Egr2 induced during DC development acts as an intrinsic negative regulator of DC immunogenicity

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Early growth response gene 2 (Egr2), which encodes a zinc finger transcription factor, is rapidly and transiently induced in various cell types independently of de novo protein synthesis. Although a role for Egr2 is well established in T-cell development, Egr2 expression and its biological function in dendritic cells (DCs) have not yet been described. Here, we demonstrate Egr2 expression during DC development, and its role in DC-mediated immune responses. Egr2 is expressed in the later stage of DC development from BM precursor cells. Even at steady state, Egr2 is highly expressed in mouse splenic DCs. Egr2-knockdown (Egr2-KD) DCs showed increased levels of major histocompatibility complex (MHC) class I and II and co-stimulatory molecules, and enhanced antigen uptake and migratory capacities. Furthermore, Egr2-KD abolished SOCS1 expression and signal transducer and activator of transcription 5 (STAT5) activation during DC development, probably resulting in the enhancement of IL-12 expression and Th1 immunogenicity of a DC vaccine. DC-mediated cytotoxic T lymphocyte (CTL) activation and antitumor immunity were significantly enhanced by Egr2-KD, and impaired by Egr2 overexpression in antigen-pulsed DC vaccines. These data suggest that Egr2 acts as an intrinsic negative regulator of DC immunogenicity and can be an attractive molecular target for DC vaccine development.

Keywords: DC development · Egr2 · Immunotherapy · Negative regulator · Th1 immunity

Introduction

Dendritic cells (DCs) consist of several phenotypic and functionally distinct subsets. Given their multiple functions in host immunity, there has been great interest in elucidating the mechanisms controlling the development of DCs from bone marrow (BM) precursor cells. However, the detailed molecular mechanisms underlying DC differentiation from BM or HSCs remain widely unknown [1].

In our previous report, we showed that a cytokine inducible SH2-domain protein, as a positive regulator, facilitates DC differentiation to become a potent stimulator of cytotoxic T lymphocytes (CTLs) [2]. Here, we focused on the function of early
growth response gene 2 (Egr2), a zinc finger transcription factor, in DC development and DC-mediated immunogenicity. In the present study, we found that Egr2 is strongly induced during the later stage of DC development and acts as an intrinsic negative regulator of DC development and DC-mediated immunogenicity.

Egr2 is well established in the growth, differentiation, and function of several cell types. Egr2 was reported to inhibit suppressor of cytokine signaling 1 (SOCS-1) expression and upregulate Bcl-2 during positive selection of thymocytes [3]. Loss of Egr2 causes downregulation of IL-7R, upregulation of SOCS-1 mRNA, inhibition of signal transducer and activator of transcription 5 (STAT5) phosphorylation, IL-7-mediated postselection survival [4], and inhibition of anergy, leading to the development of a late-onset lupus-like autoimmune disease characterized by the accumulation of interferon-γ (IFN-γ) and interleukin-17 (IL-17) producing CD4+ T cells [5–7]. IL-10-secretory regulatory CD4+CD25+LAG3+ T cells characteristically express Egr2 in inflammatory bowel disease [8]. However, a recent study indicated that Egr2 is not required to mount normal immune responses in vivo against foreign antigens, and that loss of Egr2 in T cells does not interfere with a normal response to pathogens in healthy animals [9]. Egr2 has also been implicated in hindbrain development, peripheral nerve myelination, and tumor suppression, but has not been shown to have a role in monocyte/macrophage cell fate determination [10]. A point mutation in the DNA-binding domain of Egr2 is likely involved in the development of congenital hypomyelinating neuropathy [11]. Egr2 expression requires both MAPK and calcineurin signaling pathways, promoting osteoclast survival through the MEK/ERK-dependent pathway [12]. Egr2 also plays an important role in PTEN-induced or p53-mediated cell apoptosis in cancer cells [13,14].

