CD137L-stimulated dendritic cells are more potent than conventional dendritic cells at eliciting cytotoxic T-cell responses

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Keywords: CD137, cytomegalovirus, CMV, dendritic cell, immunotherapy, T-cell responses

Abbreviations: 7-AAD, 7-aminoactinomycin D; APC, antigen-presenting cell; CD137L, CD137 ligand; cDC, classical DC; CFSE, carboxyfluorescein succinimidyl ester; CMV, cytomegalovirus; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; IFNγ, interferon γ; IL, interleukin; LPS, lipopolysaccharide; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; polyI:C, polyinosinic:polycytidylic acid; R848, Resiquimod; TLR, Toll-like receptor; TNFα, tumor necrosis factor α

Introduction

Dendritic cells (DCs) are crucial initiators of adaptive immune responses. Several heterogeneous subsets of DCs have been previously characterized, mainly distinguished on the basis of cellular origin, phenotypic profile, mechanism of pathogen recognition, biological function, and tissue localization. These include a subset of monocyte-derived DCs that arise from peripheral blood in response to inflammatory stimuli, and support tissue-resident DCs in activating naïve T cells. Such monocyte-derived DCs have been shown to be essential for mounting a protective immune response against Leishmania major in mice. Similarly, monocyte-derived DCs were found to be pivotal in generating protective responses against lepromatous leprosy. The classical protocol for generating DCs from monocyte precursors in vitro involves the step-wise differentiation of monocytes to DCs with granulocyte macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, followed by their maturation with lipopolysaccharide (LPS). Numerous inflammatory conditions can induce monocytes to differentiate to DCs, and the resultant monocyte-derived DCs exhibit unique biological activities that at least in part depends on the differentiation stimuli. Classical DCs (cDCs) are being used successfully in the clinic as a form of anticancer immunotherapy. However, the response rate of patients to DC-based therapies remains low. Thus, developing methods to generate potent DCs may translate into higher response rates and robust therapeutic benefits for cancer patients.

Two recent studies have established a novel method for generating human DCs with an enhanced immunogenic potential. CD137 ligand (CD137L) signaling has been used to derive a novel type of DCs from human peripheral blood monocytes, termed CD137L-DCs. Here, we report that CD137L-DCs induce more potent cytotoxic T-cell responses than classical DCs (cDCs). Furthermore, in exploring several DC maturation factors for their ability to enhance the potency of CD137L-DCs, we found the combination of interferon γ (IFNγ) and the mixed Toll-like receptor (TLR)7/8 agonist R848, to display the highest efficacy in potentiating the T-cell co-stimulatory activity of CD137L-DCs. Of particular importance, CD137L-DCs were found to be more efficient than cDCs in activating autologous T cells targeting the cytomegalovirus (CMV)-derived protein pp65. Specifically, CD137L-DC-stimulated T cells were found to secrete higher levels of IFNγ and killed 2–3 times more HLA-matched, pp65-pulsed target cells than T cells activated by cDCs. Finally, in addition to stimulating CD8+ T cells, CD137L-DCs efficiently activated CD4+ T cells. Taken together, these findings demonstrate the superior potency of CD137L-stimulated DCs in activating CMV-specific, autologous T cells, and encourage the further development of CD137L-DCs for antitumor immunotherapy.
recombinant CD137 or an agonistic anti-CD137L antibody induces their differentiation to DCs. These CD137L-derived DCs (CD137L-DCs) have been shown to robustly activate T cells, leading to increased cytokine secretion and strong T-cell proliferative responses in allogeneic mixed lymphocyte reactions (MLRs) as compared with cDCs. 14,15

It has previously been shown that the interaction between CD137L and CD137, which is expressed on the surface of T cells, potently enhances T-cell activation. 6-10 Concurrently, CD137L transduces a signal to APCs 20 that induces their differentiation to CD137L-DCs. 14,15 Although these studies reported promising findings on new methods to generate DCs, it remains unclear whether CD137L-DCs can either evoke improved T-cell responses or have a superior potency in an autologous setting. The present study was undertaken to address these outstanding questions. In brief, using the cytomegalovirus (CMV)-derived protein pp65 as a model antigen, we demonstrated that CD137L-DCs induce an abundant secretion of interferon γ (IFNγ) and IL-13 from autologous pp65-specific T cells, endowing them with a robust cytotoxic potential toward HLA-matched, pp65-pulsed target cells.

