OK432-Activated Human Dendritic Cells Kill Tumor Cells via CD40/CD40 Ligand Interactions

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In vivo, dendritic cells (DC) are programmed to orchestrate innate and adaptive immunity in response to pathogen-derived “danger” signals. Under particular circumstances, DC can also be directly cytotoxic against tumor cells, potentially allowing them to release tumor associated Ags from dying cells and then prime antitumor immunity against them. In this study, we describe the innate characteristics of DC (OK-DC) generated in vitro after exposure of immature human myeloid-derived DC to OK432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyogenes*. OK-DC produced proinflammatory cytokines, stimulated autologous T cell proliferation and IFN-γ secretion, expressed CCR7, and migrated in response to MIP-3β. Moreover, OK-DC displayed strong, specific cytotoxicity toward tumor cell targets. This cytotoxicity was associated with novel, OK432-induced up-regulation of CD40L on the cell surface of OK-DC, and was absolutely dependent on expression of CD40 on the tumor targets. These data demonstrate that maturation of human DC with OK432, an adjuvant suitable for clinical use, induces direct tumor cell killing by DC, and describes a novel CD40/CD40L-mediated mechanism for specific DC antitumor cytotoxicity. *The Journal of Immunology*, 2008, 181: 3108–3115.

Dendritic cells (DC) bridge the innate and adaptive immune systems by sampling the cellular environment, signaling to immune effector cells via receptor/ligand interactions or cytokines, and presenting Ag to T cells (1, 2). To orchestrate an appropriate immune response, DC must sense the presence of extrinsic threat; they recognize “danger” as pathogen-associated conserved molecules through pattern recognition receptors, including TLR (3). A range of TLR ligands has been shown to initiate the effective maturation of DC, which is essential for immune activation. In the absence of full maturation, DC can conversely induce T cell anergy, leading to tolerance rather than innate and adaptive immune priming (4).

Because DC can now be cultured in large numbers from patients as well as normal donors, particularly from myeloid precursors, there is a growing interest in using DC for clinical cellular therapy, including priming of antitumor immunity (5). In this context, the choice of reagent for DC maturation in vitro before administration becomes critical. There is a general consensus that fully activated DC, expressing appropriate markers of phenotypic activation and secreting a Th1 profile of inflammatory cytokines, will be optimal for the priming of specific T cell-mediated antitumor immunity by Ag-loaded DC (4). Similarly, DC activation can provide the appropriate signals to support initiation of earlier, innate antitumor immune priming via DC cross-talk with alternative effector cells including NK, NKT, and γδ T cells (6, 7). When designing protocols for DC culture and activation for patient use, it is clearly imperative to ensure that DC maturation is optimal and the reagents used are available and suitable for clinical use.

In this regard, OK432 is a clinical-grade, penicillin-inactivated and lyophilized preparation of *Streptococcus pyogenes*, which ligates TLR-2 and/or –4 (8), activates DC, and has potential applications for cancer therapy (9). OK432-matured DC effectively prime Ag-specific T cell responses in vitro (10), and OK432 has already been used to activate DC for clinical use (11). Importantly, OK432 has been used for many years as a direct anticancer agent, particularly in Japan, and has a well-established clinical safety profile; hence it represents a promising immunomodulator for incorporation into DC-based trial strategies. However, complete characterization of DC matured with OK432 (OK-DC), including assessment of innate immune activation, has not yet been reported.

One increasingly recognized property of DC is their ability to exert direct cytotoxicity against tumor cell targets. This has been described in rodent as well as human systems, and has been attributed to various mechanisms such as TNF, TRAIL, Fas ligand, NO, and perforin/granzyme (12–15). Although the physiological relevance of DC cytotoxicity remains uncertain, there are clear potential applications for cultured DC with the ability to kill tumor targets, particularly if this killing is specific to tumor, as opposed to normal cell, targets.