As a target gene of nuclear factor of activated T cells (NFAT), Egr2 is essential for the development of induced natural killer T (iNKT) cells, and it plays an important role in the selection, survival, and maturation of NKT cells [15], in vertebrate tendon differentiation [16], and adipogenesis [17]. In contrast, Egr2 is known not to be required for conventional T-cell [15] or monocyte/macrophage differentiation [10]. NFAT activation through the beta-glucan receptor Dectin-1 in the recognition of pathogenic fungi plays a role in the induction of Egr2 and Egr3 and Dectin-1-triggered NFAT activation regulates IL-2, IL-10, and IL-12 p70 production in zymosan-stimulated DCs [18], suggesting that NFAT activation and associated early growth response expression in myeloid cells are likely involved in the regulation of the innate antimicrobial immune response.

Despite the diverse functions of Egr2 in different cell types, its expression and roles in DC development and immunogenicity are poorly understood. In this study, we demonstrate that (i) Egr2 is markedly induced during DC development from BM stem cells, (ii) Egr2 regulates the expression of surface immune-related molecules, antigen uptake capacity, DC migration, and cytokine secretion possibly in association with SOCS-1 expression and STAT5 signaling pathways, and (iii) Egr2 inhibits the efficacy of DC-based tumor immunotherapy. Our findings suggest that Egr2 expressed in DCs plays an important role as an intrinsic negative regulator in the control of DC-based immunogenicity. Egr2 would be an attractive molecular target for DC vaccine development.

Results

Egr2 induced during BM-derived DC (BMDC) development is involved in BMDC development

During GM-CSF-mediated DC development from mouse BM cells, Egr2 expression was induced in the later stage of DC development at both the mRNA and protein levels (Fig. 1A). Intracellular staining revealed that Egr2 expression was also increased during DC development in parallel with CD11c (Fig. 1B). In addition, Egr2 was clearly detectable in steady-state mouse splenic DCs as was shown in BMDCs (Fig. 1C). Next, we examined the surface phenotypes of BMDCs in the absence of Egr2 during DC development. Egr2 was silenced by more than 80% at both protein and mRNA levels by transfection of DC precursor cells on day 4 with two Egr2-specific si-RNAs (Fig. 1D). Egr2-knockdown (Egr2-KD) increased cell apoptosis by about 5% in immature BMDCs (imDCs), but did not affect the mature BMDCs (mDCs) population (Supporting Information Fig. 1). Egr2-KD BMDCs (Egr2KD imDCs) showed increased levels of major histocompatibility complex class I and II molecules (MHC I and MHC II) and co-stimulatory molecules including CD40, CD80, and CD86 (Fig. 1E). Quantitative results of more than three consecutive experiments revealed that the levels of CD80, MHC I, and MHC II were significantly increased in Egr2KD imDCs compared with those of control DCs (con imDCs) (Fig. 1F). Even after activation with lipopolysaccharide (LPS), Egr2-KD mature BMDCs (Egr2KD mDCs) had higher levels of MHC and CD80 molecules than WT mature BMDCs (con mDCs) (Fig. 1G), indicating that Egr2 expression is likely involved in the control of DC development from BM cells.

Egr2 expression inhibits the migration and antigen-uptake capacity of BMDCs

To investigate the effects of Egr2 expression on DC migration, we first measured CCR7 expression on Egr2KD imDCs and mDCs. The CCR7+ cell population increased significantly, by about 15–20%, in both imDCs and mDCs by Egr2-KD (Fig. 2A). mDCs migration capacity toward CCL19 in transwell plates was enhanced by about 10–20% by Egr2-KD as compared with that of WT mDCs (Fig. 2B). Interestingly, even though the CCR7+ population was increased by Egr2-KD in both imDC and mDC populations, a meaningful increase in DC migration was only detected in the mDC population, which was likely due to the higher expression of CCR7 (higher MFI) in mDCs as compared with that in imDCs (Fig. 2A, left panel). Next, we measured the antigen-uptake capacity of Egr2KD imDCs and mDCs using FITC-conjugated dextran. The antigen-uptake capacity was significantly enhanced in Egr2KD imDCs as compared with that of WT imDCs (Fig. 2C). The antigen-uptake
capacity of mDCs was significantly enhanced by Egr2 silencing in repeated experiments. A higher antigen-uptake capacity is characteristic of imDCs, but was interestingly demonstrated in mDCs when Egr2 was knocked down. It is well established that the endocytic receptor DEC-205 is significantly upregulated during the maturation of BMDCs [19]. As expected, Egr2-KD enhanced the expression of DEC-205 both in imDCs and mDCs (Supporting Information Fig. 2). These data suggest that Egr2 is involved not only in the downregulation of CCR7 expression and associated DC migration, but also in disrupting the antigen-uptake capacity of mDCs during DC maturation. Detailed molecular studies will be required for further clarification. These data suggested that Egr2 expression during DC development is likely involved in the control of the immunogenicity of DCs in vivo.

