Targeting dendritic cell signaling to regulate the response to immunization

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Dendritic cells (DCs) are key regulators of the immune system; they capture antigens and then can either stimulate an immune response or induce tolerance. Our aim was to activate individual DC signaling pathways to regulate the immune response. We therefore expressed constitutive activators of mitogen-activated protein kinase (MAPK) pathways or the interferon pathway, together with tumor antigens, using lentivectors. Triggering of p38 activated DCs substantially enhanced the antitumor immune response and prolonged survival of tumor-bearing mice. Activation of extracellular signal–regulated kinase (ERK) increased TGF-β expression while expression of a constitutively activated interferon regulatory factor-3 (IRF3) stimulated IL-10 secretion by DCs. ERK and IRF3 suppressed the immune response and stimulated expansion of regulatory T cells. These results provide a toolkit to regulate immune responses to viral vector or DC immunization; vaccine responses to foreign or tumor antigens can be enhanced and harmful responses to self-antigens or introduced transgenes can be reduced. (Blood. 2008;111:3050-3061)

Introduction

A major challenge in immunization for the treatment of cancer or persistent infectious disease is to overcome an immune system that has been down-regulated after prolonged antigen exposure. This will require the development of immunization reagents that are more potent immune stimulators. On the other hand, in autoimmune or allergic disease, or in the delivery of potentially immunogenic transgenes for gene therapy, it will be helpful to have a mechanism to down-regulate an antigen-specific immune response. Much research has targeted dendritic cells (DCs) for the induction of specific immunity or antigen-specific tolerance, because DCs regulate innate and adaptive immune responses.1 DCs are widely distributed in peripheral tissues as immature DCs, exhibiting high phagocytic capacity but poor antigen presentation capacity. Antigen presentation by immature DCs results in T-cell anergy or tolerance.2,5 After encountering pathogens, DCs undergo a maturation program resulting in up-regulation of major histocompatibility complex (MHC) molecules and costimulatory molecules such as cluster of differentiation (CD) 80, CD86, CD40 and intercellular adhesion molecule I (ICAM-1). Activated DCs also secrete cytokines such as interleukin-12 (IL-12),6 critical for generation of a Th1 response, or IL-10,7 critical for a Th2 response, and migrate to secondary lymphoid organs where they present antigens to T cells.

DCs express a variety of pathogen pattern recognition receptors including the prototypical family of toll-like receptors (TLRs). A complex network of intracellular signaling molecules are recruited on TLR binding to pathogen structures through 2 adaptor proteins, MYD88 and TRIF, leading to the activation of several signaling pathways.8 Combined evidence from use of inhibitors or knockout mice has implicated the p38 mitogen-activated protein kinase (MAPK) and the NF-κB pathways in DC activation and IL-12 secretion.9-12 the extracellular signal–regulated kinase (ERK) pathway in IL-10 secretion,12,13 and IRF-3 in IFN-β induction, contributing to DC maturation.14,15 This suggests that stimulation of individual signaling pathways could generate either immunostimulatory or immunosuppressive DCs. In fact, it was recently shown that enhanced immune responses were achieved by over-expressing NF-kB–inducing kinase (NIK) in DCs using an adenovirus vector. NF-kB activation in the absence of other signaling pathways led to DC maturation and IL-12 secretion in DC cultures, and an increased immune response to the green fluorescent protein (GFP) transgene in the adenoviral vector was measured after immunization of mice.16

We therefore examined the effect of the activation of individual signaling pathways on dendritic cell function. For this purpose, we used HIV-1 based lentivectors, because they do not encode viral proteins that might affect DC function, they transduce DCs in vitro, and they target skin-derived DCs after subcutaneous injection.17 Lentivectors encoding an antigen transgene stimulate CD8 and CD4 T-cell responses to the antigen.18-20 We selected constitutively active components of the 3 main MAPK pathways (p38, JNK1, and ERK) and IFN induction pathway, a MAPK kinase 6 (MKK6EE) mutant that constitutively phosphorylates p38,21 a MAPK kinase 1 (MEK1) mutant (MEK1 NES ED) that constitutively phosphor-


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immune stimulation leads to tumor regression and increase in survival in an in vivo lymphoma model. In contrast, ERK or IRF3 activation in DCs inhibits immunization and induces expansion of CD4+ CD25+ FoxP3+ T regulatory (Treg) cells.

Methods

Approval was obtained from the University College London Animal Ethics committee.

Cells and mice

293T cells were grown in Dulbecco modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). EL4 thymoma cells stably expressing ovalbumin (OVA; EG7 cells) were grown in DMEM/10% FCS with G418 0.4 mg/mL.20 Mouse bone marrow–derived DCs were prepared from C57BL/6 mice, C57BL/6 TRIF-deficient, C57BL/6 MYD88-ESO, or MELAN-A were titrated by quantitative PCR.20 tyrosine 185 were replaced by alanines (MKK7-JNK1 AA).28 To inactivate the ubiquitin promoter with GFP or FLAG-specific M2 mAb was purchased from Sigma-Aldrich (Gillingham, United Kingdom). 20 Four days after isolation, DCs were plated at a density of 3 × 10^5 cells per well in 1 mL medium containing cytokines, and transduced with lentivectors as described in “Lentivector production, titration, and cell transduction.” Human peripheral blood monocyte (PBM)–derived HLA-A2+ DCs (huDCs) were cultured as described.19 For vaccination experiments with New York esophageal squamous cell carcinoma (NY-ESO) antigen H-2 class I knockout, HLA-A2.1 transgenic (HHD) mice were used.19,26