**Results**

**CD137L-stimulated DCs enhance the cytotoxicity of allogeneic CD8+ T cells**

Allogeneic CD8+ T cells co-cultured with CD137L-DCs have previously been shown to express higher levels of perforin than cDCs exposed to LPS and IFNγ, suggesting that CD137L-DCs may be more potent effectors than mature cDCs at inducing cytotoxic T-cell functions. 14

In order to assess this assumption, we compared co-cultures of allogeneic CD8+ T cells and CD137L-DCs or other APCs, including cDCs. Monocytes were pretreated for 7 d with either an immobilized variant of CD137 fused to a Fc fragment (CD137-Fc) to generate CD137L-DCs, or the Fc fragment alone, to generate control cells. For comparison, GM-CSF and IL-4 were used to generate immature cDCs, some of which were subsequently matured with LPS plus IFNγ for the final 18 h of culture. The efficacy of these differentially derived APCs was assayed by MLRs with allogeneic CD8+ T cells for additional proliferative responses in allogeneic MLRs as compared with cDCs. 14,15

Maturation of CD137L-DCs

Contrary to commonly used DC maturation factors (i.e., LPS and IFNγ) which give rise to CD137L-DCs that are unable to enhance CD83 expression (a DC maturation marker) and are incapable of further enhancing T-cell proliferation in allogeneic MLRs, the CD137-Fc fusion protein alone has been shown to be sufficient to generate mature and biologically potent DCs. 14 Here, we independently confirm the ability of CD137L-DCs to generate improved cytotoxic CD8+ T-cell responses as compared with mature cDCs (Fig. 1).

Nevertheless, the expression of the DC differentiation markers CD80, CD86, CCR7 and HLA-DR on CD137L-DCs could be further increased upon exposure to LPS and IFNγ 4. This implied that the potency of CD137L-DCs can potentially be enhanced by maturation, but it was unclear whether factors other than, or in addition to, LPS and IFNγ would be required. Since our ultimate goal was to explore the utility of CD137L-stimulated DCs for use in cancer immunotherapy, we decided to prospectively evaluate candidate agents that could potentially enhance the T-cell stimulatory capacity of CD137L-DCs. To this aim, we assayed a range of well-established maturation factors in different combinations (Table 1). 21-23 cDCs generated by GM-CSF plus IL-4 and matured in the presence IL-1β, IL-6, tumor necrosis factor α (TNFα) and prostaglandin E1 (PGE1), a combination of agents that in Table 1 we referred to as cocktail C2, are currently considered the “gold standard” in immunotherapy and were used as the benchmark for comparing the activity of various CD137L-DC-maturing candidates. 21

In brief, CD137L-DCs, generated as previously described from peripheral monocytes by a 6 d culture on immobilized CD137-Fc, were treated for 18 h with 7 different maturation cocktails. In basal conditions, the DC maturation marker CD83 was expressed on 39% of untreated (C0) CD137L-DCs, with a mean-fluorescence intensity (MFI) of 66 (arbitrary units). The expression of CD83 was significantly enhanced by treatment with cocktails C3 (64% CD83+ CD137L-DCs, MFI = 108) and C4 (58% CD83+ CD137L-DCs, MFI = 100). In contrast, Cl only moderately increased (58% CD83+ CD137L-DCs, MFI = 75) whereas cocktails C2, C5, C6, and C7 had no effect on, CD83 expression (Fig. 2A). A similar profile was observed for the expression of the co-stimulatory molecule CD86, such that 43% of untreated CD137L-DCs expressed CD86 (MFI = 103), while exposure to cocktails C1, C3, or C4 led to a considerable upregulation of CD86 (69% CD86+ CD137L-DCs, MFI = 178; 57% CD86+ CD137L-DCs, MFI = 165; 53% CD86+ CD137L-DCs, MFI = 139, respectively). Finally, only a slight increase in...
CD86 expression was observed in response to C2 (55% CD86+ CD137L-DCs, MFI = 122) (Fig. 2A).

The activation of antigen-specific autologous CD4+ T cells relies on the presentation of antigenic epitopes in complex with MHC class II molecules. Thus, finding that CD137L-DCs express relatively low levels of MHC class II molecules was surprising, as these cells still possess the ability to activate CD4+ T cells. With this in mind, we hypothesized that an increase in MHC class II expression on the surface of CD137L-DCs could potentially boost their ability to activate CD4+ T cells. We tested this hypothesis and found that the treatment of CD137L-DCs with cocktails C1, C3, or C4 markedly increased the expression of HLA-DR on the cell surface (70% HLA-DR+ CD137L-DCs, MFI = 116; 79% HLA-DR+ CD137L-DCs, MFI = 122; 77% HLA-DR+ CD137L-DCs, MFI = 117, respectively) as compared with control conditions (47% HLA-DR+ CD137L-DCs, MFI = 87) (Fig. 2A).

Based on the expression of CD83, CD86, and HLA-DR, we deduced that cocktails C1, C3, and C4 contain factors that promote the maturation of CD137L-DCs. To verify whether these maturation cocktails not only enhance the expression of T-cell activating molecules but also enhance the biological potency of CD137L-DCs, we next assessed the ability of differentially matured CD137L-DCs to induce T-cell proliferation in allogeneic MLRs. To this aim, C1-, C3-, or C4-matured CD137L-DCs were co-cultured with CFSE-stained allogeneic T cells for 5 d and T-cell proliferation was determined by CFSE dilution. In the absence of CD137L-DCs, there was virtually no T-cell proliferation (2% proliferation, MFI = 1877). As shown in Figure 2B, untreated (C0) CD137L-DCs induced the proliferation of 64% of T cells (MFI = 733), while C1-treated CD137L-DCs, despite expressing comparatively higher levels of CD83, CD86, and HLA-DR, were unable to drive T-cell proliferation to further levels (68% proliferation, MFI = 665). This confirmed the previous observation that the administration of LPS and IFNγ to CD137L-DCs has no substantial effect on their ability to induce allogeneic T-cell proliferation.