**Egr2 controls the IL-12 production and T-cell proliferation capacity of DCs**

Next, we assessed the level of cytokine secretion from WT and Egr2KD DCs. Egr2KD mDCs secreted more IL-12 than WT mDCs, but the secretion of other proinflammatory cytokines (TNF-α, IL-6, and IL-1β) was not affected by Egr2-KD (Fig. 3A). SOCS-1 and its associated signaling pathways were previously reported to regulate IL-12 production in DCs [20]. In addition, we demonstrated that cytokine inducible SH2-domain protein, a member of the SOCS family, is involved in the feedback inhibition of STAT5 activity [2]. We examined SOCS-1 expression and STAT5 phosphorylation in Egr2KD DCs. Egr2-KD dramatically reduced SOCS-1 expression and STAT5 activation in DCs (Fig. 3B). In the co-culture experiments with T cells, Egr2KD DCs developed significantly larger surrounding T-cell colonies than WT control DCs, and was further confirmed by MTT (3-(4,5-Dimethylthiazol-2-yI)-2,5-diphenyltetrazolium bromide) assay (Fig. 3C), indicating that Egr2 expressed during the later stage of DC development is involved in the negative regulation of DC immunogenicity to induce T-cell responses.

**Egr2KD DCs are more potent than WT DCs in inducing T-cell responses**

Next, we examined the T-cell stimulation capacity of Egr2KD DCs using ovalbumin peptide (OVA257-264 and OVA123-339) pulsed BMDCs and 5,6-carboxyfluorescein succinimidyl ester (CFSE) labeled naive splenic T cells obtained from BL6-originated OVA-specific receptor transgenic OT-1 and OT-2 mice, respectively. DC-mediated OT-1 T-cell proliferation was significantly enhanced by Egr2-KD (Fig. 4A). OT-2 T-cell proliferation was also enhanced by Egr2-KD, albeit to a lesser degree (Fig. 4B). Th1, Th2, and Th17 cytokines were assessed in the culture supernatants of OT-2 T-cell/DC co-cultures. The level of IFN-γ was significantly enhanced in the supernatants of Egr2KD DC/T-cell co-cultures as compared with that of normal DC/T-cell co-culture, while there was no significant difference in the representative Th2 (IL-4 and IL-13) and Th17 (IL-17A) cytokine levels between the experimental groups (Fig. 4C). These results suggest that Egr2 controls DC-mediated Th1 immunity. Next, in order to see whether the effect of Egr2-KD on DC-mediated T-cell proliferation was attributable to a decrease in the Treg-cell population, we analyzed the Treg cells in Egr2KD DC/T-cell co-cultures. In co-cultures with OVA257-339-pulsed DC/OT-2 T cells for 5 days at a 1:10 ratio, there was no significant difference in CD4+/CD25+/Foxp3+ T-cell populations between the Egr2KD DC/T-cell and the Egr2 WT DC/T-cell co-cultures in both syngeneic and allogeneic reactions (Fig. 4D). These data suggest that Egr2-mediated negative regulation of DC-derived Th1 immunity has little to do with Treg cells.

