-level chemokines SDF-1α and ELC, and expressed low-levels of the corresponding receptors, CXCR4 and CCR7. Stimulating these immature DCs with apoptotic tumor cells led to the secretion of SDF-1α and ELC and the up-regulation of CXCR4 and CCR7 of DCs. After continuous cultivation or conjoint with anti-CD40 or TNF-α, these DCs further expressed higher level of SDF-1α and ELC and the chemokine-receptors CXCR4 and CCR7 ($p < 0.05$). Interestingly, compared with all other mature DCs, anti-CD40-DCs produced more SDF-1α, ELC, and expressed higher levels of chemokine receptors CXCR4 and CCR7 ($p < 0.05$) (Figure 2 and Table 3).

CD40 activated DCs were able to migrate to immature DC-attracting chemokines

We next determined the effect of anti-CD40 on DC migration in response to SDF-1α and SLC using *ex vivo* chemotaxis assay. Immature DCs but not mature DCs had a strong migratory ability towards high concentrations of these chemokines. In contrast to TNF-DCs, anti-CD40-DCs still had strong migration ability to constitutive chemokines especially at high concentration (Figure 3). Furthermore, there were no significant differences of migrated cells in the underside wells among different DC groups when no chemokines were added to the bottom chamber.

CD40 activated DCs secreted more IP-10 and induced enhanced expression of CX3CR1 in activated T cells

All the results above demonstrated that anti-CD40 antibody could stimulate DC maturation and enhance their ability to migrate in response to chemokines. But effective anti-tumor immunity depends on the cooperation of APCs and active effector cells at the tumor locus. Next, we investigated whether anti-CD40 signaling active DCs attract T cells through their secretion of certain chemokines and the expression of the corresponding receptors on T cells.

To attain insights into the recruitment pathway of active T cells by DCs, we chose IP-10, which is a key chemokine known to be present at high levels at site of inflammation. By real-time PCR (Figure 4A), immunocytochemistry (immuno-reactivities were not seen when isotype control Ab was used) (Figure 4B), and ELISA (Figure 4C), all the samples showed that both the mRNA and protein levels of IP-10 in anti-CD40-DCs were higher than those of other DC groups.

To verify the effect of IP-10 on T cell trafficking, we performed blocking experiments. As described in the chemotaxis assay section above, different concentrations of anti-human IP-10 antibody were added to the bottom chamber. We found in the anti-CD40-DC group, from the density of 0 to 225 ng/ml, the chemotaxis rate descended conspicuously from 24.3% to 17.8%, and didn't descend as the density increased (Figure 4D). In contrast with anti-CD40-DCs, the TNF-DC group, when treated with the density of 0 to 175 ng/ml, the chemotaxis rate descended from 16.4% to 11.5%, and didn't descend as the density increased. These results showed that the highest effective density of the anti-CD40-DCs was higher than that of