Plasmids

To coexpress constitutive activators with GFP, IiOVA, or NY-ESO, lentivectors containing 2 promoters were constructed (dual lentivectors). IiOVA was described previously.20,27 NY-ESO encodes an Asp-Tyr-Lys-Asp-Asp-Asp-Lys peptide (FLAG)–tagged to human antigen broadly expressed in tumors.19 To introduce a second promoter encoding GFP or IiOVA, the GFP and IiOVA genes were amplified by PCR flanked by KpnI and XhoI restriction enzymes. Polymerase chain reaction (PCR) fragments were restricted with KpnI and XhoI and cloned into the plasmid pUB6V5-HisA (Invitrogen, Carlsbad, CA). The ubiquitin promoter with GFP or IiOVA genes was excised from this plasmid by digesting with SacII and XhoI, and cloned into the vector pHSV-SIN-CSGW.27 Dual lentivectors encoding inactive MAPK activators were also constructed. To inactivate MKK6/E, a point mutation replacing lysine 82 by alanine (MKK6 K82A) was introduced.25 To inactivate MKK7-JNK1, JNK1 threonine 183 and tyrosine 185 were replaced by alanines (MKK7-JNK1 AA).26 To inactivate MEK1 ΔNES ED, the ED mutations were replaced by alanines.25 To inactivate IRF3 2D, the coding region for the carboxy-terminal transactivating domain was removed. Dual lentivectors encoding activators and HA-tagged human MELAN-A30 under the control of the cytomegalovirus (CMV) promoter were generated.

Lentivector production, titration, and cell transduction

VSV-G-pseudotyped lentivectors were produced as described.20,27 Lentivectors were concentrated 100-fold by ultracentrifugation through a 20% sucrose cushion in phosphate-buffered saline (PBS) and resuspended in PBS containing 10% glycerol. Lentivector stocks for vaccination were diluted in PBS and subjected to a second ultracentrifugation step. Dual lentivectors coexpressing activators and GFP were titrated as previously described.20 Dual lentivectors coexpressing activators with IiOVA, NY-ESO, or MELAN-A were titrated by quantitative PCR.20

Antibodies

FLAG-specific M2 mAb was purchased from Sigma-Aldrich (Gillingham, United Kingdom), rabbit polyclonal antibodies specific for total and phospho-p38, total JNK and phospho-c-Jun, total and phospho-Erk and murine mAb for phospho-JNK from Cell Signaling (Danvers, MA). OVA-specific rabbit antiserum was obtained as previously described.20 For surface staining of mouse and human DCs, mouse CD11c and human/ mouse CD86, CD80, CD40, ICAM-1, MHC-I and MHC-II–specific biotinylated mAbs were purchased from e-Bioscience (San Diego, CA). Biotinylated Syrian Hamster IgG was purchased from e-Bioscience as a control. FITC mouse IgG1, kappa isotype standard, and PE-hamster IgG isotype standard were purchased from BD Pharmingen (San Jose, CA).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

Transduced cells (2 × 10^6) were lysed with 150 μL lysis buffer containing 1% NP-40 and protease inhibitors (Complete Protease Inhibitor Cocktail tablets; Boehringer Mannheim, Mannheim, Germany) in PBS. In some cases cells were previously subjected to UV irradiation (40 J UV-C per m^2) and incubated for 30 minutes at 37°C. As positive controls for detection of phosphorylated MAPKs in DCs, DC cultures were incubated with lipopolysaccharide (LPS, 10 μg/mL) and incubated for 30 minutes at 37°C. Forty micrograms of protein per sample was resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Cell staining and flow cytometric analysis

After staining with the appropriate biotinylated antibodies, DCs were incubated with streptavidin-conjugated allophtocyanin (APC). Viable stained DCs were gated in a forward/side-scan dot-plot, and APC–fluorescent intensity was plotted for GFP-positive–gated DCs. Voltage conditions were set up independently for each marker, and stained cells were analyzed in a BD LSR flow cytometer (Becton Dickinson, San Jose, CA) using appropriate channels for GFP and APC detection. As a positive control for surface staining, LPS was added to DC cultures at a final concentration of 10 μg/mL 24 hours before analyses. To stain Treg cells, the Foxp3 mouse regulatory T-cell staining kit from eBiosciences was used. Intracellular staining for the detection of TGF-β was performed using the Cytofix/Cytoperm intracellular staining kit following the manufacturer’s instructions (BD Pharmingen). Biotinylated mouse TGF-β–specific rat anti-bodies were used in the presence of Fc-receptor-specific antibodies (Becton Dickinson).

Enzyme-linked immunosorbent assay

Transduced DCs were grown to 2 × 10^6 cells/mL. Levels of cytokines IL-12, IL-10, IL-1β, IL-23, and TNF-α were quantified from DC culture supernatants by enzyme-linked immunosassay (ELISA) kits following the manufacturer’s recommendations (eBiosciences). IFN-β secretion was detected by ELISA following the manufacturer’s recommendations (PBL Biomedical Laboratories, New Brunswick, NJ). Six independent experiments were performed.