**Egr2 acts as a negative regulator of a DC vaccine in DC-based tumor immunotherapy**

To study the effects of Egr2-KD on immunogenicity of DCs in vivo, we analyzed CTL activity in mice vaccinated with Egr2KD DCs that had been pulsed with OVA peptides. The levels of IFN-γ in the spleen and the draining lymph node (DLN) of OT-1 mice vaccinated with OVA257-264-pulsed Egr2KD DCs were significantly higher than in mice vaccinated with OVA257-264-pulsed WT DCs (Fig. 5A). In the spleen and the DLN of vaccinated mice, OVA257-264-specific CTL activities were assessed with E.G7 as target cells. Antigen-specific CTL activities were significantly enhanced by the Egr2KD DC vaccine in both the spleen and DLN of vaccinated mice as compared with mice vaccinated with the WT DC vaccine (Fig. 5B). Similar patterns of T-cell proliferation (Supporting Information Fig. 3A) and CTL activity (Supporting Information Fig. 3B) were observed in OT-1 T-cell-transfused C57BL/6 mice when vaccinated with OVA peptide-pulsed Egr2KD and WT DCs (Supporting Information Fig. 3). These data imply...
that Egr2 in DCs plays an important role in the control of DC-mediated Th1 immunity and CTLs.

To determine the function of Egr2 in DC-based tumor immunotherapy, tumor-bearing C57BL/6 mice were treated twice with WT and Egr2KD OVA peptide-pulsed DC vaccines, respectively. Tumor growth was more significantly inhibited by vaccination with OVA peptide-pulsed Egr2KD DCs than with WT DCs in E.G7-derived tumor-bearing mice (Fig. 5C). However, none of the EL4 cell-derived tumor-bearing mice showed tumor suppression by DC vaccination, even with OVA peptide-pulsed Egr2KD DCs (Fig. 5D). These results indicate that Egr2KD DCs are more effective than WT DCs to induce antigen-specific antitumor immunity.
In addition, we examined the effects of Egr2-overexpression on DC maturation and DC-mediated antitumor immunity. Ectopic expression of Egr2 (Fig. 6) by transfection with the recombinant plasmid pcDNA-flag-egr2 significantly downregulated the expressions of CD80 and MHCII in mDCs (Fig. 6B and C) and IL-12 secretion (Fig. 6D). Moreover, Egr2 overexpression abrogated the therapeutic capacity of the DC vaccines for cancer immunotherapy (Fig. 7). Taken together, our data suggest that Egr2 acts as an intrinsic negative regulator of the immunogenicity of DCs.

Discussion

Early growth response genes are immediate early genes encoding transcription factors that bind to specific DNA sequences and control the transcription of their target genes. Moreover, immediate early genes are rapidly and transiently induced in various cell types independently of de novo protein synthesis. Diverse intracellular messenger systems activate these genes to link membrane events to the nucleus. The family of EGRs is composed of four members: Egr1, Egr2, Egr3, and Egr4 [21, 22]. Their expression is rapidly induced by serum and growth factors, such as nerve growth factor, EGF, and PDGF [23–25]. Recently, it was reported that Egr2 is required for T-cell anergy in vitro and in vivo [26]. Although several important functions of Egr2 are established in other cell types and T cells, its role in DCs has not been reported yet.

In this study, we first demonstrated the specific roles of Egr2 during DC development and associated immune responses. We found that Egr2 expression increases at both the RNA and protein level during DC development from BM precursor cells. Egr2 expression in steady state was confirmed in mouse splenic DCs as well (Fig. 1A–C). Egr2-KD enhanced the expression of MHC I/II molecules and other co-stimulatory molecules on the surface phenotypes of DCs (Fig. 1E and F). In addition, Egr2-KD DCs showed enhanced migratory capacity and antigen-uptake capacity (Fig. 2). These data suggested that Egr2 acts as an intrinsic negative regulator of the immunogenicity of DCs.