While analyzing the properties of OK-DC we found that, as expected, OK432 was an effective DC maturation agent, increasing the expression of Ag presentation and costimulatory markers and inducing the production of inflammatory cytokines. Remarkably,
OK-DC were significantly more potent at inducing proliferation and IFN-γ secretion by autologous T cells than LPS/IFN-γ-matured DC (LPS/IFN-DC). OK-DC/T cell cocultures also displayed innate killing of a range of tumor targets; however, the majority of this cytotoxicity resided in neither the CD4 nor CD8 T cell fraction of these cultures. Instead, OK-DC (but not LPS/IFN-DC) directly killed tumor, but not normal, cell targets. Target cell death was cell:cell contact dependent, but was not related to target Fas ligand or TRAIL sensitivity. Instead, killing was associated with up-regulation of CD40L on OK-DC, and dependent on CD40 expression or TRAIL sensitivity. Instead, killing was associated with up-regulation of CD40L on OK-DC, and dependent on CD40 expression on tumor cell targets.

Materials and Methods

Cell culture

CD14+ cells, isolated from human PBMC by MACS selection (Miltenyi Biotec), were cultured with 800 U/ml GM-CSF (Schering-Plough) and 0.05 mg/ml IL-4 (R&D Systems) in X-Vivo medium (Cymbrex) with 1% human AB serum (Sigma-Aldrich) for 5 days to generate immature DC (IDC), and matured for a further 2 days in 10 μg/ml OK432 (Chugai Pharmaceuticals) to generate OK-DC. Reference maturation stimuli tested comprised 250 ng/ml LPS (Sigma-Aldrich) and 1000 U/ml IFN-γ (Sigma-Aldrich). Autologous T cells were isolated from the CD14 negative PBMC fraction using The Pan T Cell Isolation Kit II (Miltenyi Biotec); CD8 and CD4 T cells were positively selected using CD4/CD8 microbeads (Miltenyi Biotec). T2 (T cell) and K562 (CML) cell lines were grown in RPMI 1640 (Life Technologies), 10% FCS (Harlan Sera-Labs), and 1% glutamine (Life Technologies). Bladder tumor cell lines EJ, RT112, and 253J cells were grown in a 50:50 ratio of RPMI 1640 (Life Technologies), 10% FCS, and 1% glutamine. SW480 cells (colorectal tumor cells) were grown in DMEM, 10% FCS, and 1% glutamine; parental RT112 and SW480 cell lines were transduced to express CD40 using a retroviral vector (16, 17). Human foreskin fibroblast (HFF) and normal human urothelial (NHU) cells were grown as previously described (16).

Flow cytometry

All Abs were obtained from B.D. Pharmingen except anti-CD40L, which was from R&D Systems. All analysis was performed using a FACSCalibur cytometer (BD Biosciences).

DC phagocytosis

IDC, LPS/IFN-DC, or OK-DC were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich) for 30 min at 37°C and cells were washed three times in FACS buffer before cell acquisition using a FACSCalibur cytometer (BD Biosciences). Control IDC (not incubated with FITC dextran) were acquired at the same time to allow background levels of fluorescence to be determined.

ELISA

DC were seeded at 200,000 cells/ml, and supernatant collected after 48 h. IL-12p40, TNF-α, IFN-γ, IL-10, and IL-6 were determined using matched pair Abs (BD Pharmingen) following standard protocols.

DC migration

In brief, 2 × 10^5 DC were seeded into transwells (Nunc, Fisher Scientific) in triplicate in a 24-well plate (Corning Lifesciences, Schiphol-Rijk, Netherlands), above wells containing X-Vivo medium/1% human AB serum with or without 0.5 μg/ml MIP-3β (R&D Systems). Plates were incubated for 3 h. Transwells were then carefully removed and discarded, and all well contents harvested. Cells were stained for CD11c and analyzed using a FACSCalibur cytometer as above; data was acquired from each tube for 1 min. The number of CD11c+ events over this time for each replicate and condition was calculated.

T cell proliferation assay

IDC, LPS/IFN-DC, and OK-DC were cocultured at 1 DC: 10 T cell ratio for 5 days. Cells were pulsed with [3H]thymidine (0.5 μCi/10^6 well) for 18 h and harvested onto filter mats using a TOMTEC harvester 96 MachIII. [3H]Thymidine incorporation was determined using a Wallac Jet 1459 microbeta scintillation counter and microbeta Windows software (Wallac).

