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ORIGINAL ARTICLE

OK-432 synergizes with IFN-γ to confer dendritic cells with enhanced antitumor immunity

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Generation of functional dendritic cells (DCs) with boosted immunity after the withdrawal of initial activation/maturation conditions remains a significant challenge. In this study, we investigated the impact of a newly developed maturation cocktail consisting of OK-432 and interferon-gamma (IFN-γ) on the function of human monocyte-derived DCs (MoDCs). We found that OK-432 plus IFN-γ stimulation could induce significantly stronger expression of surface molecules, production of cytokines, as well as migration of DCs compared with OK-432 stimulation alone. Most importantly, DCs matured with OK-432 plus IFN-γ-induced maintained secretion of interleukin-12 (IL-12)p70 in secondary culture after stimulus withdrawal. Functionally, OK-432 plus IFN-γ-conditioned DCs induce remarkable Th1 and Tc1 responses more effectively than OK-432 alone, even more than the use of α-type-1 cytokine cocktail. As a result, DCs matured with OK-432 plus IFN-γ can prime stronger cytotoxic lymphocyte (CTL) and natural killer (NK) cell response against tumor cells *in vitro*. Peripheral blood mononuclear cells activated by DCs matured with OK-432 plus IFN-γ also showed greater tumor growth inhibition *in vivo* in null mice. Molecular mechanistic analysis showed that DC maturation using IFN-γ in concert with OK-432 involves the activation of p38 and nuclear factor-kappa B (NF-κB) pathways. This study provided a novel strategy to generate more potent immune segments in DC vaccine.

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Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) and are widely used for vaccination in cancer immunotherapy.[1–3] It has been found that mature DCs (MDCs) are superior to immature DCs (iDCs) to activate $CD4^+$ Th1 cells and to prime $CD8^+$ CTLs.[4,5] MDCs have demonstrated enhanced specific antigen presentation (signal 1) and upregulated expression of co-stimulation molecules (signal 2) to T cells for their antigen-specific proliferation and activation.[4–7] Furthermore, MDCs have revealed enhanced cytokine production (signal 3), which stimulates $CD4^+$ T cells to develop into Th1, Th2 or Treg cells, respectively, and determines the functional polarization of T cells to induce either protective immunity or immune tolerance.[4–7]

For cancer rejection, Th1 response is desirable whereas Th2 or Treg responses are considered deleterious. In this regard, bioactive IL-12p70 secreted by DCs has a key role in mediating the polarization of Th1 $CD4^+$ cells and the generation of potent $CD8^+$ CTL responses.[8–10] Furthermore, it was showed that IL-12 can activate antigen-specific $CD8^+$ T cells directly to undergo optimal clonal expansion; develop effector functions and establish a memory population.[11–13] Thus, high level of IL-12 production is an important factor for successful DC vaccine preparation.[14] Unfortunately, in spite of many reagents used to induce DCs to secrete a high level of IL-12, its expression is often transient and DCs frequently become refractive to subsequent induction of IL-12, a phenomenon termed 'exhaustion' or 'paralysis'.[15,16] This is especially true in the case of serum-free medium culture system for DC generation.[17–19] Therefore, optimizing *ex vivo* DC maturation conditions to induce DC maturation but prevent its exhaustion so that they can keep producing high levels of IL-12 after *in vivo* administration represents a significant challenge.

In previous studies, we and others have reported that OK-432, a penicillin-killed and lyophilized preparation of a low-virulence strain of *Streptococcus spyogenes* (group A),[20] was a strong activator to induce DC maturation.[21–28] However, most of these studies reported were on the production of IL-12p70 by OK-432-matured DCs during culture. The usefulness of OK-432 for the maturation of DCs for clinical

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application has not yet been analyzed in depth. It remains unknown whether OK-432-matured DCs that have produced large amounts of bioactive IL-12 during the *ex vivo* culture in serum-free medium are still capable of secreting IL-12p70 after stimulus withdrawal. In this study, we found that human monocyte-derived DCs (MoDCs) matured with OK-432 and IFN-γ could preserve their ability to secondarily secreting a high level of bioactive IL-12 in serum-free medium culture system after stimulus withdrawal. Such DCs induced Th1 responses as well as antigen-specific CTL immunity. These results may help to design novel strategies for the generation of more potent DCs to induce therapeutic T-cell responses against cancer.

RESULTS

Monocyte-derived DCs matured with OK-432 plus IFN-γ demonstrated continuous production of inflammatory cytokines in serum-free conditions after stimulus withdrawal

We tested the abilities of different maturation stimuli to activate human MoDCs. Several previous studies have showed that IFN-γ alone could not induce DC maturation and IL-12p70 production by DCs.[29–31] Instead, we used α-type-1 cytokine cocktail (αDC1) as a control, which contains poly I:C, IL1-β, tumor necrosis factor-α (TNF-α), IFN-α and IFN-γ. The αDC1 cocktail has shown to be able to induce a high level of IL-12p70 production and migratory responses of DCs as well as CTL responses against tumor.[19,32–34] As shown in Figure 1a, stimulation with OK-432 only, OK-432 combined with IFN-γ or α-type-1 cytokine cocktail (αDC1) all upregulated the expression of surface markers, for example, CD40, CD80, CD83, CD86 and HLA-DR on DCs. The surface expression level between OK-432 plus IFN-γ group and αDC1 group was comparable. However, DCs matured with OK-432 combined with IFN-γ showed significantly higher CD40, CD80, CD86 and HLA-DR expression than DCs matured with OK-432 alone.

Another vital aspect of DC maturation is their ability to secrete inflammatory cytokines that directly influence T-helper and killer cell function. We therefore tested the capacity of DCs stimulated with different maturation stimuli to secrete IL-12p70, TNF-α and IL-6. As shown in Figure 1b, in primary culture, OK-432 combined with IFN-γ stimulation resulted in the highest production of IL-12p70, TNF-α and IL-6 by DCs compared with OK-432 alone or α-type-1 cytokine cocktail activation in serum-free conditions. The abilities of inflammatory cytokine production by MDCs after stimulus withdrawal are considered more critical to induce T-cell differentiation *in vivo*. We therefore tested these cytokine productions by the MDCs in the secondary culture without any cytokine and stimuli. As shown in Figure 1c, DCs matured with OK-432 alone or α-type-1 cytokine cocktail produced very low levels of IL-12p70 during the second culture. However, OK-432 combined with IFN-γ conditioned DCs and preserved their ability to secondarily secrete a high level of IL-12p70 when the stimuli were withdrawn. Similarly, OK-432 combined with IFN-γ conferred DCs to produce significantly higher levels of TNF-α and IL-6 than OK-432 alone or α-type-1 cytokine cocktail in the second culture medium. These results indicate that OK-432 combined with IFN-γ stimulation can generate fully MDCs in serum-free conditions, and the resultant MDCs are capable of continuously producing inflammatory cytokines, for example, IL-12, TNF-α and IL-6 without further stimulation.

DCs matured with the combination of OK-432 and IFN-γ showed increased migration

Migration capacity of MDCs is generally considered as an important factor for vaccine development due to the concern for the injected DCs to migrate to the lymph nodes. We tested the CCR7 expression of MDCs and their migration toward CCR7 ligands, CCL19 and CCL21, in this study. As shown in Figures 2a and b, respectively, OK-432 stimulation alone enhanced CCR7 expression and migratory capacity of MDCs toward CCR7 ligands, CCL19 and CCL21, compared with iDCs. Importantly, OK-432 combined with IFN-γ stimulation further increased the CCR7 expression and migratory capacity of DCs significantly. The levels of CCR7 expression (Figure 2a) and the numbers of migrated DCs (Figure 2b) stimulated with OK-432 plus IFN-γ were significantly higher than DCs stimulated with OK-432 alone and comparable to the αDC1 stimulation group.