In Figure 3, Cytokine production and T-cell stimulation capacity of mDCs in association with Egr2 expression. (A) Th1 and proinflammatory cytokines were assessed by ELISA from the culture supernatants of Egr2-KD and WT C57BL/6-originated BMDCs matured by LPS (200 ng/mL) for 24 h. Data are shown as mean ± SD of six samples pooled from three experiments performed. (B) The effects of Egr2-KD on STAT5 activation and SOCS-1 expression were assessed by Western blotting. β-actin was used as a loading control. (C) OT-1 T cells were co-cultured with WT and Egr2-KD OVA257–264 peptide-pulsed DCs. Representative bright field image of T-cell colonies (OT-1) on day 3 of DC/T-cell co-culture (left) and the T-cell proliferation capacity of WT and Egr2-KD DCs were assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and are represented as mean ± SD of six samples pooled from three independent experiments (right). *p < 0.05, **p < 0.01 compared with control DCs, Student’s t-test.
Molecular immunology

Mice, cell lines, and reagents

Six- to eight-week-old female BALB/c (H-2b) or C57BL/6(H-2d) mice and C57BL/6-background (H-2d) OT-1 (OVA257–264-specific CD8+ T-cell receptor transgenic) and OT-2 (OVA-specific CD4+ T-cell receptor transgenic) mice were used. OT-1/OT-2 mice used in the experiments were Rag-1/Rag2 normal. All mice were maintained in the animal care facility of Sungkyunkwan University according to the University Animal Care and Use guidelines. EL4 (C57BL/6 mouse derived thymoma) and E.G7 (OVA-expressing EL4) cells were obtained from the American Type Culture Collection (ATCC). CFSE (Molecular Probes) was used. Anti-SOCS1 antibody, anti-phospho-tyrosine-STAT5, and anti-pan-STAT5 antibodies were purchased from Cell Signaling Inc. Anti-β-actin (Sigma), HRP-conjugated anti-rabbit and anti-mouse IgGs (Sigma) were used in this study. Murine GM-CSF was obtained from Creagen Inc., Korea. Egr2 mAb was obtained from EPITOMICS. We used PE-conjugated mAb to CCR7 from BioLegend, and 24-well transwell chambers (8 μm pore size) from Corning Costar, Cambridge, MA, USA. Ovalbumin agonistic peptides (OVA257–264, SINFKEL and OVA323–339; ISQAVHAAHAEINEAGR) synthesized based on sequence information [28] were provided by Peptron (Dajeon, Korea). FITC- or PE-conjugated mAbs for DC-specific surface markers were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). FITC- or PE-conjugated CD4, CD8, Foxp3, and DEC-205 antibodies and Cytokine ELISA kits for murine IL-6, IL-10, IL-12p70, IL-17A, TNF-α, and IFN-γ were purchased from BioLegend. ELISA kit for IL-13 was obtained from Abcam. Dextran-FITC (40 000 Da) and LPS (from Escherichia coli O111:B4) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and mCCL19 was from Peprotech (Korea).

BMDCs (imDCs and mDCs) and splenic DCs

BMDCs were generated from BM progenitor cells, as described previously [2]. Briefly, BM cells were collected from the femurs of gating cells (left), and data are shown as mean ± SD of six samples pooled from three independent experiments performed. (B) The T-cell population in DC/T-cell co-cultures was assessed by intracellular Foxp3 staining from the co-cultures of OVA323–339 peptide-pulsed mDCs (C57BL/6) and OT-2 T cells at a 1:10 ratio for 5 days. For allo Treg-cell population in allogeneic reaction, BALB/c (H-2b) derived mDCs were co-cultured with OT-2 T (H-2b) cells for 5 days at a 1:10 ratio, and then CD25−Foxp3+ Treg cells were assessed by flow cytometry as reported previously [2, 35, 36]. As a control, T cells alone were treated with anti-CD3 mAb (0.5 μg/mL). The values on the dot blot indicate the percentage of gating cells (left), and data are shown as mean ± SD of three samples pooled from three independent experiments (right). (A–C) * p < 0.05, ** p < 0.01 compared with control DCs, Student’s t-test.