Cytotoxicity assays

Target cells were labeled with 51Cr; cocultured at different E:T ratios with either T cell/DC cocultures, isolated CD4+ or CD8+ T cells, or DC for 4 h (T cell) or 20 h (DC); and a standard 51Cr release assay was performed (18). For DC, shorter killing assays over 4 h were also performed, which showed similar results, although overall levels of death were lower (data not shown). The JAM cytotoxicity test for DNA fragmentation was also used as previously described (19). To examine the contact dependence of DC killing, labeled cell targets and DC were separated using a 0.4-μm transwell membrane.

Anti-CD40L blocking Ab experiments

51Cr cytotoxicity assays of OK-DC against CD40-expressing RT112 were performed as above at an E:T ratio of 10:1, with addition of an anti-CD40L Ab (1 μg/ml or 5 μg/ml), or 1 μg/ml isotype control (R&D Systems). DC were preincubated with Ab for 30 min before addition of tumor cells to the killing assay.

Results

OK432 induces maturation of human myeloid DC

Human myeloid IDC were treated with OK432 for 48 h to generate OK-DC, or LPS/IFN-γ to generate LPS/IFN-DC, and the surface expression of MHC class I and class II, CD1a, CD80, CD86, CD83, and CD54 were examined by flow cytometry. Figure 1A demonstrates that, in general, OK432 stimulated equivalent or greater phenotypic DC maturation than LPS/IFN-γ. No changes in CD56 and CD11c expression were identified (data not shown). To confirm that OK432 induced DC maturation, phagocytosis of FITC dextran by IDC, LPS/IFN-DC, and OK-DC was determined, as mature DC are less effective at phagocytosis than IDC. Consistent with phenotypic maturation, we found that IDC were more efficient at phagocytosis than either LPS/IFN-DC or OK-DC (Fig. 1B).

To further determine whether OK-DC were functionally active, we next examined the ability of OK432 to induce the secretion by DC of a range of proinflammatory cytokines. OK432, like LPS/IFN-γ, induced production of TNF-α, IL-6, and IL-12, which were not secreted by IDC (Fig. 1C). In contrast, IL-10 (an immunosuppressive cytokine that can inhibit the generation of successful antitumor immunity) was not induced by OK432, although it was by LPS/IFN-γ (Fig. 1C).

OK432 enhances DC migration

For DC-based immunotherapy to be effective, it is important for DC to traffic to appropriate sites for interaction with immune effector cells. In particular, DC migration to lymph nodes, where they can prime innate and adaptive immune responses, from sites of Ag acquisition such as the tumor itself, will be essential for successful immunotherapy. One cell surface marker of DC migratory capacity is CCR7, which can promote DC migration from peripheral tissues to lymph nodes (20). Figures 2, A and B shows that, although both LPS/IFN-γ and OK-432 up-regulated CCR7 on DC, only OK-DC efficiently migrated toward a gradient of MIP-3β/CCL-19, a potent chemoattractant for DC (21). The poor correlation between CCR7 expression on LPS/IFN-DC and their migratory capacity in this assay may be due to the complexity of the functional interactions between chemokines and their multiple potential receptors. Nevertheless, these data show that OK-DC are likely to migrate effectively to lymph nodes in vivo.

OK-DC potently stimulate autologous T cells

Previous studies have demonstrated that peptide-pulsed OK432-treated DC can prime Ag-specific T cells (10), a result we have also been able to replicate in our laboratory (data not shown). Interestingly, more recent studies have shown that a distinct, non-Ag-specific innate killing by T cells can alternatively be stimulated...
upon short term culture of T cells with cytokines (22, 23). We therefore set out to address whether our cytokine-secreting OK-DC could similarly stimulate innate T cell functions, including cytotoxicity. First, T cell proliferation stimulated by autologous IDC, OK-DC, or LPS/IFN-DC was investigated using [3H]thymidine incorporation. LPS/IFN-DC was taken forward as the maturation comparator in these coculture experiments as we had established this as our previous optimal combination for DC phenotype.