Vaccination and enzyme-linked immunosorbent spot analyses

C57BL/6 or HHD mice were subcutaneously vaccinated with lentivector preparations diluted in PBS. Spleens were collected at days 10 or 12 postvaccination and enzyme-linked immunosorbent spots (ELISpots) from splenocytes stimulated in vitro with MHC class I and class II OVA peptides (257-264 and 323-339 purchased from Proimmune, Oxford, United Kingdom), or with MHC class I NY-ESO peptide (157-165 when indicated) were performed as previously described.30 For boosting experiments, mice vaccinated with the appropriate lentivectors were subcutaneously injected with 5 μg OVA (Sigma-Aldrich) in Freund incomplete adjuvant 1 month after priming, followed by ELISPOT 10 days after boosting.

Tumor experiments

Groups of 5 mice were subcutaneously injected with 2 × 10^6 EG7 cells. When tumor growth was apparent, mice were subsequently vaccinated with 10^6 DCs transduced with the indicated lentivectors. A second vaccination was carried out 1 week later and tumor growth was monitored. Tumor scores were calculated by multiplying tumor length by width. Mice were killed when tumor scores higher than 150 to 160 mm^2 were reached. Tumors were excised postmortem and processed for immunoblot analyses.

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In vitro T-cell expansion by human DCs

PBM-derived HLA-A2+ huDCs were transduced with lentivectors. The nonadherent fraction of peripheral blood was depleted of CD4 T cells using CD4-specific paramagnetic Dynabeads CD4 (T helper/inducer) as recommended by the manufacturer (Invitrogen). CD4 T cells were kept to supplement CD8 T-cell cultures with huDCs. When indicated, huDCs were matured using IFN-γ (150 U/mL) and IL-1α (50 U/mL), and loaded with HLA-A2 MELAN-A peptide (27-35 aa; 50 μg/mL) in the presence of β-2 microglobulin (3 μg/mL). Then, huDCs were washed twice with medium and plated in M24 well plates at a density of 2 to 3 × 10⁵ cells per well in medium containing 3% AB human serum (HS). Responder cells (CD8 T cells supplemented with 10% CD4 T cells) were added to DC cultures at a DC:T cell ratio of 1:10. DC-T cell cultures were kept in medium containing 3% HS supplemented with IL-7 (20 ng/mL) and IL-12 (100 pg/mL) for 7 days. After that, the medium was replaced with medium containing 3% HS supplemented with IL-7 (10 ng/mL) and IL-15 (2 ng/mL). Three days later, lymphocyte proliferation was assessed by counting viable T cells in each culture. Total CD8 T cells and MELAN-A–specific CD8 T cells were quantified by APC-conjugated pentamer staining (Pro5 recombinant human MHC pentamer A*0201) together with FITC-conjugated human CD8-specific antibody, as recommended by the manufacturers (Proimmune). Viable CD8 T lymphocytes were gated on a forward/ side-scatter dot-plot, and APC-fluorescent intensity was plotted as a function of FITC intensity. Isotype control antibodies were used to set up gates in dot-plots. The percentage of CD8 T cells specific for MELAN-A (27-35) were quantified from 3 independent T-cell cultures.

Statistical analyses

Normality of MFIs was confirmed using the χ² test. Multiple comparisons between samples were performed by one-way analysis of variance (ANOVA), and pair-wise comparisons were performed using the Tukey test when appropriate. In some cases, due to the inherent variability between experiments, samples were compared by a 2-way ANOVA using experiments as a random criterion. Cytokine levels estimated by ELISA from DC culture supernatants and Treg expansion were also statistically compared by these tests. For survival in tumor experiments, the log-rank test from Kaplan-Meier plots was performed. Mean survival times were
also normally distributed and compared between groups by one-way ANOVA and pair-wise comparisons.

Results

Expression of MAPK constitutive activators by lentivectors specifically activates MAPKs

To characterize the role of MAPKs and IRF3 in immune regulation, constitutive activators were expressed in DCs using lentivectors (Figure 1A). To determine that GFP was a valid marker for activator expression, we first generated a control dual lentivector encoding GFP under the control of the SFFV promoter and cherry FP under the control of the ubiquitin promoter. Lentivectors were then used to transduce mouse dendritic cells and coincident expression of GFP and cherry FP was detected (data not shown). In the final constructs a GFP transgene was expressed from the human ubiquitin promoter and the activator was expressed from the SFFV promoter (dual lentivectors, Figure 1A). A lentivector encoding 2 GFP genes was used as a control.

To confirm that each activator could phosphorylate endogenously expressed MAPKs, human 293T were transduced with the dual lentivectors. The expression of activators was evaluated by immunoblot using a FLAG-specific antibody (Figure 1B) and levels of phosphorylated p38, JNK1, and ERK were assessed by immunoblot using specific antibodies (Figure 1C). As positive controls for p38 and JNK1 phosphorylation, cells were subjected to UV irradiation. Specific p38 phosphorylation was observed in UV-irradiated cells and in MKK6EE-expressing cells (Figure 1C). Likewise, phosphorylated MKK7-JNK1 and phosphorylated cJun were detected in UV-irradiated cells and in cells expressing MKK7-JNK1 (Figure 1C). Phosphorylated ERK was detected after