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Figure 5. An Egr2<sup>KD</sup> DC vaccine is more potent than a WT DC vaccine in DC-based tumor immunotherapy. (A) OT-1 mice were immunized twice at 1-week intervals with 1 × 10<sup>6</sup> OVA<sub>257-264</sub> pulsed Egr2<sup>KD</sup> (si-Egr2) or WT (si-con) mDCs of C57BL/6 mice. Cells in the spleen and DLN of vaccinated mice were obtained and cultured in the presence of the OVA<sub>257-264</sub> for 5 to 7 days. The levels of IFN-γ in culture supernatants were assessed in triplicates on day 2 of culture by ELISA and data are shown as mean ± SD of six samples from two independent experiments performed. (B) CTL activity in the spleens and DLNs of vaccinated OT-1 mice was assessed by flow cytometer using CSFE-labeled E.G7 or EL4 as target cells. Quantitative CTL activities are shown as mean ± SD (n = 3). DLN: draining lymph nodes. *p < 0.01, compared with the group of mice vaccinated with OVA<sub>257-264</sub>-pulsed control (si-con) DCs, Student’s t-test. (C, D) C57BL/6 mice were inoculated s.c. with E.G7 and EL4 tumor cells (5 × 10<sup>5</sup>) into the right flank area, respectively, and then immunized twice on days 3 and 10 with 1 × 10<sup>6</sup> cells of OVA peptide-pulsed Egr2<sup>KD</sup> (si-Egr2) or WT (si-con) mDCs (derived from C57BL/6 BM cells). The upper panel shows representative images of (C) E.G7 and (D) EL4 tumor-bearing mice, respectively, on day 20 after tumor inoculation. Tumor growth was monitored and represented as mean ± SD of n = 5 mice from one experiment and representative of two experiments performed (bottom). *p < 0.05, Student’s t-test.
Figure 6. The effects of Egr2 overexpression on DC differentiation and maturation of Egr2 expression plasmid (pCDNA3-flag-egr2) and control vector (pCDNA-flag) were transfected into DC precursor cells on day 4 and then harvested on day 6 as a source of imDCs. mDCs were prepared by stimulation of imDCs with LPS (200 ng/mL) for 24 h. (A) Egr2 expression in transformed mDCs was assessed by Western blot using anti-Flag and anti-Egr2 Abs. (B) The surface phenotype of transformed mDCs was assessed by flow cytometer. (C) The MFI of selective DC surface markers from more than three individual experiments is shown as mean ± SD of nine samples. *p < 0.05, **p < 0.01 compared with control vector transfected DCs, Student’s t-test. (D) IL-12 level was assessed by ELISA from the culture supernatant and shown as mean ± SD of nine samples pooled from three independent experiments. *p < 0.05, Student’s t-test.

Figure 7. DC vaccines for tumor immunotherapy impaired by Egr2 overexpression C57BL/6 mice were inoculated s.c. with E.G7 tumor cells (5 × 10⁵) into the right flank area and then immunized twice on days 3 and 10 with 1 × 10⁶ cells of OVA peptide-pulsed pCDNA3-egr2-transfected mDCs (OVA-mDC-Egr2) or control pCDNA3 vector transfected mDCs (OVA-mDC-con) generated from C57BL/6 BM cells. (A) Representative images of E.G7 tumor-bearing mice after DC vaccination were taken on day 20 after tumor injection. (B) Tumor growth was monitored and represented as mean ± SD of n = 3 mice from one experiment representative of two experiments performed. **p < 0.01, Student’s t-test.
Transfection of DCs with small interfering RNA (si-RNA)

si-RNAs targeting murine Egr2 were designed by BLOCK-IT RNAi Designer (Invitrogen) and Dharmacon RNAi Technologies (Thermo Scientific) to achieve full specificity of si-RNA without any “off-target-effects” such as the induction of type 1 interferon. Two Egr2-specific si-RNAs (5′-gctgtagaggaatc-3′ (si-Egr2-1), 5′-cggagagatgtagatca-3′ (si-Egr2-2)) and one control si-RNA (5′-ccctcgccagatagaccttctt-3′) were used. DC precursor cells on day 4 or 5 from BM stem cells were transfected with Egr2 si-RNA (si-Egr2) or control si-RNA (si-con) using lipofectamine RNAiMAX/Gene porter transfection kit (Life Technologies) as described previously [2]. Briefly, 100 μL of serum-free RPMI 1640 medium containing 5 μL of 20 μM annealed si-RNA were mixed with the same volume of serum-free RPMI 1640 medium containing 5 μL of Lipofectamine RNAiMAX (Invitrogen). After incubation for 20 min at room temperature, 200 μL of the mixture were added to 2 mL of DC culture. After 4 h of incubation, an equal volume of RPMI 1640 supplemented with 10% FCS was added to the culture. Around 24–48 h later, cells were washed and used as a source of Egr2KD immature DCs (Egr2KD imDCs) for subsequent experiments.