**FIGURE 1.** OK432 activates human monocyte derived DC. Human monocyte derived DC were either left untreated (IDC), treated with 250 ng/ml LPS/1000U/ml IFN-γ (LPS/IFN-DC), or treated with 10 μg/ml OK432 for (OK-DC) for 48 h. A, DC were harvested and surface expression of CD1a, CD80, CD86, CD83, CD54, MHC class I, and MHC class II analyzed by FACS (data representative of four independent donors is shown). B, Control IDC (no FITC dextran), IDC, LPS/IFN-DC, and OK-DC (all with FITC dextran) were incubated for 30 min, washed, and uptake of FITC dextran was determined by flow cytometry (representative of n = 3). C, DC supernatants were harvested and the production of TNF-α, IL-6, IL-10, and IL-12 was examined by ELISA (representative of n = 3; error bars = SEM).

**FIGURE 2.** OK432 enhances DC migration. A, Surface expression of CCR7 on IDC, LPS/IFN-DC, and OK-DC was analyzed by FACS (representative of n = 3). B, Two × 10⁵ DC were placed in the upper chamber of an 8-μm transwell and migration toward 0.5 μg/ml MIP-3β determined (representative of n = 3; error bars = SD).
activation/cytokine induction during head-to-head comparisons of a number of reported DC maturation agents (CD40L, LPS alone, CpG, poly I:C, TNF-α, and a cytokine mixture comprising TNF-α, IL-1β, IL-6, and PGE₂ (Ref. 24 and data not shown). Fig. 3Ai shows that OK-DC were significantly better at stimulating autologous T cell proliferation than IDC or LPS/IFN-DC. Illustrative photographic images of IDC/T cells and OK-DC/T cells after coculture for 6 days. B, IFN-γ secretion in DC:T cell cocultures was measured by ELISA (representative of n > 4). C, Killing of T2 and K562 tumor cell targets by IDC/T cell (diamonds) and OK-DC/T cells (squares) cocultures was determined by 51Cr release assay (representative of n > 4). D, Either CD4 or CD8 T cells were positively selected from IDC/T cell and OK-DC/T cell cocultures and killing of T2 and K562 tumor cell targets measured using 51Cr release assay (at a 100:1 E:T ratio). Killing of positively selected CD4+ and CD8+ isolated cells and remaining nonselected cells was determined (representative of n = 2; all error bars = SD).

FIGURE 3. OK-DC potently stimulates autologous T cells. DC were cocultured with autologous T cells for 5 days at a 1:10 DC:T cell ratio. Ai, Overnight proliferation was measured by addition of tritiated thymidine (representative of n > 4). ii, Images of IDC/T cells and OK-DC/T cells after coculture for 6 days.

Next, cytotoxicity after T cell coculture with IDC or OK-DC, in the absence of Ag, was examined by 51Cr release assay. Killing of T2 and K562 tumor cell targets after coculture of T cells with IDC or OK-DC for 5 days suggested that OK-DC stimulated greater innate T cell cytotoxicity against these tumor cell targets (Fig. 3C). OK-DC/T cell cocultures also killed a range of other tumor cell lines, while poorly proliferative LPS/IFN-DC/T cells were as ineffective as IDC/T cells (data not shown). Because generation of innate T cell cytotoxicity upon coculture with DC has not previously been reported, we were next interested in determining which T cell population was responsible for tumor cell killing. CD4 and CD8 T cells were isolated after coculture with OK-DC and their cytotoxicity against T2 or K562 tumor cell targets determined (Fig. 3D). Surprisingly, it appeared that neither the CD4 nor CD8 T cells were responsible for the majority of innate killing observed, as a significant cytotoxic component was always found in those cells remaining after CD4 or CD8 isolation. Because the OK-DC themselves always remained in the cytotoxic fraction, and direct killing by DC has been previously reported (12–15), this led us to question whether the OK-DC were directly responsible for tumor cell target death. In initial experiments, we positively selected CD86-expressing cells from the T/OK-DC cocultures (as a marker expressed by DC but not T cells), and found that indeed significant cytoxicity resided in this CD86+ fraction (data not shown). We therefore went on to investigate further the cytotoxic potential of OK-DC alone, without any prior culture with T cells.
Tumor specific killing by OK-DC