DCs matured with OK-432 plus IFN-γ demonstrated significantly enhanced potential to induce Th1/Tc1 cytokine production

To analyze the effect of maturated DCs on T-cell polarization, we tested the Th1 and Th2 cytokine production by $CD4^+$ T cells stimulated with different MDCs. As shown in Figure 3a, after 7 days of coculture, all three kinds of MDCs enhanced IFN-γ secretion by $CD4^+$ T cells compared with the use of iDCs. However, DCs matured with OK-432 plus IFN-γ stimulated $CD4^+$ T cells, which produced significantly higher level of IFN-γ than T cells stimulated with OK-432 alone- or α-DC1-maturated DCs. Intracellular cytokine analysis confirmed the tendency (Figure 3b). On the other hand, iDCs and α-DC1-matuared DCs stimulated IL-5 production by $CD4^+$ T cells. However, stimulation with DCs matured with OK-432 alone or OK-432 plus IFN-γ showed significantly decreased IL-5 production by $CD4^+$ T cells (Figure 3a). We observed a very similar IL-10 production pattern to IL-5 (Figure 3a). Together, these results indicated that DCs matured with OK-432 combined with IFN-γ could prime $CD4^+$ cells to a Th1 phenotype.

We then tested IFN-γ production by $CD8^+$ T cells activated with different MDCs. Similar to $CD4^+$ T cells, IFN-γ secretion by $CD8^+$ T cells activated with DCs maturated with OK-432 plus IFN-γ was significantly higher than $CD8^+$ T cells activated with DCs maturated with OK-432 alone or with α-DC1 (Figure 3c). We further detected the IFN-γ production of $CD8^+$ T cells using intracellular cytokine analysis. The results in Figure 3d showed the same tendency as in Figure 3c. These results indicated that DCs matured with OK-432 combined with IFN-γ are superior to activate type-1 $CD8^+$ as well as $CD4^+$ T-cell responses.

In addition to T cells, NK cells can also be activated by DCs.[35–37] Thus, we evaluated the stimulation effects of different MDCs to NK cells. As shown in Figure 3e, similar to T cells, NK cells activated with DCs maturated with OK-432 plus IFN-γ produced significantly higher amounts of IFN-γ and TNF-α than NK cells activated with DCs maturated with OK-432 alone or with the α-type-1 cytokine cocktail, αDC1.

DCs matured with OK-432 plus IFN-γ show significantly elevated ability to induce CTL and NK cell responses to tumor *in vitro*

To analyze the tumor cell killing capacity of T cells primed with different MDCs, MDCs were pulsed with AFP peptides and then cocultured for 7–10 days with autologous $CD8^+$ or $CD3^+$ T cells purified from the peripheral blood mononuclear cells (PBMCs) collected from the same $HLA\text{-}A2^+$ donor. HLA-A2- and AFP-positive hepatocellular carcinoma (HCC) cells lines BEL-7402 were used as target cells to test the cytotoxicity of the CTLs. As shown in Figure 4a, killing capacity of $CD8^+$ AFP-primed CTLs stimulated with DCs maturated with OK-432 alone and α-DC1 was comparable but higher than T cells stimulated with iDC. Of note, $CD8^+$ T cells stimulated

with peptide-pulsed DCs maturated with OK-432 plus IFN-γ displayed significantly higher cytotoxicity than all other groups. However, if $CD8^+$ T cells were stimulated with DCs without peptide pulsing (Figure 4b), the cytotoxicity was significantly reduced, indicating that the killing was antigen-specific. Of note, we found that when stimulated with OK-432 plus IFN-γ matured DCs without peptide, the resultant $CD8^+$ T cells still showed a high-level cytotoxicity than other groups when peptide was not used, suggesting that OK-432 plus IFN-γ matured DCs may induce some nonspecific cytotoxic activity of CTL as well. The killing capacity of $CD3^+$ T cells showed a similar tendency (Figures 4c and d).

We further investigated the stimulation effects of different MDCs to NK cells for their killing of tumor cells. In these experiments, DCs were not pulsed with peptide. As shown in Figure 4e, after coculturing

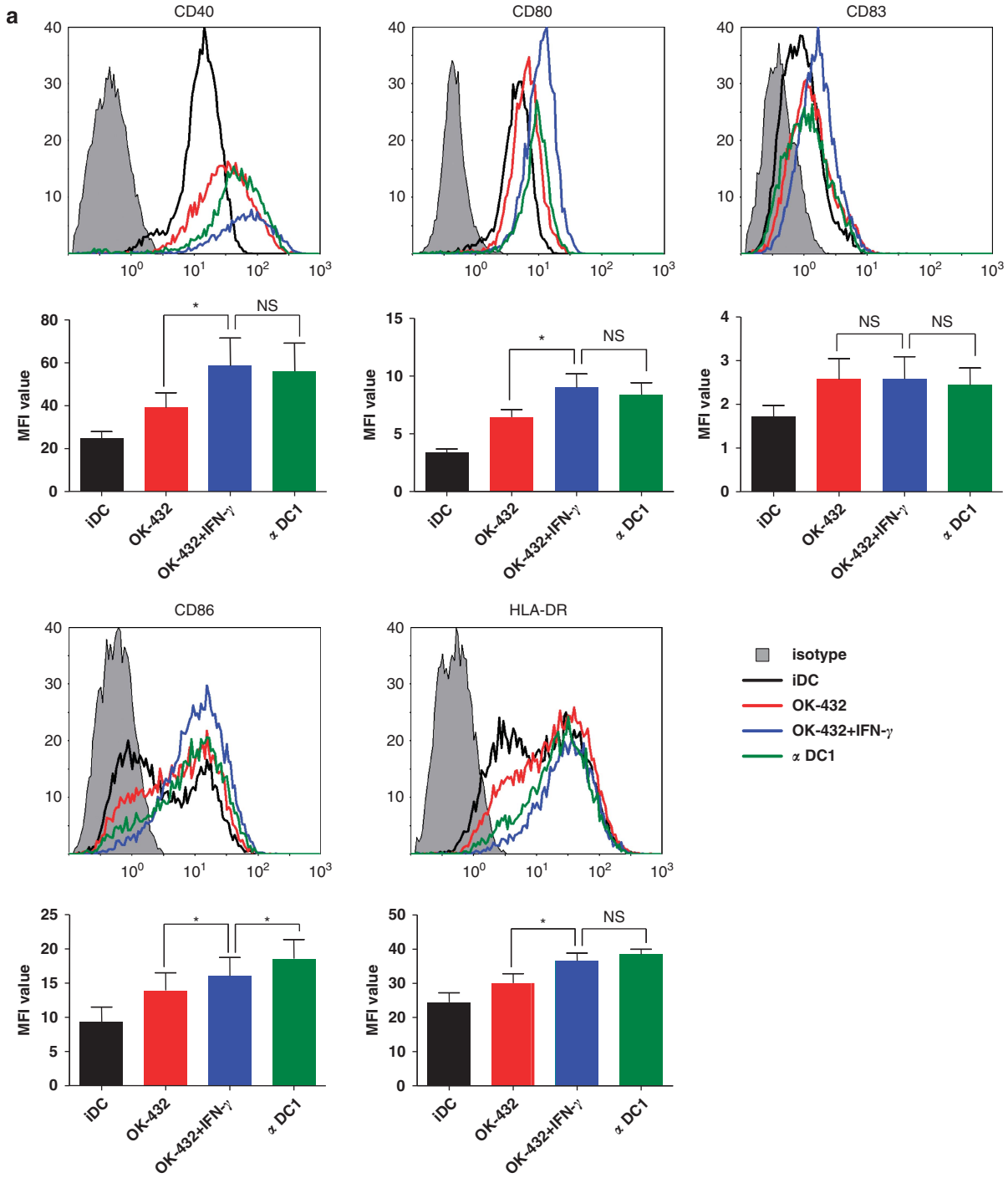
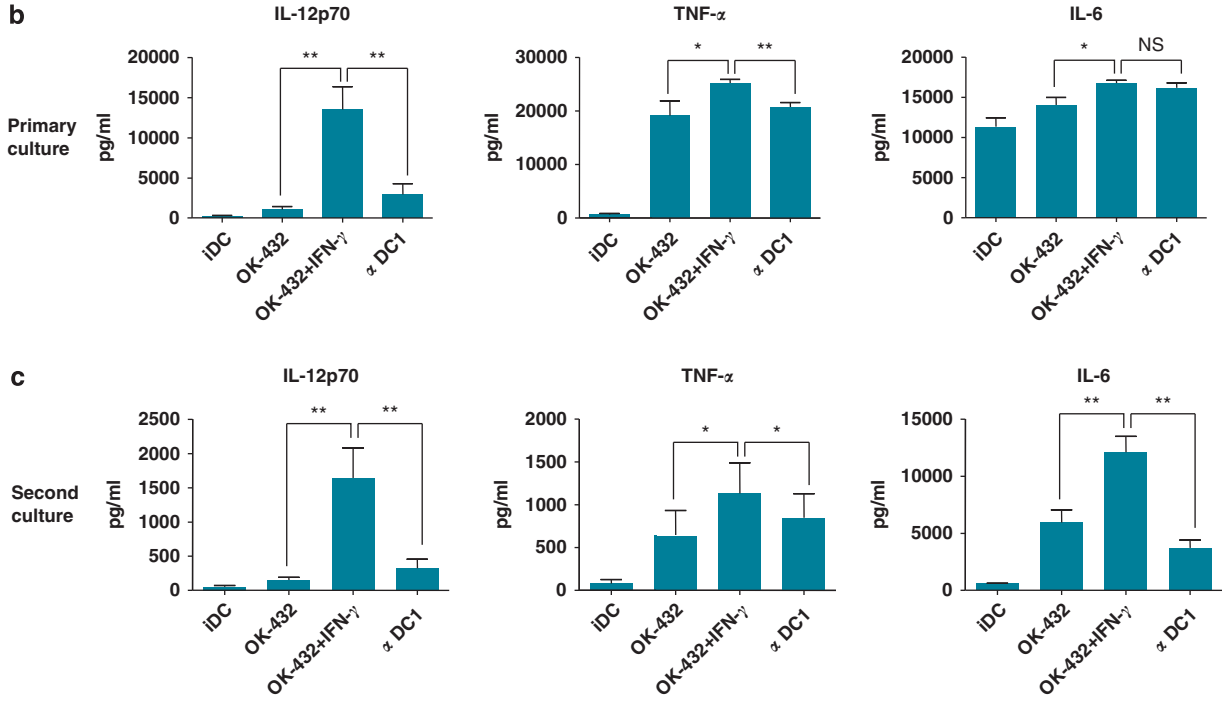