Figure 2. Expression of MAPK constitutive activators differentially regulates DC maturation phenotype. BM-derived DCs were transduced with dual lentivectors and grown for 6 days at 37°C. Surface staining was performed for each DC marker. (A) Dot plots from flow cytometric analyses for CD40 in DCs transduced with the indicated lentivectors (top of each panel). CD40 APC fluorescence levels (y-axis) were represented as a function of GFP fluorescence (x-axis). Quadrants were established based on fluorescence of the isotype control in untransduced cells. Percentage of cells and mean fluorescence intensities are indicated in each quadrant. LPS represents DCs treated with LPS 24 hours before staining. (B) Dot plots from flow cytometric analyses for CD40 as shown in panel A. The 2 top panels represent DCs transduced with lentivectors encoding only GFP (left) or lentivectors encoding MEK1 /H9004 NES ED using the same settings as in panel A. The 2 bottom panels represent DCs transduced with the lentivectors shown in the top, but measured on a more sensitive flow cytometry (FACS) setting to visualize CD40 down-regulation on both transduced and nontransduced cells. (C) Histograms from dot plots as shown in panel A gated on GFP-positive transduced DCs. The number of events is represented as a function of log fluorescence intensity for the DC markers shown. I indicates the distribution from the isotype control. The horizontal line indicates the cells considered to be positive, which is set to exclude 95% of cells stained with the isotype control. Percentage of positive DCs and mean fluorescent intensities are indicated within each histogram, for DCs transduced with lentivectors encoding the indicated activators. The first row of histograms show untransduced DCs (UT, filled histogram), DCs transduced with a control lentivector only expressing GFP (GFP, thin line), and DCs treated with LPS (LPS, dotted line). The second row shows histograms from DCs transduced with lentivectors encoding only GFP (GFP, filled histogram), expressing MKK6EE (MKK6, thin line), or treated with LPS (dotted line). The third row shows histograms from DCs transduced with a control lentivector expressing GFP (GFP, filled histogram), expressing MKK7-JNK1 (JNK, thin line), or treated with LPS (dotted line). The fourth row shows histograms from DCs transduced with a control lentivector expressing GFP (GFP, filled histogram), expressing MEK1 /H9004 NES ED (MEK, thin line), or treated with LPS (dotted line).
the cells reached confluence only when MEK1 ΔNES ED was expressed (Figure 1C).

Bone marrow (BM)–derived mouse DCs were then transduced with dual lentivectors and the effects on endogenous MAPKs assessed. As a positive control for p38 and JNK1 activation, DCs were treated with LPS overnight. All activators were expressed in DCs as evaluated by immunoblot (Figure 1D). MKK6EE and MKK7-JNK1 specificities were confirmed by immunoblot using specific antibodies for phosphorylated p38 and JNK1 (Figure 1E). High levels of phosphorylated ERK were detected in all cells. However, an increase in phosphorylated ERK was observed in MEK1 ΔNES ED-expressing DCs (Figure 1E). IRF3 2D expression was detected in DCs (Figure 1D) and this did not result in MAPK phosphorylation (Figure 1E).

Expression of MAPK constitutive activators differentially regulates DC maturation phenotype

To characterize the effects of MAPK activators and IRF3 2D on DC maturation, transduced DCs were labeled with antibodies specific for the classical maturation/activation markers CD86, CD80, CD40, ICAM-I, MHC I, and MHC II, and analyzed by flow cytometry. To quantify and compare the effects of each activator on DC phenotype, GFP-positive transduced DCs were gated for analysis (Figure 2). As expected, 80% to 100% of DCs expressed CD80, ICAM-I, MHC I (not shown), and MHC II (not shown), while CD40 was expressed at low levels (Figure 2B,C). DCs expressing only GFP were phenotypically comparable to control untransduced cells (Figures 2A,C, 3), suggesting that DC transduction with lentivectors did not induce maturation in vitro, as already reported.32,33 CD86, MHC I, and MHC II levels were not significantly changed after transduction with any of the lentivectors (Figure 3). MKK6EE expression significantly up-regulated CD80 (P = .02), CD40 (P = .02), and ICAM-I (P = .04; Figures 2A,C, 3). Expression of MKK7-JNK1 also resulted in CD80 and ICAM-I up-regulation (P = .04 and P = .02, respectively; Figures 2, 3). MEK1 ΔNES ED expression markedly down-regulated CD40 (P = .01; Figures 2B,C, 3); DCs expressing IRF3 2D were phenotypically comparable to GFP-expressing DCs (Figures 2A,3 and results not shown). Thus, constitutive activation of MAPKs resulted in differential surface expression of DC maturation markers, with p38 and JNK1 leading to a more activated phenotype and ERK down-regulating CD40.

Cytokine production by DCs expressing MAPK and IRF3 constitutive activators

DC maturation triggered by TLR ligands results in secretion of proinflammatory and regulatory cytokines, through the activity of NF-κB, MAPKs, and IRFs. To test whether constitutive activation of MAPKs and IRF3 resulted in secretion of specific cytokine profiles, cytokine levels were quantified in supernatants from transduced DC cultures by ELISA. Levels of IL-12, TNF-α and IL1-β were measured because these cytokines are strong inducers of Th1 responses. IL-10 levels were quantified as this is a potent immunomodulatory cytokine leading to either Th2 responses or tolerance. IL-23 levels were also measured, as this is a potent immunomodulatory cytokine through the activation of a novel subset of IL-17–secreting T helper cells (Th17 cells).34 No significant secretion of IL-12 or TNF-α was observed by expression of MAPK and IRF3 constitutive activators (P = .12, P = .32; Figure 4A). These results indicated that single activation of MAPKs is not sufficient for IL-12 and TNF-α secretion, in contrast to selective NF-κB activation where IL-12 and TNF-α secretion was induced.16 Similarly, no significant secretion of IL1-β and IL-23 was observed (results not shown). As expected, IRF3 2D expression induced significant secretion of IFN-β compared with background levels (P = .02; Figure 4A). IRF3 2D expression also induced IL-10 secretion from transduced DCs (P = .009) to levels statistically comparable to LPS treatment (P = .72; Figure 4A).
IRF3 2D expression in DCs induces IL-10 secretion through a MYD88-dependent pathway, whereas ERK up-regulates TGF-β expression