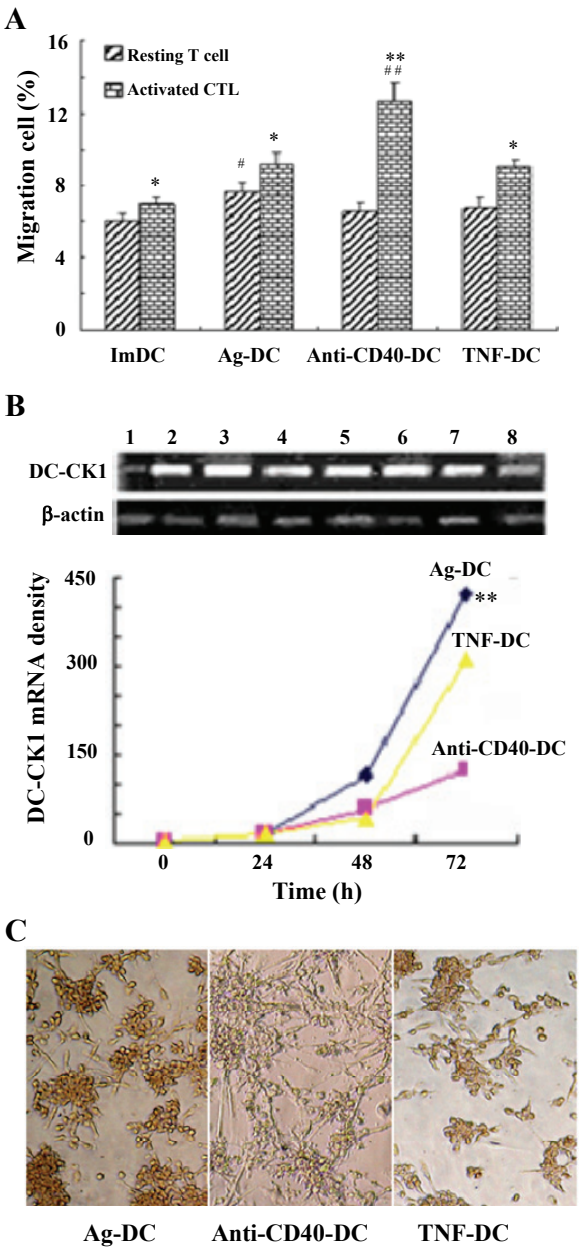

Figure 5. Anti-CD40-DCs had the greatest ability to attract active T cells but not resting T cells. (A) The ability of DC supernatant to attract T cells. DCs and T cell groups were set up as described in Materials and Methods. *$p < 0.05$, **$p < 0.01$ *vs* resting T cells. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ *vs* other T cell groups. (B) Quantitative real-time PCR analysis of DC-CK1. DC precursors obtained from PBMCs were cultured with IL-4 and GM-CSF for 7 days, then co-cultured with apoptotic Raji cells for 72 h. At 24 h of the co-culture, the anti-CD40 antibody or TNF-α were added. Lane 1, ImDCs (day 7); Lane 2, Ag-DCs (24 h); Lane 3, Ag-DCs (48 h); Lane 4, Anti-CD40-DCs (24 h); Lane 5, TNF-DCs (24 h); Lane 6, Ag-DCs (72 h); Lane 7, Anti-CD40-DCs (48 h); Lane 8, TNF-DCs (48 h). (C) Immunocytochemical analysis results demonstrated that DC-CK1 protein was expressed by DCs. The Ag-DCs had the strongest expression of DC-CK1. Isotype control staining revealed no immunoreactivities (data not shown). Original magnification: 200×. The representative results from 3 independent experiments with similar results are shown.

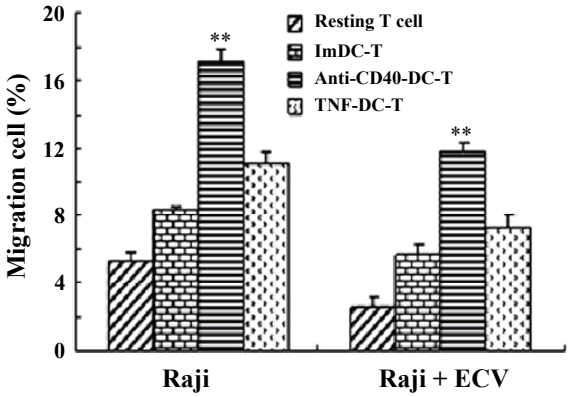

Figure 6. Anti-CD40-DC-T cells had the strongest chemotactic response to tumor supernatant and greatest transmigration ability through ECV cells. Six hundred μl of tumor cell (Raji) supernatant were added to each of the lower wells. T cells was added to upper wells with or without an endothelial monolayer. After 3 h of incubation, the cells in the lower wells were collected and counted with FACS. $^{**}p < 0.01$ *vs* other groups.