Figure 1 Cell surface marker expression and cytokine secretion of MDCs. Human MoDCs were generated in CellGro serum-free DC medium containing GM-CSF and IL-4 for 5 days. After maturation with different stimuli as indicated for another day, DCs were collected and used for analysis. (**a**) The expression of cell surface molecules was measured using the flow cytometry and the means fluorescent intensity (MFI) of four experiments is shown. (**b**) Cytokine secretion of differently matured DCs in the primary culture system. (**c**) Cytokine secretion of MDCs in the secondary culture system after stimulus withdrawal. $^*P<0.05$, $^{**}P<0.01$.



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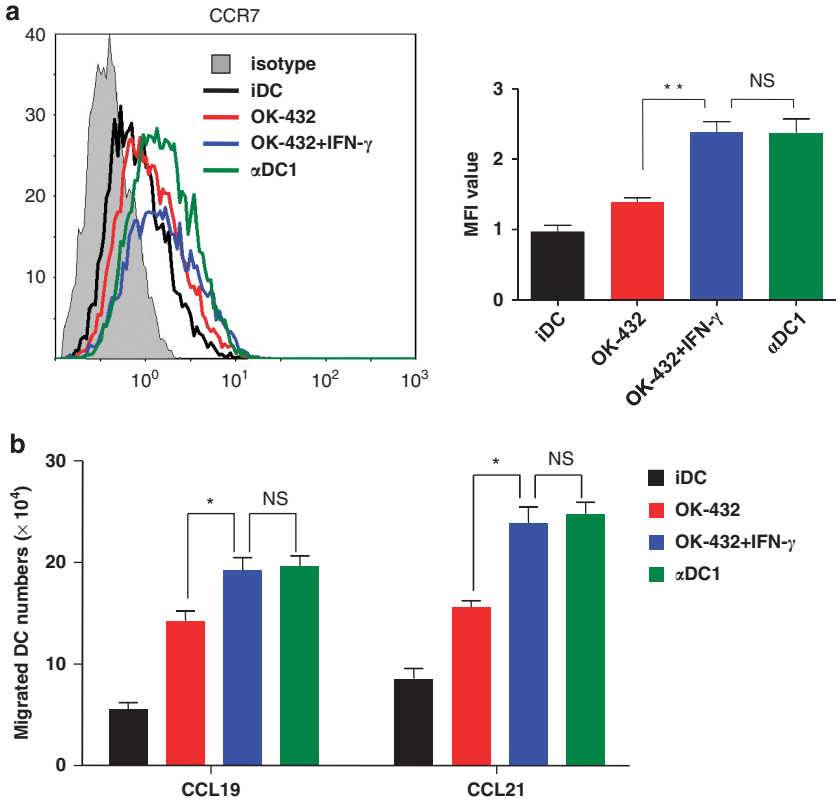


Figure 2 The migration capacity of MDCs. (**a**) DCs matured with the combination of OK-432 and IFN-γ showed significantly increased expression of CCR7. CCR7 expression of matured DCs was detected by flow cytometry and the means fluorescent intensity (MFI) of four experiments is shown. (**b**) DCs matured with the combination of OK-432 and IFN-γ showed significantly increased migration towards CCR7 ligands CCL19 and CCL21. The migratory capacity of matured DCs to CCL19 and CCL21 was measured by transwell assay. Bars represent s.e. on the mean of four experiments. $^{*}P<0.05$, $^{**}P<0.01$.