Previous reports have demonstrated that IL-10 and IFN-β secretion are linked by a common pathway through MYD88 and TRIF, downstream of TRAF3.35,36 To find out whether IRF3 2D was directly inducing IL-10 secretion and to characterize the mechanism of induction, BM-derived DCs from wild-type, MYD88-deficient and TRIF-deficient mice were transduced with lentivectors expressing GFP, IRF3 2D, and an inactive IRF3 mutant with a deletion of the carboxy-terminal transactivation domain (IRF3ΔC, Figure 4B). As already reported,36 wild-type DCs secreted IL-10 after LPS treatment, while IL-10 secretion was severely impaired in MYD88-deficient DCs (Figure 4B). Interestingly, IRF3 2D expression resulted in IL-10 secretion in TRIF-deficient DCs but IRF3 2D expression did not lead to IL-10 secretion in MYD88-deficient DCs (Figure 4B). The role of IFN-β secretion in IL-10 induction was assessed using DCs from mice deficient for the β1 chain of the type I IFN receptor. IRF3 2D induced IL-10 in these cells, although IL-10 induction by both IRF3 2D and LPS was reduced compared with wild-type controls (Figure 4B), demonstrating that IFN feedback plays a role in IL-10 induction.

The level of TGF-β expression was also measured as this is a key inducer of Tregs,37 that can be produced by myeloid DCs.38 ERK activation in DCs by MEK1ΔNES ED expression selectively up-regulated the level of TGF-β expression (Figure 4C).

MAPK and IRF3 constitutive activators regulate in vivo immune responses

As MAPK and IRF3 constitutive activation resulted in DCs with differing phenotypic characteristics, we decided to test whether this translated to differences in DC function in vivo. Therefore, these activators were coexpressed with an antigen to examine whether they could enhance or suppress immune responses. We used the
Figure 5. Coexpression of MAPK and IRF3 constitutive activators with an antigen transgene modulates CD4 and CD8 immune responses to lentivector vaccination. (A) Schemes of the lentivector constructs and inactive MAPK activators are shown. Activators and their inactive mutants were cloned under the control of the SFFV promoter, while the IiOVA antigen was expressed from the ubiquitin (UBIQ) promoter. Inactivating mutations are indicated within each bar. Expression levels of each constitutive activator and inactive mutants were compared by FLAG immunoblot of 293T cells transduced with the lentivectors at a multiplicity of transduction of 2. Immunoblots were also probed with an OVA-specific antibody (α-OVA). (B) Groups of 6 mice were vaccinated with lentivectors encoding only IiOVA and dual lentivectors encoding IiOVA together with the MKK6EE (MKK6) and the inactive mutant MKK6 K82A (bottom of the graph), at the indicated doses. OVA-specific CD8 T cells in spleen were evaluated by IFN-γ ELISPOT and represented as column graphs. Each column shows the mean from 3 independent experiments together with error bars (SD). Relevant statistical comparisons are shown within each graph, between indicated samples and vaccination with lentivectors encoding only IiOVA. Significant (* P < .05) and highly significant (*** P < .001) differences are shown in the graphs. Ns, nonsignificant differences. The rest of the graphs show vaccination results performed with lentivectors encoding MKK7-JNK1 and MKK7-JNK1 AA, MEK1 NES ED and MEK1 NES AA, or IRF3 2D and IRF3 ΔC. (C) Groups of 9 mice were vaccinated with the indicated dual lentivectors encoding IiOVA together with each MAPK and IRF3 activator. OVA-specific MHC class I and class II primary responses in spleen were primed with CD8 and CD4 OVA epitope peptides and evaluated by IFN-γ ELISPOT and represented as column graphs. Each column represents the mean with error bars (SD). Relevant statistical comparisons are shown within each graph between indicated samples and unprimed mice vaccinated only with OVA protein. Ns indicates nonsignificant differences; ** very significant differences (P < .01); *** highly significant (P < .001) differences. Secondary immune responses were evaluated 1 month after vaccination (lentivector vaccines, bottom of the graphs) using 5 μg of OVA per mouse in Freund incomplete adjuvant (Boost, bottom of the graphs). – indicates absence of vaccination or boosting. (D) Groups of 3 mice were vaccinated with PBS or lentivector vaccines coexpressing IiOVA and the indicated activators on top of the panels. Dot plots of CD4+CD25+ T lymphocytes from spleen are shown, with Foxp3 fluorescence intensity represented as a function of CD4 fluorescence on a logarithmic scale. Quadrants were established from isotype controls. Percentages of CD4+CD25+Foxp3+ T lymphocytes relative to CD4+ primary CD25+ T lymphocytes are shown within the upper right quadrant. Below the percentages, MFIs from Foxp3+ cells are shown. (E) The mean percentage of Tregs from mice immunized with the lentivector vaccines (bottom of the graph) is represented as a column graph (left), together with error bars (SD). The mean of Foxp3 expression levels (MFIs) within CD4+CD25+Foxp3+ T cells from mice immunized with the lentivector vaccines is represented as a column graph (right), together with error bars (SD). Selected statistical comparisons between indicated samples and the group vaccinated with lentivectors encoding only IiOVA are shown, and were performed as described in “Statistical analysis.” *, significant differences (P < .05); **, very significant differences (P < .01).
model synthetic antigen containing OVA MHC class I and class II epitopes fused to a MHC class II invariant chain (IiOVA; Figure 5A). Each constitutive activator was paired with a corresponding inactive mutant as a control (Figure 5A). Lentivectors were administered to mice by subcutaneous vaccination because dermal DCs are targeted through this immunization route and we have now demonstrated superior immunization after subcutaneous injection, compared with the intravenous immunization we previously described. As previously described direct injection of a lentivector encoding IiOVA resulted in OVA-specific CD8 T-cell expansion (Figure 5B). Interestingly, coexpression of the p38 activator MKK6EE or MKK7-JNK1 with IiOVA led to a significant increase of OVA-specific CD8 T cells compared with the lentivector expressing only IiOVA. This enhancement was particularly evident when the lowest doses were administered (P < .001), and it was abrogated when the corresponding inactive mutants were used (P = .06, P = .1; Figure 5B). Furthermore, significant decreases of OVA-specific CD8 T cells were observed when MEK1 ΔNES ED or IRF3 2D were coexpressed with IiOVA; (P < .001; Figure 5B). In fact, an almost complete suppression was achieved at the lowest lentivector dose when MEK1 ΔNES ED and IiOVA were coexpressed (P < .001; Figure 5B). Thus, MKK6EE and MKK7-JNK1 expression resulted in a significant enhancement of MHC class I antigen presentation after lentivector vaccination, while MEK1 ΔNES ED and IRF3 2D strongly suppressed lentivector-induced immune responses.