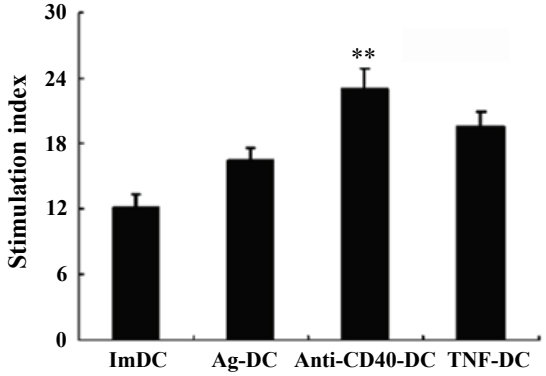

Figure 7. Anti-CD40-DC supernatant had the strongest ability to induce allogenic T cell proliferation. T cells (2×10^4/well) were cultured in triplicate in the presence of 4×10^2 DCs in 96-well plates for 74 hours at 37°C in humidified air with 5% CO_2 with the addition of ^{3}H-TdR (3.7×10^4 Bq) in the last 18 hours of incubation. The incorporation of ^{3}H-TdR (mean ± SD) was measured and stimulatory index (SI) values were calculated and presented. DCs were grouped as described above. $^{**}p < 0.01$ *vs* other groups.

TNF-DCs and suggested that the concentration of IP-10 secreted by anti-CD40-DCs was more than TNF-DCs. Interestingly, anti-CD40-DCs also induced more CXCR3 expression on activated T cells than other DC groups by flow cytometry (Figure 4E). Thus, anti-CD40-DCs are potentially the most potent cells to attract more active T cells through the interaction of IP-10 with CXCR3.

CD40 activated DCs had the greatest propensity to attract T cells but not resting T cells

To test this hypothesis, we investigated whether anti-CD40-DCs had a stronger ability to attract active or resting T cells. We detected the chemotactic capacity of T cells in DC supernatant as described above. As shown in Figure 5A, all groups of activated T cells had greater migration ability than resting T cells. Anti-CD40-DCs are most potent in attracting activated T cells. By contrast, Ag-DC supernatant had the strongest ability to attract resting T cells. To further explore the mechanism whereby Ag-DCs attract resting T cells, we focused on DC-CK1 because accumulating evidence indicates that this is a key chemokine for DCs in attracting resting T cells. By real-time PCR (Figure 5B) and immuno-cytochemistry (Figure 5C), we found that Ag-DCs secreted much more DC-CK1 than other mature DC groups.

T cells activated by anti-CD40-DCs showed the greatest migration to tumor supernatant

To further investigate the particularity and potential clinical use of anti-CD40-DC-T cells, we detected T cell migration to tumor supernatant. As shown in Figure 6, activated T cells showed greater migration to tumor supernatant than resting T cells. Among the activated T cells, those T cells induced by Anti-CD40-DCs showed the highest migration to tumor supernatant and transmigration ability through endothelial cells (ECS) cultured on Transwell membrane filters.

Anti-CD40-DCs supernatant had the strongest ability to induce allogenic T cell proliferation

To further explore the proliferative stimulatory capacities of the different DC groups, allogenic MLR was performed by ^{3}H-TdR incorporation assay at the DC:T cell ratio of 1:50. The results shown in Figure 7 indicated the stimulatory index of 5C11 activated DCs were the strongest of all the groups, including TNF-α stimulated DCs. These results further indicate that the CD40 signal is much more efficient than conventionally used TNF-α to induce DC vaccines.

CD40-DCs had stronger that NF-κB activity than TNF-DCs

It is widely accepted the production of chemokines is regulated by the NF-κB pathway. To further elucidate the mechanism causing the difference between anti-CD40 and TNF-α on mDCs, we measured the P50 and P65 NF-κB subunits activity after 5C11 or TNF-α stimulation. As shown in Figure 8, the cytoplasmic protein levels of NF-κB subunits were enhanced in mDCs after treatment with 5C11 compared to TNF-α. Nucleoprotein of NF-κB subunit was only detectable in mDCs after 5C11 but not TNF-α treatment. These results suggest that the different functional effect of CD40 and TNF-α signals on mDC chemokine secretion may be caused by their different NF-κB activation activities.