Flow cytometry analysis

For phenotypic analysis, direct immunofluorescence staining was performed as described previously [29]. Cells were stained in FACS buffer for 20 min at 4 °C with the appropriate antibodies: FITC-labeled rat anti-mouse CD14 (rmC5–3), anti-mouse CD86 (GL1), anti-mouse I-A/1-E(2G9), anti-mouse MHC I (H-2K), PE-labeled hamster anti-mouse CD11c (HL3), anti-mouse CD80 (16–10A1), rat anti-mouse-CD40 (3/23) (BD Pharmingen), and anti-mouse-CCR7 together with PE- or FITC-labeled isotype control antibodies. After washing, cells were then analyzed by FACS Calibur (BD) using CellQuest or FlowJo software. For intracellular Egr2 staining, cells pre-stained with PE-labeled hamster anti-mouse CD11c antibody were fixed and permeabilized by using the BD Cytofix/Cytoperm™ kit (BD Bioscience Pharmingen). Cells were then stained with rabbit anti-mouse Egr2 antibody (Abcam) or rabbit isotype control antibody in BD Perm/wash buffer for 1 h, followed by staining with second FITC-labeled goat anti-rabbit IgG antibody. After washing with BD Perm/wash buffer, cells were analyzed by flow cytometer.

T-cell proliferation assay

Egr2-normal and Egr2KD DCs were first prepared by transfection of DC precursor cells on day 4 or 5 with control si-RNA or Egr2-si-RNAs, and then cultured 2 more days before harvesting as Egr2KD imDCs. These imDCs were matured by culturing with LPS (200 ng/mL) for 24 h (for mDCs). The Egr2KD or WT mDCs were then pulsed with 1 μg/mL OVA peptide (OVA257–264 or OVA323–339) for 1 h. OVA peptide-pulsed DCs were washed three times with cold phosphate-buffered saline (PBS) to remove remnant-free peptides, and then used for T-cell proliferation assays. T cells were isolated from the spleen or DLN of OT-1 and OT-2 mice as described [30]. Briefly, mouse spleens or DLNs were homogenized in RPMI medium, passed through a 70 μM nylon cell strainer (BD Falcon), and treated with ACK lysing buffer (Lonza). T cells were purified from the spleen or DLN using a mouse CD4+ and CD8a+ T-cell Isolation Kit II (Miltenyi Biotech), and then labeled with CFSE (1 μM). CFSE-labeled T cells were co-cultured with OVA peptide-pulsed DCs at different ratios for 3–4 days. Cells were harvested and analyzed by flow cytometer.

Antigen-uptake assay

BMDCs were generated from BM of C57BL/6 mice. On day 4, Egr2 and control si-RNAs were transfected into DC precursor cells. After 48 h, cells were stimulated by LPS (200 ng/mL) for 24 h. Cells were harvested, and 2 × 105 cells were equilibrated at 37 or 4 °C, respectively, for 45 min in FACS tubes, pulsed with 1 mg/mL FITC-conjugated dextran for 1 h, and then stopped with cold staining buffer. Washed cells were stained with PE-conjugated anti-CD11c and analyzed using a FACS Calibur flow cytometer.