Secondary CD8 and CD4 immune responses were then examined. Mice were initially immunized with lentivectors; MKK6EE also significantly enhanced the primary CD4 T cell response (data not shown) and MEK1 ΔNES ED and IRF3 2D expression strongly reduced the CD4 primary responses (data not shown). Vaccinated mice were then immunized one month later with OVA protein (5 μg per mouse) in Freund incomplete adjuvant. A very significant increase of CD8 T cells was observed when MKK6EE was coexpressed with IiOVA during priming (P = .006; Figure 5C). In contrast, coexpression of MEK1 ΔNES ED or IRF3 2D with IiOVA during priming led to a significant impairment of OVA-specific CD8 and CD4 T-cell expansion (Figure 5C; P < .001, P = .005). To examine the mechanism of this impairment, expansion of Tregs in splenocytes was assessed 10 days after subcutaneous vaccination. Tregs are characterized by expression of CD4, CD25, and the transcription factor forkhead box p3 (Foxp3). The number of Tregs was consistently increased when MEK1 ΔNES ED and IRF3 2D were expressed (P = .04; Figure 5D,E), and an increase in Foxp3 expression measured by MFI was also observed (P = .001; Figure 5D,E). In contrast, the number of Tregs and Foxp3 expression levels were comparable to controls vaccinated with PBS or expressing only IiOVA when inactivated mutants were used for vaccination (Figure 5D,E). These results suggested that expression of ERK and IRF3 constitutive activators in lentivector vaccines expanded CD4+ CD25+ Foxp3+ Tregs that might at least partly explain the suppression of lentivector-induced immune responses.

**MKK6EE expression in DCs enhances antitumor immune responses**

As MKK6EE was the best MAPK activator at inducing DC maturation in vitro and enhancing in vivo immune responses, we tested whether its expression in DCs would trigger enhanced antitumor immune responses. Mice were subcutaneously injected with EL4 cells expressing OVA that quickly develop into aggressive lymphomas. When tumors were detected, mice were vaccinated with DCs transduced with lentivectors encoding either MKK6EE or MKK6 K82A together with IiOVA. As controls, injections with immature DCs, DCs loaded with class I OVA peptide (peptide-loaded DCs), or LPS-matured peptide-loaded DCs were performed. The latter group represents the most common protocol that has been used in clinical trials of DC-based immunotherapy. Mice injected with EG7 cells and vaccinated with unloaded DCs or with peptide-loaded DCs showed quick tumor growth that led to their sacrifice within the first 2 weeks (Figure 6A,B). In the group vaccinated with LPS-treated peptide-loaded DCs, only one mouse showed complete tumor regression and prolonged survival (Figure 6A,B). In contrast, all mice vaccinated with DCs coexpressing MKK6EE and IiOVA showed efficient tumor regression (complete in 3 mice), and a significant increase in survival (P = .03; Figure 6A,B). Mice vaccinated with DCs expressing the inactive MKK6 K82A showed only a partial antitumor response, with a survival pattern not statistically different from the other controls (Figure 6A,B). Interestingly, when tumors reappeared after regression OVA expression was lost, most likely leading to immunologic escape (Figure 6C).