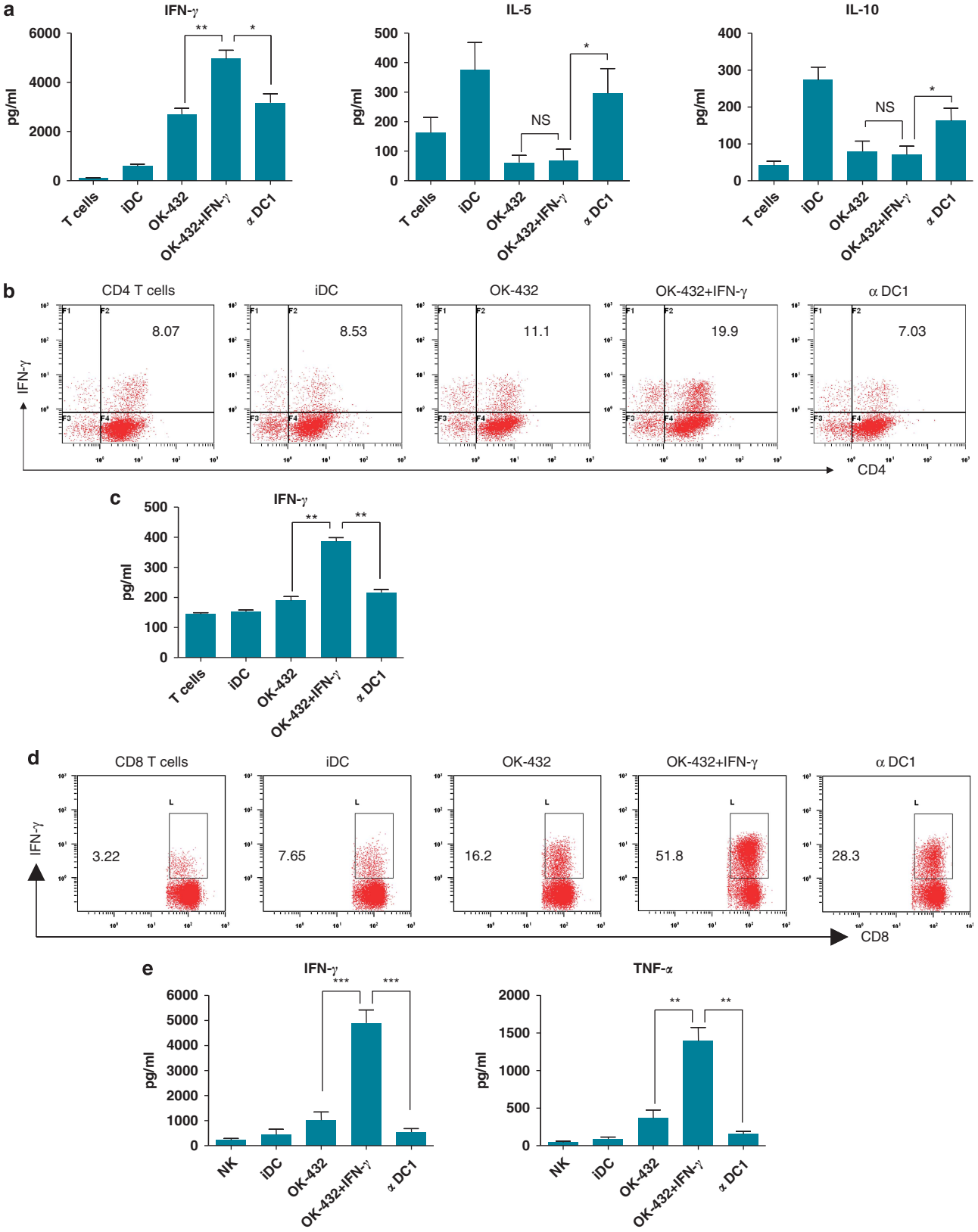


Figure 3 OK-432 plus IFN-γ-matured DCs significantly enhanced Th1/Tc1 and NK cell polarization. (**a**) Purified $CD4^+$ T cells were cocultured with different matured DCs as indicated for 7 days. The supernatants were then collected for IFN-γ, IL-5 and IL-10 detection. (**b**) Intracellular IFN-γ assay of $CD4^+$ T cells by flow cytometer. (**c**) Purified $CD8^+$ T cells were cocultured with different matured DCs as indicated for 7 days. The supernatants were then collected for IFN-γ assay. (**d**) Intracellular IFN-γ assay of $CD8^+$ T cells. (**e**) Purified NK cells were incubated with different matured DCs for 2 days, and supernatants were collected for IFN-γ and TNF-α production assay. Bars represent s.e. on the mean of four experiments. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

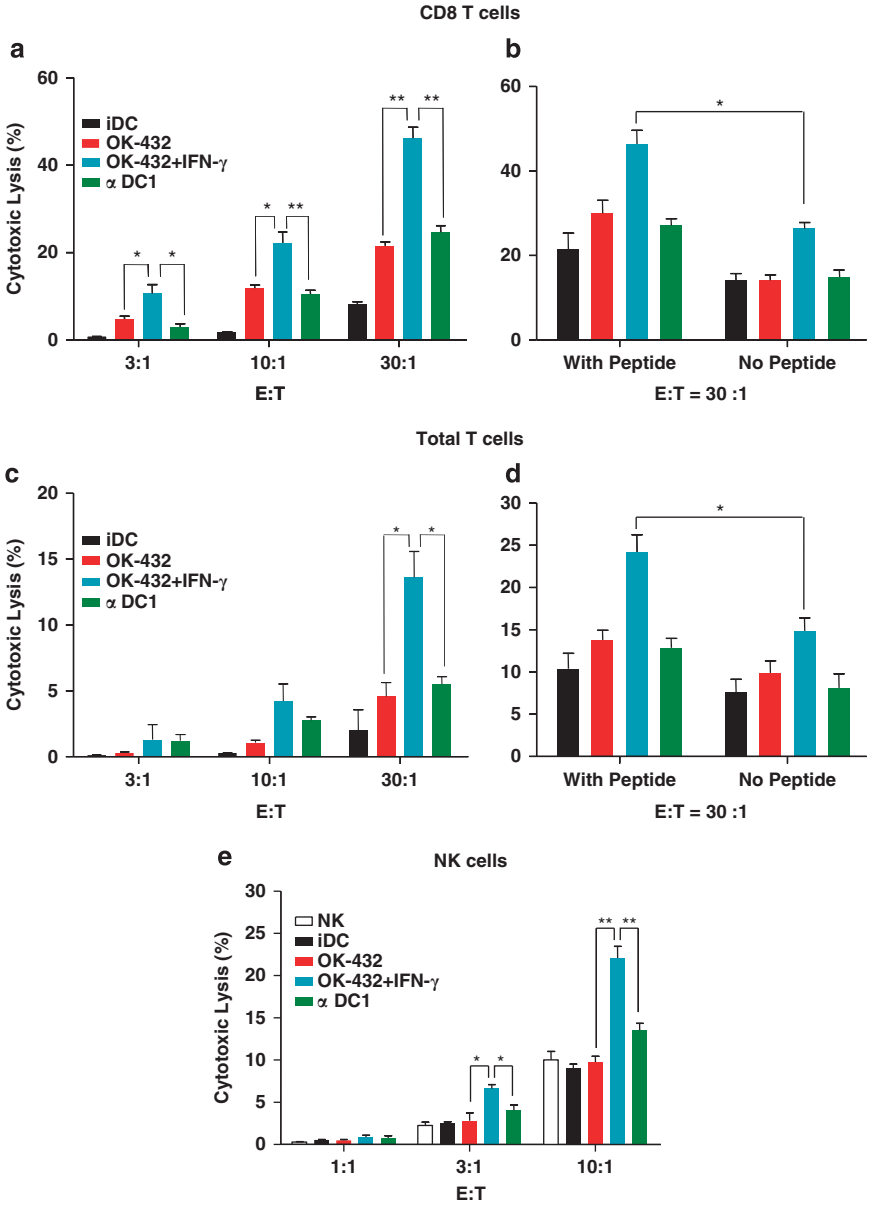


Figure 4 OK-432 plus IFN-γ could MDCs which in turn significantly induce CTLs and NK cells against tumor *in vitro*. (**a**, **b**) Purified $CD8^+$ T cells were cocultured with different matured DCs which were pulsed with HLA-A2-restricted AFP peptide (**a**) or not (**b**) for 7 days. The primed CTLs were then collected for cytotoxicity against AFP and HLA–A2–positive HCC cells BEL-7402. (**c**, **d**) Purified total T cells were cocultured with different matured DCs, which were pulsed with HLA-A2-restricted AFP peptide (**c**) or not (**d**) for 7 days. The primed CTLs were then collected for cytotoxicity to AFP and HLA-A2-positive HCC cells BEL-7402. (**e**) Purified NK cells were incubated with different matured DCs as indicated for 2 days and used for cytotoxicity to HCC cells BEL-7402. Bars represent s.e. on the mean of four experiments. $^*P<0.05$, $^{**}P<0.01$.

for 2 days, neither OK-432 alone nor α-DC1–matured DC stimulation modulated the killing of NK cells to BEL-7402 cells compared with iDC stimulation. However, when NK cells were stimulated with DCs maturated with OK-432 plus IFN-γ, the killing ability of NK cells to BEL-7402 cells was increased significantly compared with any other groups. These data indicate that OK-432 combined with IFN-γ could mature DCs to significantly elevate their ability to induce CTL responses as well as NK cell killing.