DC were again either left untreated (IDC), treated with LPS-IFN-γ (LPS/IFN-DC), or OK432 (OK-DC), and cytotoxicity toward a range of tumor cell targets (T2, K562, EJ, and 253J cells) and normal cells (HFF and NHU) was examined using a 51Cr release assays (Fig. 4, A and B, respectively). OK-DC effectively killed all four tumor cell targets investigated while the cytotoxicity of IDC was negligible (Fig. 4A). Some killing by LPS/IFN-DC was observed, but only at low levels. In contrast to killing of tumor targets, no DC killed normal NHU or HFF cell targets (Fig. 4B). These studies demonstrate, for the first time, that OK432 stimulated human myeloid DC to specifically kill tumor, but not normal cell, targets.

Mechanism of OK-DC tumor specific killing

Various cellular mechanisms have been implicated in DC cytotoxicity, including via TRAIL and Fas, both of which are members of the TNF superfamily (12–14). In the current studies, however, levels of OK-DC killing of TRAIL-sensitive (EJ) vs TRAIL-insensitive (253J) (16), or Fas-sensitive (T2) (25) vs Fas-insensitive (K562) (26) cell lines (Fig. 4A) were similar, suggesting that a different mechanism was responsible for OK-DC cytotoxicity. Secreted factors, such as TNF-α, have also been implicated in OK-DC killing (12). Therefore, to determine whether OK-DC killing was contact dependent or mediated by the release of a soluble factor(s), OK-DC and tumor targets were cultured in the presence or absence of a transwell membrane. OK-DC killing of T2 tumor cell targets is shown in Fig. 5A, which demonstrates that cytotoxicity is clearly dependent on cell:cell contact.

When considering alternative surface molecules that might account for OK-DC contact-dependent, tumor-specific killing, we noted that CD40, like Fas and TRAIL, is a member of the TNF receptor superfamily, has also been implicated in cell death pathways, in addition to its central role in immune activation. The functional outcome of CD40/CD40L interactions is complex and often dependent on cell lineage and differentiation (27). For example, CD40-expressing epithelial cells can secrete proinflammatory cytokines and proliferate in response to CD40 ligation (28, 29). In contrast, in carcinoma cells, membrane-presented, surface CD40L induces tumor cell-specific apoptosis (17, 30) via a signaling mechanism involving TNFR-associated factor (TRAF)3

A  

FIGURE 4. Tumor specific killing by OK-DC. IDC, LPS/IFN-DC and OK-DC were cocultured at different ratios with 51Cr labeled T2, EJ, K562, and 253J targets (A), or labeled normal HFF and NHU cell targets (B) for 20 h. DC cytotoxicity was determined using a standard 51Cr release assay (representative of n = 4; all error bars = SD).

B  

FIGURE 5. OK-DC express CD40L and kill via a contact dependent mechanism. A, IDC and OK-DC were cocultured with 51Cr labeled T2 cell targets (10:1 E:T ratio) for 20 h either in the presence or absence of a 0.4 μm transwell membrane. % killing of T2 was determined using a 20 h 51Cr release assay (representative of n = 2; error bars = SD). B, Surface expression of CD40 on T2, K562, EJ, and 253J cells was analyzed by FACS. C, Surface expression of CD40L on IDC, LPS/IFN-DC, and OK-DC was analyzed by FACS (representative of n = 4).
and JNK/AP-1 activation (19). Significantly, CD40 ligation on the normal NHU and HFF cell targets resistant to OK-DC killing in Fig. 4B is known not to be associated with cell death (19, 30). The tumor-specific nature of epithelial CD40/CD40L-mediated killing led us to question whether this could be the mechanism responsible for OK-DC cytotoxicity. To address this we first determined whether each of the tumor cell targets from Fig. 4A expressed CD40, and would therefore potentially be responsive to CD40L-induced killing. CD40 expression on K562, T2, 253J, and EJ tumor cell targets is shown in Fig. 5B, which confirms that each cell line expresses CD40 on their surface. For OK-DC cytotoxicity to occur via CD40L/CD40 interactions up-regulation of surface CD40L on DC in response to OK432 is also required; Fig. 5C demonstrates that this does indeed occur. Potentially consistent with their poor killing by this mechanism (Fig. 4A), LPS/IFN-DC, in contrast to OK-DC, expressed only very low levels of CD40L.