To further prove that this methodology can be extended to clinically relevant human tumor antigens, NY-ESO, expressed in melanoma and other tumors was coexpressed with MAPK and IRF3 2D activators in dual lentivectors (Figure 6D). NY-ESO-specific class I immune responses were then evaluated in a humanized HLA-A2.1 transgenic mouse model. Direct lentivector vaccination led to an efficient NY-ESO-specific CD8 T-cell expansion, which was significantly enhanced when MKK6EE was coexpressed (P = .03; Figure 6D). Coexpression of MEK1 ΔNES ED or IRF3 2D with NY-ESO led to a highly significant suppression of lentivector-mediated NY-ESO-specific CD8 T-cell expansion (P < .001; Figure 6D).

**Constitutive activation of MAPK p38 enhances antigen presentation by human DCs**

The effectiveness of MKK6EE in enhancing antigen presentation in human DCs was then evaluated. Firstly, the capacity of MKK6EE to induce huDC maturation was tested in peripheral blood monocyte–derived huDCs. The ubiquitin promoter was not active in huDCs, so this promoter was substituted by CMV promoter (Figure 7A). The maturation phenotype of transduced huDCs was characterized by surface staining of CD80, CD40, and ICAM-I, previously shown to be selectively up-regulated by MKK6EE in mouse DCs (Figures 2,3). MKK6EE expression significantly up-regulated the 3 maturation markers (P = .02, P = .01, P = .04, respectively; Figure 7A). Then, in vitro expansion of CD8+ T cells from HLA-A2+ human lymphocytes by MKK6EE-expressing huDCs was evaluated. For this purpose, the human MELAN-A melanoma antigen was coexpressed in dual-lentivectors together with MKK6EE or MKK6 K82A (Figure 7B). MELAN-A was chosen in this assay because MELAN-A–specific CD8 T cells are present at a detectable frequency in peripheral blood of naive HLA-A2+ donors. HLA-A2 huDCs were transduced with these lentivectors and their capacity to expand CD8 T cells was compared with that of cytokine-matured (IFN-γ and IL-1α) DCs loaded with class I MELAN-A peptide. As a negative control, peptide-loaded immature DCs were used. Expansion of CD8 T lymphocytes was evaluated, and MELAN-A–specific CD8 T cells were quantified by pentamer staining (Figure 7C,D). MKK6EE expression led to a highly significant increase in CD8 T-cell expansion (P < .001) and also in MELAN-A–specific CD8 T cells (P < .001; Figure 7D). Thus, MKK6EE expression in
Figure 6. Constitutive p38 activation in DCs enhances antitumor immune responses and increases survival. (A) Groups of 5 mice were injected with OVA-expressing EG7 lymphoma cells. After tumor growth, mice were injected with immature DCs (only DC), DCs loaded with class I OVA peptide (DC + OVA PEP), LPS-matured peptide-loaded (DC + OVA PEP + LPS), or with DCs transduced with lentivectors coexpressing the inactive MKK6 K82A mutant or MKK6EE with IiOVA. Tumor growth was quantified as tumor size with error bars (SD). GFP represents lentivectors expressing only GFP; K82A, MKK6, MEK, and IRF3 represent lentivectors coexpressing MKK6 K82A, MKK6EE, MEK1 and IRF3 2D with NY-ESO.

Discussion

By the use of constitutive activators, we have shown that MAPKs differentially regulate DC maturation in cell culture. MAPK p38 activation up-regulated CD80, CD40, and ICAM-I. Interestingly, ERK activation resulted in a marked CD40 down-regulation. The control of CD40 levels on the DC surface could be relevant in vivo, because CD40 strengthens the immunologic synapse between DCs and T cells during antigen presentation and induces IL-12 secretion from DCs on T cell contact.42 Thus, the balance of p38 and ERK activation can determine the extent of CD40 surface levels that will contribute to the potency of T-cell stimulation.

There is considerable evidence linking ERK activation to IL-10 induction by DCs.12,13,35,36,44 However, we found that ERK activation alone did not stimulate IL-10 secretion by DCs, suggesting that ERK activation is necessary but not sufficient for IL-10 production. However, ERK activation did up-regulate TGF-β expression in DCs. Interestingly, ERK activation has been linked to TGF-β up-regulation in macrophages.45,46 It will be of interest to determine whether TLR ligands that stimulate multiple signals including ERK also induce TGF-β up-regulation in DCs. Interestingly, IFR3 activation led to IFN-β secretion and also significant IL-10 secretion. A previous report showed that IL-10 and IFN-β secretion are coinduced by a pathway involving TRAF3, the TRIF adaptor.35 Our results show that IFR3 itself can induce IL-10 secretion by a pathway that is TRIF-independent. Furthermore, IL-10 induction by IFR3 is also independent of IFN-β secretion, but it does require MYD88. This suggests that a direct intracellular interaction between the IFR3 2D mutant and MYD88 might occur in myeloid DCs, as previously reported for IRF7.47

Coexpression of p38 or JNK1 activators with antigen transgenes, including clinically relevant human tumor antigens, significantly enhanced CD8- and CD4-mediated T-cell responses after lentivector vaccination. This is in agreement with our in vitro data showing up-regulation of CD80, CD40, and ICAM-I when p38 or JNK1 were activated in DCs, because these costimulatory molecules are engaged in the immunologic synapse between DCs and T cells. Significant tumor regression and increase in survival was achieved in an in vivo lymphoma model by cellular vaccination with MKK6EE-expressing DCs. In fact, although tumors reappeared after regression, these tumors had lost OVA expression providing evidence for strong immunoselection of tumor cells lacking OVA expression. In addition, MKK6EE coexpression with human NY-ESO resulted in a significant enhancement of NY-ESO–specific class I immune responses in a humanized HLA-A2.1 transgenic mouse model, frequently used for preclinical evaluation of antitumor therapies. Finally, MKK6EE also induced huDC maturation, and its coexpression with human MELAN-A significantly enhanced MELAN-A–specific CD8 T-cell expansion in vitro. These combined results from mouse models and huDC data show that this strategy could be applied to clinical cancer immunotherapy.
MKK6 K82A with MELAN-A; MKK6 represents huDCs coexpressing MKK6 EE with MELAN-A.