PBMCs activated with DCs maturated with OK-432 combined with IFN-γ show significantly increased antitumor activity *in vivo*

In order to confirm the superior tumor killing capacity of T cells stimulated with DCs maturated with OK-432 plus IFN-γ, we carried out antitumor assays *in vivo*. Autologous unsorted nonadherent fraction of PBMCs was cocultured with AFP peptide-pulsed iDCs or different MDCs as indicated for 7 days. PBMCs without DC stimulation were used as control (T cells only). These activated or nonactivated PBMCs were then mixed with the HCC BEL-7402 cells and were injected subcutaneously into the nude mice. As shown in Figure 5a, PBMCs activated by DCs maturated with OK-432 plus IFN-γ delayed tumor growth remarkably. On day 38 after injection, mice were killed and the tumors were collected. The size of tumor with the injection of PBMCs activated by DCs maturated with OK-432 plus IFN-γ was the smallest (Figure 5b). Quantatively, the weight of tumor with the injection of PBMCs activated by DCs maturated with OK-432 plus IFN-γ was significantly lighter than any

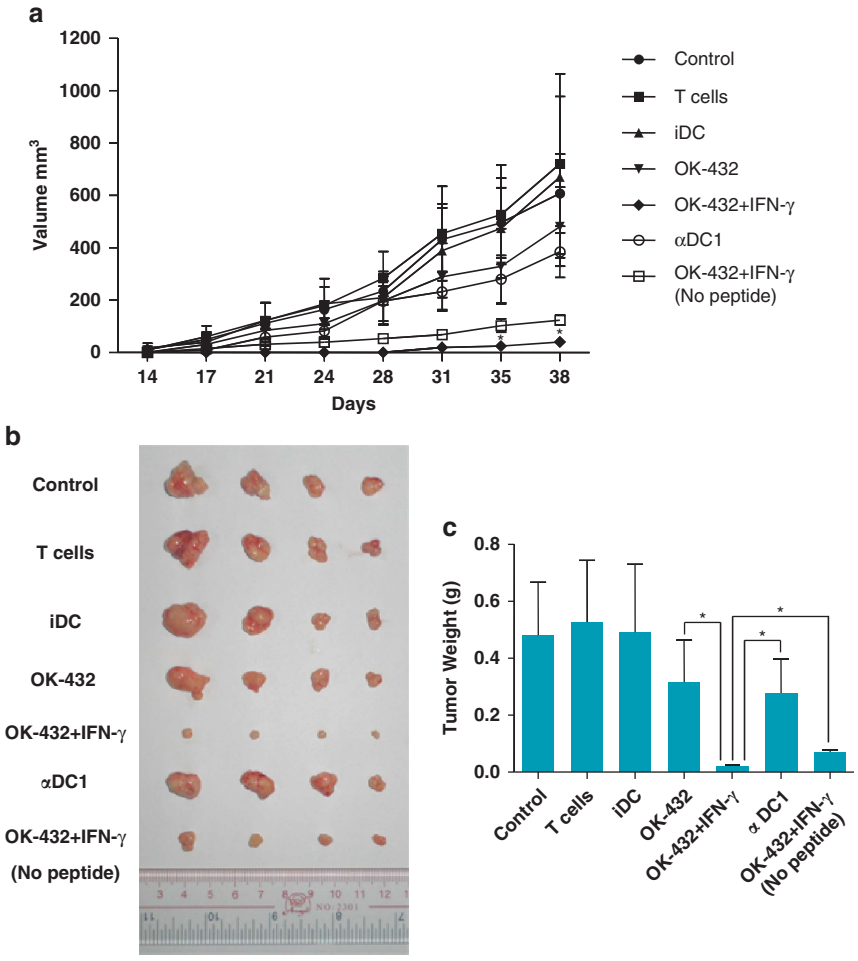


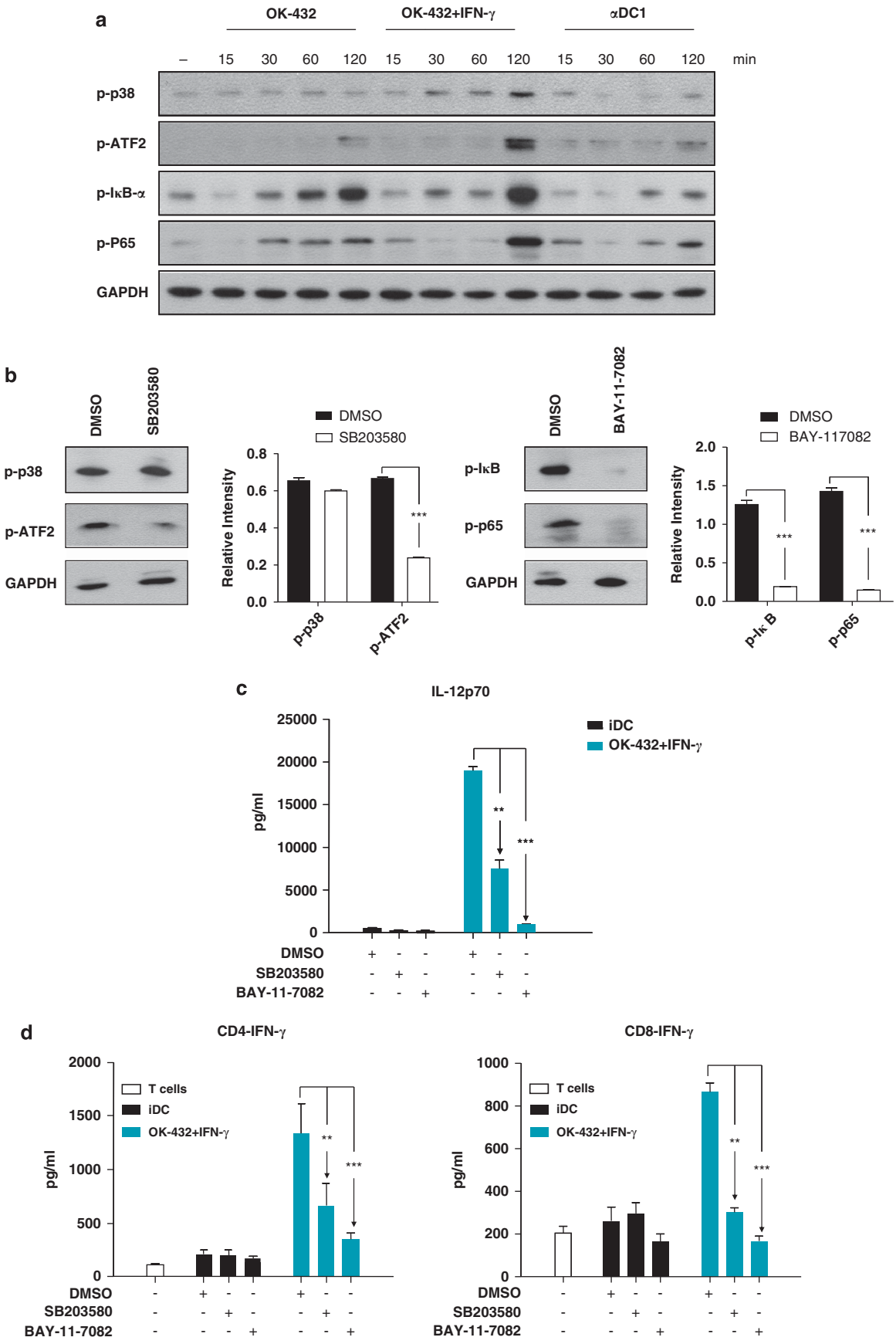
Figure 5 OK-432 combined with IFN-γ could maturate DCs to confer PBMCs with significantly higher antitumor activity *in vivo*. (**a**) Nonadherent PBMCs were cocultured with different groups of DCs pulsed with APF peptide at ratio T:DC = 10:1 for 7 Days. A total of 3×10^7 of the PBMCs with or without DC activation were then mixed with 1×10^6 BEL-7402 HCC cells and were injected subcutaneously into the nude mice. Control mice were not treated. Furthermore, mice treated with T cells activated with OK-432 plus IFN-γ matured DC without peptide pulsing were used as an additional control. Tumor growth was recorded and shown (*$P<0.05$, compared with any other groups on the same date). (**b**) On day 38, mice were killed and the tumors were taken out to take pictures as shown. (**c**) The weight of tumor in different groups was measured and compared. Bars represent s.e. on the mean of three measurements. *$P<0.05$.

other groups (Figure 5c). Furthermore, in line with the *in vitro* results, the OK-432plus IFN-γ without peptide-pulsing group showed significantly lower suppression effect on tumor growth than OK-432 plus IFN-γ with peptide-pulsing group, but still showed remarkable tumor growth suppression, confirming that OK-432 plus IFN-γ matured DCs not only induced antigen-specific CTL response, but also induced nonspecific antitumor cytotoxic activity. These *in vivo* data provided more experimental evidence to support the conclusion that DCs maturated with OK-432 combined with IFN-γ are significantly more potent in priming immune effector cells in antitumor immunity.