Discussion

DCs are the most potent APCs *in vivo* in the context of their ability to activate naïve T cells. As a member of the TNF-TNFR costimulatory molecule super family, CD40/CD40L is a key signal for DC maturation into fully functional APCs (14-16). On the other hand, whether DCs can fully mature depends on their ability to undergo *in vivo* migration. The *in vivo* migration of DCs is a complicated and

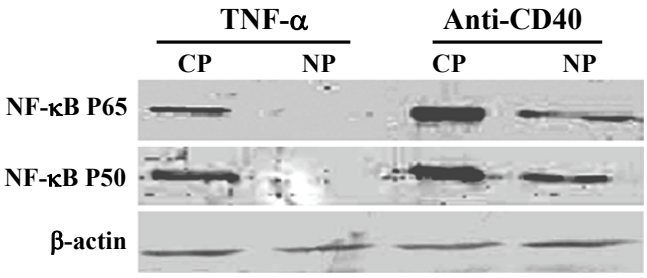

Figure 8. Nuclear translocation of NF-κB subunits in generated DCs. Cultured with apoptotic Raji cells for 24 hours, 1×10^7 GM-CSF and IL-4 generated mDCs were treated with 5C11 or TNF-α for additional 48 hours, and then analyzed by Western blotting as described in Materials and Methods. CP, cytoplasm protein; NP, nuclear protein.

successive process in which a series of chemokines and accessory receptors play critical roles (29, 30). Thus, we investigated the impact of CD40/CD40L costimulatory signal on the function of mDCs and DC-activated T cells as well as their chemokine secretion and chemokine receptor expression.

PBMC-derived immature DCs, that express low-levels of costimulatory molecules and high-levels of inflammatory chemokine receptors CCR1, CCR2, etc.; can respond to inflammatory chemokine RANTES, etc. (31). These immature DCs have potent ability to migrate, to ingest and dispose antigens into inflammatory loci where high-level inflammatory chemokines are produced. After peripheral immature DCs arrive at the inflammatory locus and ingest antigen, they receive stimuli from a variety of maturation factors and gradually mature. Mature DCs secrete large quantities of inflammatory chemokines, down-regulate their expression of inflammatory chemokine receptors through autocrine effect, and show a marked decrease in chemotactic response to inflammatory chemokines. This allows mature DCs to migrate away from the inflammatory locus after antigen ingestion. At the same time, these mature DCs increase their secretion of constitutive chemokines, up-regulate the expression of chemokine receptors CXCR4 and CCR7, and migrate to secondary lymphatic organs attract resting T cells and stimulate T cell maturation and polarization, switch on all kinds of immunological response, and finally get themselves real maturation (32).

Previous studies revealed that immature DC vaccines exhibited strong chemotaxis toward the tumor locus along with powerful antigen presenting ability (33, 34). Yet, when immature DCs moved to tumor locus, they were influenced by multi-negative regulatory factors, especially IL-10, present in the tumor microenvironment (35). Although they could ingest tumor antigens, they were restricted at the tumor locus, and lost their ability to move to secondary lymph organs. Although conventional mature DC vaccines which were cultivated by antigen-load could partially withstand the negative regulation of tumor microenvironments, they still lost part of their ability to exit the tumor locus. Thus, conventional mature DCs can't effectively migrate to tumor locus, and attract activated T cells to the tumor locus. The effective anti-tumor immunity depends on the cooperation of APCs and effector cells like active T cells at tumor locus. It would be the possible reason that immature DCs and conventional cultivated mature DC vaccines was not satisfactory in clinical use. Therefore, further study of the DC migration ability under different maturation stimulating conditions should be important for the design of effective tumor immunotherapy (36, 37).