To confirm that CD40 expression was absolutely required for OK-DC killing, we tested two CD40-negative tumor cell lines, SW480 and RT112, and their equivalent stable CD40-expressing transductants (Fig. 6A) as targets in OK-DC cytotoxicity assays. Fig. 6B shows that CD40- SW480 and RT112 cells were not sensitive to OK-DC killing, while SW480 and RT112 CD40+ transfectants were. We confirmed this result, using the JAM test as an alternative measure of cytotoxicity (19) (Fig. 6C). Using this assay killing, as assessed by DNA fragmentation, was again greater against the CD40 expressing tumor targets than the parental CD40 negative cells. Finally, we were able to show that OK-DC cytotoxicity against CD40-expressing RT112 targets was significantly reduced in the presence of an anti-CD40L blocking Ab, compared with an isotype control Ab (Fig. 6D). These studies demonstrate that OK432 stimulates DC to become tumor-specific killers via ligation between CD40L on the DC and CD40 on the tumor cell, representing a novel mechanism of DC-mediated cytotoxicity.

**Discussion**

Depending on their microenvironment DC may dampen, modulate, or activate various immune responses. Among the many maturation protocols used to activate DC in vitro, OK432 has shown recent promise as a clinically viable TLR agonist (9). As expected for a DC maturation agent, in this study OK432 up-regulated expression of the DC activation markers MHC I, MHC II, CD80, CD86, CD54, and CD83 (Fig. 1A), reduced DC phagocytosis (Fig. 1B), and induced secretion of the inflammatory cytokines IL-12, TNF-α, and IL-6 (Fig. 1C). OK-DC also displayed increased surface expression of CCR7, migrated toward MIP-3β in vitro (Fig. 2), and stimulated proliferation and IFN-γ production upon coculture with autologous T cells in the absence of Ag (Fig. 3, A and B). This OK-DC-induced T cell activation was significantly greater than that induced by DC matured with LPS/IFN-γ.
On finding that T cells cocultured with OK-DC were cytotoxic against tumor cells (Fig. 3C), we initially suspected that this represented innate T cell killing as previously reported on T cell stimulation with cytokines such as IFN-γ, IL-2, and IL-15 (22, 23). However, as the main cytotoxic fraction of the cocultures was in neither the CD4 nor CD8 fraction (Fig. 3D), we considered instead whether the OK-DC were themselves directly cytotoxic.

Recently, several reports have shown that, under special circumstances or during an intermediate phase, DC can, like NK cells, be directly cytotoxic (14, 15, 31, 32). When OK-DC were cocultured with a range of tumor cell targets (T2, K562, EJ, and 253J) they too were cytotoxic (Fig. 4A); neither IDC nor LPS/IFN-DC showed significant killing ability. Significantly, OK-DC did not kill normal N hu or HFF (Fig. 4B). These cytotoxic OK-DC are distinct from recently described IFN-γ-producing killer DC (6), as they do not directly secrete IFN-γ themselves, and do not express any surface NK markers (data not shown). The tumor specificity of OK-DC killing (Fig. 4), its contact dependence (Fig. 5A), and its lack of discrimination between Fas and TRAIL insensitive and sensitive targets (Fig. 4A), led us to seek an alternative mechanism for OK-DC cytotoxicity, including other potential members of the cytotoxic TNF ligand family.