DC maturation with OK-432 and IFN-γ is associated with the activation of p38 and NF-κB pathways

In the following experiments, we investigated the potential mechanisms involved in OK-432/IFN-γ-induced maturation of DCs at the molecular level. As shown in Figure 6a, all three maturation conditions can enhance the phosphorylation of p38 and downstream molecular ATF2. After 120 min, OK-432 combined with IFN-γ stimulation displayed the strongest p38 and ATF2 phosphorylation. Similarly, OK-432 combined with IFN-γ stimulation also lead to the strongest IκB-α and p65 phosphorylation.

To verify that the p38 and NF-κB pathways are involved in the activation of DCs by OK-432 combined with IFN-γ, we used specific inhibitors to treat DCs. As shown in Figure 6b, SB203580 (p38 pathway inhibitor) treatment effectively inhibited the ATF2 phosphorylation but not p38 itself phosphorylation because SB203580 binds to the ATP-binding pocket of p38 kinase and inhibits its activity, but does not prevent it from being phosphorylated. BAY-11-7082 (NF-κB pathway inhibitor) treatment effectively inhibited both IκB and p65 phosphorylation (Figure 6b). In addition, SB203580 and BAY-11-7082 significantly inhibited the IL-12p70 production by DCs matured with OK-432 plus IFN-γ (Figure 6c). BAY-11-7082 displayed higher efficiency to decrease IL-12p70 production than SB203580, suggesting that the NF-κB pathway may have a more important role in the activation of DCs under OK-432 plus IFN-γ stimulation. In addition, we found that both SB203580 and BAY-11-7082 significantly decreased the ability of DCs matured with OK-432 plus IFN-γ to induce IFN-γ production by $CD4^+$ and $CD8^+$ T cells (Figures 6d and e), suggesting that p38 and NF-κB pathways are both involved in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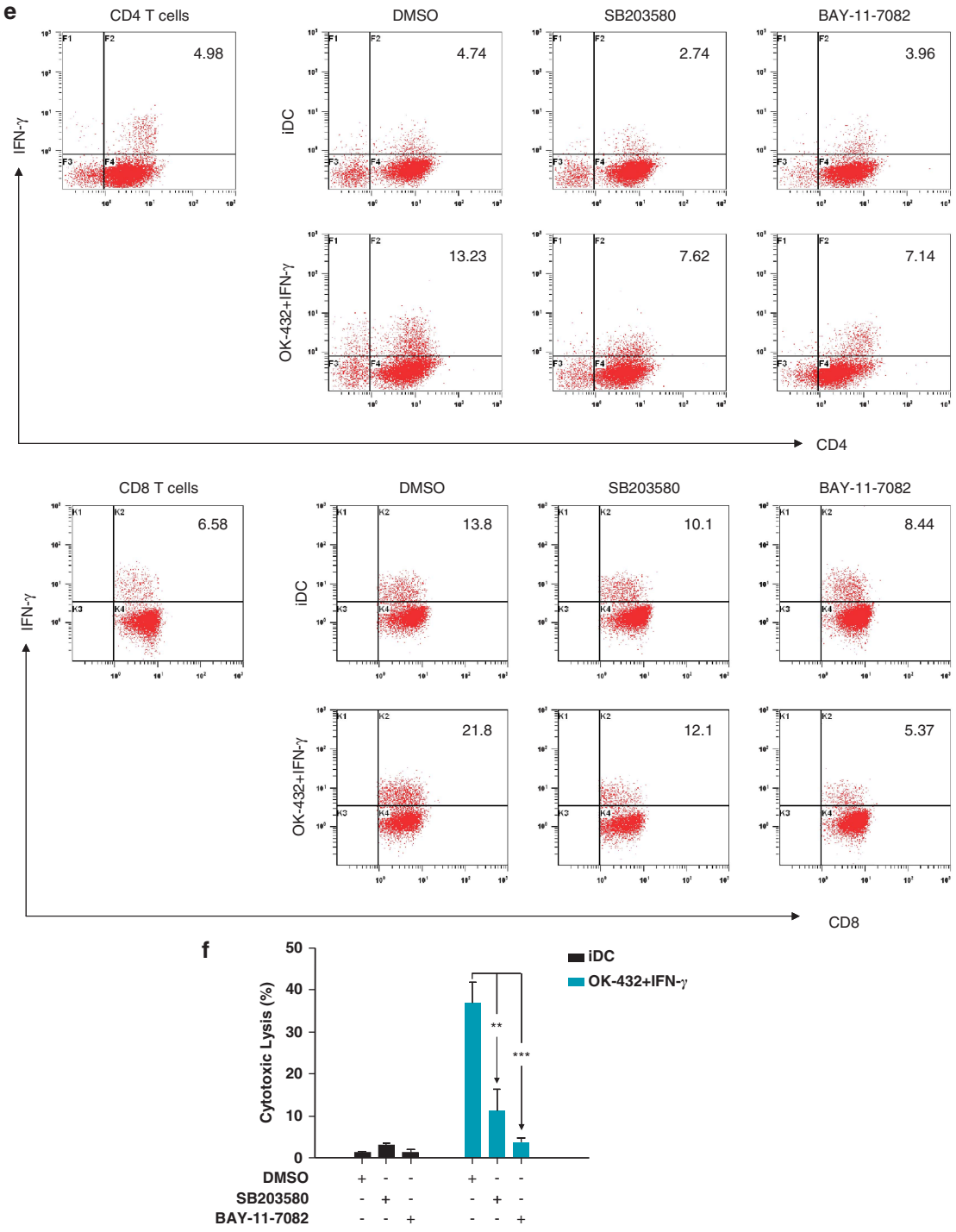

Figure 6 Continued.

Figure 6 Activation of p38 and NF-κB pathways in OK-432/IFN-γ matured DCs. (**a**) Various stimuli as indicated were added to the iDC for DC maturation. DCs were collected at indicated time points and lysed using 1 × SDS loading buffer. p-p38, p-ATF2, p-IκB-α and p-p65 proteins were detected by western blot. GAPDH was used for loading control. (**b**) Before OK-432/IFN-γ maturation, iDCs were pretreated with the p38 pathway-specific inhibitor SB203580 (10 μM) or the NF-κB pathway specific inhibitor BAY-11-7082 (10 μM) for 30 min. After 2 h maturation, DCs were collected and the p-p38, p-ATF2, p-IκB-α and p-p65 proteins were detected, respectively, by western blot. (**c**) Detection of IL-12 secretion by OK-432/IFN-γ matured DCs with or without inhibitor treatment. (**d**) Detection of IFN-γ production by $CD4^+$ T cells or $CD8^+$ T cells stimulated with OK-432/IFN-γ-matured DCs with or without inhibitor treatment. (**e**) Intracellular IFN-γ assay of $CD4^+$ T cells or $CD8^+$ T cells stimulated with OK-432/IFN-γ matured DCs with or without inhibitor treatment. (**f**) Cytotoxicity assay of $CD8^+$ T cells activated by OK-432/IFN-γ matured DCs with or without inhibitor treatment. Bars represent s.e. on the mean of four experiments. $^{**}P<0.01$, $^{***}P<0.001$.

the priming of Th1 and Tc1 responses induced by DCs matured with OK-432 combined with IFN-γ. Finally, cytotoxicity analysis of $CD8^+$ T cells demonstrated that the tumor killing capacity of T cells was significantly blocked when the DCs were treated with SB203580 or BAY-11-7082 (Figure 6f), confirming that p38 and NF-κB pathways have pivotal roles in DC maturation with OK-432 plus IFN-γ.