Immature huDCs loaded with class I MELAN-A peptide; mDCp indicates cytokine-matured huDCs loaded with class I MELAN-A peptide; K82A represents huDCs coexpressing activation causes TGF-

In agreement with this, we have shown that 2 distinct signaling pathways in DCs lead to antigen-specific suppression; ERK

Figure 7. MKK6EE expression enhances human (hu)DC maturation and expands MELAN-A–specific CD8 T cells in vitro. (A). The structure of the lentivector constructs used for transduction of huDCs is shown on top. The ubiquitin promoter and IiOVA gene were replaced with the CMV promoter and GFP gene. Human DCs were transduced with dual lentivectors coexpressing MKK6EE with GFP and grown for 6 days at 37°C. Surface staining was performed for the indicated DC marker. Histogram plots gated on GFP-positive transduced DCs are shown. The number of events is represented as a function of log fluorescence intensity for the DC markers shown. LPS indicates the distribution from the isotype control. The horizontal line indicates the cells considered to be positive, which is set to exclude 95% of cells stained with the isotype control. Percentage of positive DCs and mean fluorescent intensities are indicated within each histogram. LPS indicates immature huDCs treated with LPS overnight previous to surface staining. GFP indicates huDCs transduced with lentivectors encoding only GFP. Levels of DC maturation markers were estimated from 3 independent experiments by surface staining and flow cytometry, as shown in the histograms above. Mean fluorescent intensities for each marker from GFP-expressing transduced huDCs were represented as percentages compared with levels after LPS treatment. Means are shown as column graphs with error bars (SD), for the indicated markers within the graph. Lentivectors encoding the indicated activators used for transductions are shown on the bottom of the graphs. Selected statistical comparisons between indicated samples and GFP-expressing huDCs are shown. *, significant differences (P < .05). (B) The structure of the lentivector constructs used for transduction of huDCs is shown on top. The ubiquitin promoter and IiOVA gene were replaced with the CMV promoter and MELAN-A gene. Expression of the indicated activators and MELAN-A was confirmed by immunoblot using FLAG-specific or HA-specific antibodies, as indicated on the left. kDa, kilodaltons. (C) Dot-plots from CD8 T cell cultures representing fluorescence intensity from APC-conjugated MELAN-A–specific MHC I pentamer as a function of CD8 fluorescence intensity in logarithmic scale. These T cell cultures were stimulated with immature huDCs loaded with MELAN-A peptide, cytokine-matured peptide-loaded huDCs and huDCs transduced with lentivectors coexpressing MKK6 K82A or MKK6EE with MELAN-A, as shown within each graph. The percentage of events within each quadrant and the absolute number of CD8 T cells are shown. (D) The graph on the left shows CD8 T cell numbers in cultures with the indicated huDC stimulator cells. Each column represents the mean from 3 independent experiments with error bars (SD). Relevant statistical comparisons are shown within each graph. The percentage of CD8 T cells with increased Foxp3

In contrast, ERK or IRF3 activation significantly suppressed lentivector-induced immune responses for 2 antigens, IiOVA and human NY-ESO. Tregs are the main effectors in immune suppression and tolerance, and we have shown increased numbers of CD4+ CD25+ Foxp3+ T cells with increased Foxp3 levels in splenocytes after vaccination with lentivectors encoding ERK and IRF3 activators. Tregs are known to be induced after antigen presentation by immature or tolerogenic DCs. In agreement with this, we have shown that 2 distinct signaling pathways in DCs lead to antigen-specific suppression; ERK activation causes TGF-β up-regulation and CD40 down-regulation, while IRF3 activation causes IL-10 secretion. Either pathway leads to systemic Treg expansion.

Our results are highly relevant for improved vaccine design. They suggest that incorporation of a p38 activator into a vaccine vector designed to transduce DCs could improve immune responses. We have shown that this type of p38 activator can also be expressed in DCs to be injected as a cell-based therapy for cancer. For future implementation of this therapy in patients, it will be possible to combine a p38 activator with coexpression of 2 or more tumor antigens to reduce immunologic escape of tumor cells. On the other hand, tolerance to pathogenic auto-antigens or allergens could be induced by injection of DCs or vectors expressing activated ERK or IRF3. In addition, one of the major challenges of gene therapy is to avoid undesired immune responses against corrected cells that can occur, particularly if antigen-presenting cells are transduced. Therefore, coexpression of transgenes with modulators of DC maturation such as activators of ERK or IRF3 could suppress immune responses against the transgene. Clearly there is a risk in expression of potential oncopgenes, such as MAPK
activators, in a vaccine vector. DCs are not likely to be susceptible to transformation, due to their terminally differentiated state and limited lifespan. However, other cells such as lymphocytes will be transduced by a lentivector with a VSV-G envelope and constitutive promoters. To address this problem we have now developed a lentivector with a DC-specific dectin-2 promoter, and potential oncocenes could therefore be expressed in this way.

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