CD40/CD40L engagement has previously been shown to specifically kill tumor cells, although not in the context of DC effectors. Membrane-presented, but not soluble, CD40 agonists can induce apoptosis in carcinoma, but not normal, CD40-expressing homologous epithelial cells (19). Membrane-presented CD40L triggers cell death in malignant human urothelial cells via a direct mechanism involving rapid up-regulation of TRAF3 protein, without concomitant up-regulation of TRAF3 mRNA, followed by activation of the JNK/AP-1 pathway and induction of the caspase-9/caspase-3-associated intrinsic apoptotic machinery. The current study clearly shows that OK-DC killing is restricted to CD40-expressing (K562, T2, 253J, EJ; Fig. 5B), as opposed to CD40-negative (RT112, SW480; Fig. 6A), tumor targets. Critically, and consistent with OK-DC expression of CD40L (Fig. 5C), transfected RT112 and SW480 expressing CD40 acquire sensitivity to OK-DC (Fig. 6, B and C), and OK-DC-mediated killing of CD40-expressing RT112 cells is blocked by an anti-CD40L Ab (Fig. 6D). We therefore conclude that CD40/CD40L interactions represent a novel pathway by which DC activated in this way can specifically kill tumor cells. CD40/CD40L is not likely to be the only/exclusive mechanism for OK-DC killing (note low-level target cell death with LPS/IFN-DC in Fig. 4A, and some killing by OK-DC of CD40− RT112 targets in the JAM assay in Fig. 6C). Nevertheless, the clear differential between killing of CD40 positive and negative targets in Fig. 6 validates CD40 activation by membrane CD40L as the major and novel killing mechanism for these cytotoxic DC. It should also be noted that, although OK-DC express both CD40 and CD40L, they do not kill each other (i.e., there is no evidence of cell fratricide); this is demonstrated by low-level uptake of tritiated thymidine by OK-DC when cultured alone (data not shown), suggestive of limited proliferation rather than cell death.

To date, we have been unable to find another DC maturation protocol which up-regulates CD40L and confers target CD40-dependent tumor cytotoxicity. Our main maturation comparator so far has been LPS/IFN-γ, as we have previously established this, in our hands, as optimal for DC phenotypic activation and cytokine production; however neither LPS/IFN-DC nor DC matured with a cytokine mixture comprising TNF-α, IL-1β, IL-6, and PGE2 (24) were cytotoxic (data not shown). We are currently testing DC matured with a wider variety of TLR ligands, cytokines, and CD40L, as well as further subtypes of both mouse and human DC, such as plasmacytoid DC.

There are clear potential applications for cytotoxic OK-DC. DC can acquire tumor-associated Ags from within tumors and prime therapeutic antitumor immunity, particularly when used in combination with an additional modality, such as chemotherapy or radiotherapy, to trigger additional cell death (33, 34). If OK-DC can be delivered intratumorally or locoregionally into involved sites, their direct and specific killing of tumor cells may release tumor-associated Ags for T cell priming; further work is required to address these questions in animal models. Critically, however, the clinical practicality of OK432 should allow early translation to testing in patients. In this regard, we have recently completed a pilot clinical study, injecting OK-DC intramyocardially into the feet of patients with advanced cancer (but not with involved lymph nodes), in which we showed efficient tracking of injected cells to draining pelvic nodes (E. West, R. Morgan, K. Scott, A. Merrick, A. Lubenko, D. Pawson, P. Selby, P. Hatfield, R. Prestwich, S. Fraser, et al., submitted for publication). We are now planning to develop this strategy to target tumor-affected nodes, where OK-DC may be able to kill and engulf malignant cells to generate systemic antitumor immunity.

In summary, this study describes a preparation of mature, myeloid-derived human DC (OK-DC) which exert specific cytotoxicity against tumor cell targets via a novel CD40/CD40L-mediated mechanism. Tumor cell killing by OK-DC may be particularly suitable for clinical application and development, as OK432 has been safely administered to cancer patients for many years (35, 36).

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Disclosures

The authors have no financial conflict of interest.

References