In vitro chemotaxis assay in transwell system

Chemotaxis of BMDCs was measured by DC migration through a polycarbonate filter of 8 μm pore size in 24-well transwell chambers (SPL, Korea and Corning Costar, Cambridge, MA, USA). BMDCs were generated from the BM of C57BL/6 mice. On day 4 of culture, cells were transfected with Egr2 and control si-RNA. After 48 h, cells were stimulated with LPS (200 ng/mL) for 24 h, followed by PBS washing. The lower chambers contained CCL19 (300 ng/mL) diluted in 0.6 mL serum-free RPMI 1640, and DCs (1 × 105 cells in 0.1 mL) resuspended in serum-free RPMI 1640 medium were placed in the upper chambers. Plates were incubated at 37 °C CO2 incubator for 3 h to allow cell migration. Migrated DCs were harvested from the lower chambers, and assessed by FACS Calibur flow cytometer.

Quantitative RT-PCR

Total RNAs were purified from cells using TRIzol (Invitrogen). cDNA was synthesized using RevertAid™ H Minus Reverse transcriptase and Oligo dT primers (Fermentas). Quantitative PCR was performed using the SYBR green PCR Master mix (Qiagen), and RT-PCR was performed using the Maxime RT-PCR kit (iNtRON Biotechnology, Inc.). The following primers were used: Egr2 (S) 5′-cactgtaagcgcaacctt-3′, (AS) 5′-aactgtgcttctgtt-3′; GAPDH (S) 5′-aactgtgcttctgtt-3′, (AS) 5′-tccacccagctttgta-3′; β-actin (S) 5′-gtatgcctcgggtgta-3′, (AS) 5′-tcctgccctgctattc-3′.
Western blot analysis

Western blot analysis was performed as described previously [31]. In brief, cells were washed in cold PBS, and lysed with a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM DTT, 30 mM NaF, 10 mM Na3VO4, 0.5% NP40, and a protease inhibitor cocktail (Pierce). Whole cell lysates were normalized with Bradford reagent (Bio-Rad), and 40–120 μg of the lysates were subjected to 8–12% SDS-PAGE and transferred to a PVDF membrane (Millopire). For immunoblottinf, membranes were blocked for 1 h at room temperature with 5% nonfat dry milk in 0.5% TBST. Membranes were washed four times in TBST and incubated overnight at 4°C with optimal concentrations of primary antibodies diluted in 4% nonfat dry milk. Following four additional washes in TBST, the membranes were further incubated for 45 min with HRP-conjugated secondary antibodies. Bound antibodies were detected using chemiluminescent HRP substrate (Millipore, USA) and autoradiographic film (Agfa Healthcare NV, Belgium).

CTL assay and ELISA for IFN-γ

OT-1 mice were injected twice at a 1-week interval with OVA257–264 peptide-pulsed control (si-con) or Egr2KO (si-Egr2) DCs. Spleen and DLNs were harvested from injected mice on day 14, and splenocytes and DLN cells were cultured for 7 days in the presence of 10 μg/mL OVA257–264 in 6-well plates (2 × 10⁶ per well), and then co-cultured with target cells (EL4 and E.G7) labeled with 1 μM CFSE at different ratios for 4 h. After PI staining, CTL activity was assessed by flow cytometer as described [32,33]. The level of IFN-γ in the second culture supernatant was assessed by ELISA.

DC-based tumor immunotherapy

C57BL/6 mice were injected with EL4 or E.G7 cells (5 × 10⁵ cells per mouse) subcutaneously (s.c.) into the right flank region [27]. Tumor-bearing mice were vaccinated twice on days 3 and 10 after s.c. tumor inoculation with WT, Egr2KO, or Egr2-overexpressing DCs (1 × 10⁶ per mouse) pulsed with OVA peptide (10 μg/mL). Tumor growth was monitored every 2–3 days starting from day 7 using a caliper. Tumor mass was calculated as reported previously [34]: \( V = (A^2 \times B)/2 \), where A is the length of the short axis (width) and B is the length of the long axis.

Statistical analysis

All experiments were repeated at least three times with consistent results. Statistical data are presented as means ± SD. Comparisons of group means were assessed with Student’s t-test. A p-value of less than 0.05 (p < 0.05) was considered statistically significant.

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