DISCUSSION

OK-432 has been shown to be able to induce DC maturation through TLR-4.[24,25] OK-PSA, a lipoteichoic acid-related molecule which was isolated by affinity chromatography of a butanol extract of OK-432, and the OK-432 derived DNA were identified as the active components of OK-432.[38,39] These active components of OK-432 were found effective as DC-maturating agent to induce Th1 response *via* TLR.[25,39] However, several studies have found that single TLR ligand stimulation failed to generate full MDCs as they cannot maintain stable IL-12p70 production, especially in serum-free medium.[17–19] In this study, we found that although OK-432 single stimulation could enhance DC surface marker expression as well as pro-inflammatory cytokine production in the primary culture, the IL-12p70 production was decreased significantly to a low/no level in the secondary culture system when stimulus was withdrawn. However, when IFN-γ was added in addition to OK-432, the IL-12p70 secretion could not only be increased significantly in the primary culture, but was also maintained in the secondary culture with no more stimuli. These results are consistent with previous reports, indicating that IFN-γ could assist other TLR ligands such as lipopolysaccharides, poly I:C or monophosphoryl lipid A to enhance IL-12 production of DCs.[18,19,40] IFN-γ alone did not induce functional IL-12p70 secretion by DCs,[29–31] because it only induced p35 expression but not p40 subunit.[41] Nevertheless, if, and only when toll-like receptor (TLR) ligands are presented, could IFN-γ enhance both p35 and p40 expression.[42–44] Thus, these data indicate that IFN-γ may function as a co-stimulatory factor to cooperate with TLR ligands, for example, OK-432, as we reported herein, to optimize IL-12p70 production by DCs.

We also found that DCs matured with OK-432 plus IFN-γ could more effectively induce Th1 and Tc1 responses, presumably due to their stronger IL-12p70 production. NK cells activated by DCs matured with OK-432 plus IFN-γ also revealed improved immune activity, evidenced by significantly higher levels of IFN-γ and TNF-α production. It is worth noting that TNF-α and IFN-γ production from NK cells may provide additional helper for DC activation, which forms a positive feedback loop to enhance Th1 and CTL responses.[45] These results thus suggest that OK-432 plus IFN-γ-matured DCs not only induce T-cell responses directly, but also effectively activate innate NK cell response and therefore provide additional help to optimize adaptive immunity. In line with these findings, *in vitro* tumor killing experiments demonstrated that T-cell as well as NK cell cytotoxicity was significantly upregulated by DCs matured with OK-432 plus IFN-γ. Importantly, these data were validated by *in vivo* antitumor activity of the PBMCs stimulated with autologous DCs maturated with OK-432 plus IFN-γ. Collectively, these date provide experimental evidence to support the conclusion that OK-432 plus IFN-γ may represent an ideal combination to activate DCs to induce superior antitumor immune responses.

From *in vitro* tumor killing experiments (Figure 4), we noted that T cells, either purified $CD8^+$ T cells or purified total $CD3^+$ T cells, primed with OK-432 plus IFN-γ-matured DCs without peptide pulsing still showed a high cytotoxicity toward tumor cells. One reason for this phenomenon might be the contamination of NK cells. Particularly, in *in vivo* experiments (Figure 5), the T cells primed with DCs were not purified. Alternatively, unpulsed DCs may have induced non-antigen-specific antitumor cytotoxic activity of T cells, which was reported by previous studies.[46,47] To identify the molecular mechanisms responsible for the enhanced activation effects of OK-432 plus IFN-γ to DCs, we found in this study that the p38 and NF-κB pathways are involved. Both p38 and NF-κB pathways have been identified to have pivotal role in increasing the expression of co-stimulatory molecules and pro-inflammatory cytokines.[26,48–51] How IFN-γ in concert with a TLR ligand, for example, OK-432, could result in p38 and NF-κB pathway activation is not clear. α-type-1 cytokine cocktail (αDC1), which also contain IFN-γ but a different TLR ligand, for example, Poly I:C, did not show the same enhanced effects as OK-432 plus IFN-γ. These results suggest that the observed difference may be owning to different TLR ligand stimulations, for example, OK-432 for TLR-4 but Poly I:C for TLR-3.[52,53] The underlining mechanisms need to be further investigated.

Altogether, we have described in this report a new clinical grade and cost-effective cocktail only consisting of OK-432 and IFN-γ to generate MDCs with promising potential to stimulate antitumor immunity. This simple cocktail can effectively induce high levels of MHC and co-stimulatory molecule expression and stable bioactive IL-12p70 production as well as a high migratory ability of DCs. These regulations have resulted in upregulated Th1, CTL and NK cell responses *in vitro*, and significantly improved the antitumor activity *in vivo*. These data may provide useful information for the development of novel strategies for cancer immunotherapy.

METHODS

Generation of MoDCs

PBMCs were isolated from buffy coats (Guangzhou Blood Center, Guangzhou, China) by Ficoll-Hypaque gradient centrifugation. PBMCs were then plated in 75-cm^2 culture bottles at 1×10^8 cells and allowed to adhere to plastic for 1 h. Nonadherent cells were removed and cultured in Quanta-007 lymphocyte medium (PAA, Freiburg, Germany) containing 20 U ml^{-1} IL-2 (Peprotech, Suzhou, China) for T-cell or NK cell isolation. Monocyte-derived DCs were generated in CellGro DC medium (CellGenix, Freiburg, Germany) supplemented with 1000 U ml^{-1} recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF) and 400 U ml^{-1} recombinant human IL-4 (both from Life Technologies, Guangzhou, China). iDCs were collected on day 5. MDCs were generated by stimulating iDC with (i) 0.1 KE ml^{-1} (10 μg ml^{-1}) OK-432 (Shandong Lukang Pharmaceutical, Jining, China) alone, or (ii) OK-432 plus 1000 U ml^{-1} IFN-γ (Peprotech, Suzhou, China), or (iii) α-type-1 cytokine cocktail which contains 25 μg ml^{-1} poly I:C (Sigma, Shanghai, China), 3000 U ml^{-1} IFN-α, 25 ng ml^{-1} IL1-β, 100 ng ml^{-1} TNF-α (both from Peprotech) and 1000 U ml^{-1} IFN-γ (αDC1 group) for 24 h. In some experiments, iDCs were pretreated for 30 min with either 10 μM SB203580 (Sigma), 10 μM BAY-11-7082 (Sigma) or 1% dimethylsulphoxide (DMSO) as control for the inhibitors (These treatment will not significantly affect the viability of DCs, Supplementary Figure S1 online), and were then matured with different stimuli as listed above.

Phenotyping of DCs

Differently matured DCs were collected and stained with following antibodies: anti-CD40 (FITC), anti-CD80 (APC), anti-CD83 (FITC), anti-CD86 (APC), anti-HLA-DR (APC) and anti-CCR7 (PE) (all from Biglegand, Beijing, China). Isotype-matching antibodies were used as controls. After incubating at 4 °C for 30 min and washing with phosphate-buffered saline (PBS), DCs were fixed with 4% paraformaldehyde and then analyzed using a Cytomics FC500 Flow Cytometry (Beckman Coulter, Shanghai, China). Data analysis was performed using the CXP Analysis software (Beckman Coulter).