In our experiments, PBMCs were isolated from heparinized whole blood of healthy volunteer donors and immature DCs were cultivated for 7 days. After these cells ingested specific apoptotic tumor cells, we added either exogenous CD40 stimulatory mAb 5C11 or TNF-α as an auxiliary stimulatory signal in order to study the influence of different exogenous signals on DC maturation and chemotaxis. Results showed that mature DCs stimulated by different signals had different chemokine secretions and chemokine receptor expression patterns. CD40 stimulatory mAb 5C11 had the strongest ability to stimulate DC secretion of the constitutive chemokines SDF-1α and ELC, along with their receptors CXCR4 and CCR7, respectively. Previous reports had shown that CCR7 had a strong function of resisting down-regulating effects mediated by the corresponding ELC (38). Our results corroborate these findings. Constitutive chemokines can not down-regulate their corresponding receptors through an autocrine pathway. We speculate that the high level expression of inflammatory chemokine receptors CCR1 and CCR2 does occur during the process of DC maturation, but CXCR4 and CCR7 expressions are better indicators of DC maturation.

Thus, the strong chemotactic migration of immature DCs to the tumor locus is accounted for by the heightened expression of receptors specific for chemokines found in the tumor microenvironment. Immature DCs move up concentration gradients of these chemokines to arrive at the tumor locus. As they mature, the expression of CCR1 and CCR2 on DC is down-regulated and the expression of CXCR4 and CCR7 is up-regulated. We presumed that this causes DCs to be chemotactically drawn toward constitutively secreted chemokines in secondary lymphatic tissues while at the same time eliminate the positive chemotaxis to the tumor site. This presumption is based on the relative abundance of ligand binding sites. When a receptor is expressed at high levels, more of its ligand can bind and cause cell signaling, while lower levels of receptor mean less ligand binds, yielding fewer cell signals. It is probable that when CCR1 and CCR2 are expressed at high levels in immature DCs, this is what helps them localize to tumor loci. Moreover, when CCR1/2 are down-regulated and CXCR4 and CCR7 are up-regulated, it is probable that this accounts for DCs moving away from tumor loci and toward secondary lymphatic tissues. Our results indicated that anti-CD40-DCs express more CXCR4 and CCR7 than other mature DC groups. Therefore, we anticipated that these DCs would have the greatest likelihood of migrating to lymphatic tissues.

To investigate the migration capacity and potential clinical use of anti-CD40-DCs, we focused on DCs' migration ability. The results showed that when compared with TNF-α stimulated mature DCs, anti-CD40-DCs demonstrated a stronger ability to migrate in response to SDF-1α and SLC signaling. This could possibly be related to the high level expression of CXCR4 and CCR7 after 5C11 stimulation and the subsequent sensitivity of these cells to their corresponding chemokine ligands. In addition, our experiments indicated that 5C11 could help DCs to attract active T cells in three major ways: through secreting IP-10, up-regulating CXCR3 expression on active T cells, and enhancing the chemotactic affinity of active T cells for chemokines at the tumor locus. Anti-CD40-DCs, which secreted the highest level of IP-10, had a strong chemotactic ability to attract anti-CD40-DC-activated T cells which expressed high levels of the corresponding receptor CXCR3. At the same time, anti-CD40-DCs demonstrated a robust resistance to multiple negative-regulatory mechanisms present in the tumor microenvironment, a strong chemotactic affinity for tumor supernatant, and a T cell stimulatory capacity which surpassed that of conventional TNF-α stimulated DCs. Therefore, anti-CD40-DCs would effectively attract the migration and infiltration of anti-CD40-DC-T cells to the tumor locus, thereby colocalizing APCs and effector cells to generate an effective anti-tumor immune response.

In summary, recent published data indicate tumor antigen loaded DC vaccination can be an effective vaccine method to induce therapeutic and protective anti-tumor immunity (39, 40). A key finding of our work is that anti-CD40 Ab treatment can significantly enhance DC functions, including migration, production of high levels of chemokines, and T cell activation, compared with that of TNF-α treatment. These anti-CD40 Ab-induced DCs will prove to be invaluable for improving DC-based vaccines for tumor therapy.

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