Analysis of inflammatory cytokine production of MDCs

iDCs (3×10^5) were cultured in 48-well plate in 1 ml medium with different maturation stimuli as listed above. After 24 h, the supernatants were collected for cytokine analysis in primary culture. DCs were then collected and washed with PBS to remove residual cytokines as well as maturation stimuli and were transferred to new plates in fresh medium without any cytokines such as GM-CSF, IL-4 or any of the maturation stimuli listed above. DCs were cultured for another 24 h. The supernatants were then collected for secondary cytokine production. IL-12p70, TNF-α and IL-6 in the supernatants of differently treated DC cultures were measured using CBA kits (BD, Shanghai, China) according to the manufacturers protocols.

DCs migration assay

For migration studies, 24-well plates were filled with 600 μl fresh Quanta-007 lymphocyte culture medium in the presence of 500 ng ml^{-1} CCL19 or CCL21 (both from Peprotech). Different MDCs (5×10^5) in 100 μl fresh CellGro DC medium were added to 5 μm Transwell inserts (Costar, Corning, NY, USA); placed into the 24-well plates, and incubated for 6–8 h at 37 °C. The medium in the lower chambers was then concentrated to 50 μl, and cells were counted with a hemocytometer. Samples were analyzed in duplicates.

T-cell and NK cell purification

Autologous total T cells ($CD3^+$), $CD4^+$ T cells, $CD8^+$ T cells and NK cells ($CD56^+$) were purified from the nonadherent fraction of PBMCs collected from the same donor by negative selection using the Human T Lymphocyte Enrichment Set-DM, Human CD4 T Lymphocyte Enrichment Set-DM, Human CD8 T Lymphocyte Enrichment Set-DM and Human NK Enrichment Set-DM (all from BD), respectively, according to the manufacturer's protocols. After purification, the purity of the $CD3^+$ T, $CD4^+$ cells was over 95%, and the purity of the $CD8^+$ T cells and NK cells were over 90%, respectively, according to the flow cytometry assays.

Analysis of inflammatory cytokine production by T cells or NK cells

A total of 3×10^5 iDCs were maturated with different stimuli as listed above for 24 h. The matured DCs were then collected, washed extensively, and incubated with 1.5×10^6 purified autologous $CD4^+$ T cells or $CD8^+$ T cells (T:DC = 5:1) in 48-well plates. Half of the medium was replaced every other day by fresh Quanta-007 lymphocyte culture medium containing IL-2 (20 U ml^{-1}) and IL-7 (Peprotech 5 ng ml^{-1}). On day 7, the supernatants were collected. The concentrations of IFN-γ, IL-5 and IL-10 were measured by CBA kits (BD) according to the manufacturers protocols.

For intracellular cytokine analysis, purified autologous $CD4^+$ or $CD8^+$ T cells were coincubated with different MDCs at 1:5 ratio in a 48-well plate in Quanta-007 lymphocytes culture medium. On day 7, the active T cells were restimulated with paraformaldehyde (PMA) (Sigma, Shanghai, China, 50 ng ml^{-1}) and ionomycin (Sigma 500 ng ml^{-1}) for 4 h in the presence of Brefeldin A (Biolegend, Beijing, China, 10 μg ml^{-1}). The cells were then collected, washed and fixed with 4% PMA) for 5 min at room temperature and permeabilized with Permeabilization Buffer (eBioscience, San Diego, CA, USA) for another 10 min at room temperature. Cells were then labeled with anti-IFN-γ (APC) and analyzed by flow cytometer.

Generation of CTLs

To generate antigen-specific CTLs, 3×10^5 matured DCs were pulsed with HLA-A2–restricted AFP-peptide mixture: hAFP137-145 (PLFQVPEPV), hAFP158-166 (FMNKFIYEI) and hAFP 325–334 (GLSPNLNRFL) at 20 μg ml^{-1} for each peptide for 2 h. DCs without peptide pulsing were used as controls. A total of 1.5×10^6 purified autologous total T cells or $CD8^+$ T cells were then cocultured with peptide-pulsed MDCs in Quanta-007 lymphocyte culture medium containing IL-2 (20 U ml^{-1}) and IL-7 (5 ng ml^{-1}). Half of the medium was replaced every other day with fresh medium. On day 7, the T cells were collected and used for cytotoxicity analysis.

Cytotoxicity assay of T cells and NK cells

Cytotoxic activity of activated CTLs or NK cells was analyzed using LDH release assay (CytoTox 96 kits; Promega, Beijing, China) according to the protocol. AFP- and HLA-A2-positive BEL-7402 hepatocellular carcinima cells were used as target cells. The E:T cell ratios were 30:1, 10:1 and 3:1 for T cells and 10:1, 3:1 and 1:1 for NK cells, respectively.

Detection of antitumor activity *in vivo*

Female BALB/c nude mice, aged 4–5 weeks, were purchased from Medical Experimental Animal Center of Guangdong Province and maintained under pathogen-free conditions in accordance with the institutional guidelines, and all mice experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University Cancer Center. Human unsorted autologous PBMCs are cocultured with AFP peptide-pulsed-iDCs or different MDCs at ratio 5:1 for 7 days. In total, 1×10^6 BEL-7402 tumor cells mixed with 3×10^7 different activated PBMCs and then injected subcutaneously into mice. Mice were randomly divided into seven groups of four mice each. The groups included were as follows: (1) Control group: BEL-7402 tumor cells mixed with PBS; (2) T cells group: BEL-7402 cells mixed with PBMCs without being cocultured with DCs; (3) iDC group: BEL-7402 cells mixed with PBMCs cocultured with iDC; (4) OK-432 group: BEL-7402 cells mixed with PBMCs cocultured with OK-432-matured DCs; (5) OK-432 + IFN-γ group: BEL-7402 cells mixed with PBMCs cocultured with OK-432 plus IFN-γ matured DCs; (6) αDC1 Group: BEL-7402 cells mixed with PBMCs cocultured with α-type-1 cytokine cocktail matured DCs; (7) OK-432 + IFN-γ without peptide-pulsing group: BEL-7402 cells mixed with PBMCs cocultured with OK-432 plus IFN-γ matured DCs without peptide pulsing. Tumor volumes were measured with caliper and estimated as tumor volume (mm^3) = length × width × width/2. Animals were killed 6 weeks after cell injection, and tumors were collected for weighting.

Western blot detection

iDCs (3×10^5) were exposed to different stimuli as listed above for maturation. Thereafter, cells were collected and washed twice with PBS and lysed with 40 μl of 1 × SDS loading buffer. The lysates were heated at 100 °C for 10 min, and 5 μl samples were run on a 12% SDS-polyacrlyamide gel electrophoresis (PAGE) gel and transferred to a polyvinylidine difluoride membrane (Millipore, Guangzhou, China). Membranes were then blocked for 1 h in PBST containing 5% skim milk, and probed with rabbit monoclonal antibodies against phosphorylated forms of IκB-α, p65, p38 MAPK and ATF2 (all from Epitomics, Hangzhou, China, 1:1000 diluted in blocking buffer) for 2 h at 37 °C. The membranes were then washed three times with PBST for 10 min and incubated with horseradish peroxidise (HRP)-conjugated goat-anti-rabbit secondary antibody (Santa Cruz, CA, USA, 1:2000 diluted in blocking buffer) at 37 °C for 1 h. After being washed three times, the membranes were developed by an enhanced chemiluminescence system (Cell Signal, Shanghai, China). Antibody to GAPDH (Epitomics) was used as control.

Statistical analysis

The two-tailed unpaired Student's *t*-test was used to assess differences between two experiment groups. All calculations were performed with the software Graphpad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). The results were considered significantly different when *P*-value was less than 0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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