Enhanced T-cell proliferation could be documented in the presence of CD137L-DCs treated with either C3 (81% proliferation, MFI = 444) or C4 (80% proliferation, MFI = 463), evincing that these maturation cocktails give rise to CD137L-DCs that
Figure 2. Maturation of CD137L-derived dendritic cells. (A–C) Monocytes were treated with immobilized CD137-Fc for 6 d and then exposed to the indicated maturation cocktails (see also Table 1) for 18 h, followed by a phenotypic and functional characterization. (A) Cells were immunostained for the CD83, CD86, and HLA-DR cell-surface expression and analyzed by fluorescence cytometry. Unshaded and gray histograms represent the unstained (control) and stained samples, respectively. Values associated with each histogram indicate the percentages of positive cells and mean fluorescence intensity (MFI). Bar charts on the right report the mean percentage of positive cells and mean MFI ± SD of data acquired from 2 different donors. (B) Upon 18 h of treatment with cocktails C0, C1, C3, or C4, CD137L-derived dendritic cells (CD137L-DCs) were co-cultured for additional 5 d with carboxyfluorescein succinimidyl ester (CFSE)-stained allogeneic T cells at a ratio of 1:10. T-cell proliferation was then quantified by fluorescence cytometry based on CFSE dilution. Numbers in the histograms and bar charts represent percentages of proliferating cells and CFSE MFI. (C) Supernatants from the co-cultures of mature CD137L-DCs and allogeneic T cells were harvested and the levels of interferon γ (IFNγ) were quantified by ELISA. Means ± SD of triplicate measurements are depicted. **P < 0.01 (two-tailed, unpaired Student t test). This experiment has been performed independently twice, yielding comparable results. PHA, phytohemagglutinin; Rst, resting.
are more potent T-cell activators than untreated CD137L-DCs ($P < 0.01$; Figure 2B).

Next, as an additional measure of CD137L-DC potency and as a first indication of their ability to induce T$_{\gamma}1$ response, we determined the levels of IFN$\gamma$ secreted in the supernatants of differentially matured CD137L-DCs co-cultured with T cells. The levels of IFN$\gamma$ detected in supernatants of co-cultures containing C3-exposed CD137L-DCs were 5 times higher (1265 ± 243 pg/mL) than those of co-cultures involving control (C0) CD137L-DCs (241 ± 74 pg/mL). Similarly, IFN$\gamma$ levels were 3 times higher (720 ± 241 pg/mL) in the supernatants of T cells exposed to C4-treated CD137L-DCs than in control co-cultures. In sharp contrast, there was no significant increase in IFN$\gamma$ secretion by T cells co-cultured with C1-treated CD137L-DCs (287 ± 40 pg/mL), (Figure 2C). Taken together, these findings suggest that the secretion of IFN$\gamma$ by T cells correlates with their proliferation rate, and that although cocktails C1, C3, and C4 enhance the expression of DC activation marker, only C3 and C4 also boost the ability of CD137L-DCs to stimulate T cells.

The maturation cocktails C3 and C4 differ by the presence of polyinosinic: polycytidylic acid (poly I:C), which is only included in the latter (Table 1). Poly I:C is a Toll-like receptor (TLR) 3 agonist capable of inducing the production of IL-12p70 in DCs. Despite the presence of poly I:C, however, no IL-12p70 could be detected in the supernatants of C4-treated CD137L-DCs, nor in any of the other conditions (data not shown). Since, poly I:C apparently did not functionally contribute to CD137L-DC maturation, we surmise that the remaining components, i.e., those in common with cocktail C3, are the key mediators of this process.

A TLR7/8 agonist and IFN$\gamma$ are essential and sufficient to functionally mature CD137L-DCs

We identified cocktail C3, consisting of IL-1$\beta$, TNF$\alpha$, IFN$\gamma$, PGE$_2$, and R848 (a mixed TLR7/8 agonist) as the most potent and suitable cocktail for maturing CD137L-DCs. Since reverse signaling through activated CD137L on the surface of peripheral monocytes has been shown to induce the secretion of inflammatory cytokines including TNF$\alpha$ and IL-1$\beta$, we reasoned that these potentially autocrine signaling components of C3 could be omitted without deleterious effect. Our aim was to systematically eliminate superfluous components of C3 in order to define a minimal maturation cocktail exhibiting high potency, to facilitate the future clinical use of CD137L-DCs (Table 2).

As expected, the absence of IL-1$\beta$ and TNF$\alpha$ in the C3-derived cocktail C3A made no difference in the induction of the differentiation and activation markers CD83, CD86, and HLA-DR, as compared with the complete C3 cocktail (Fig. 3A). Also, T cells co-cultured with CD137L-DCs matured by either C3 or C3A exhibited no apparent difference in their proliferation rates and IFN$\gamma$ secretion ability (Fig. 3B and 3C). Altogether, these results suggest that the autocrine secretion of TNF$\alpha$ and IL-1$\beta$ by CD137L-DCs is sufficient to promote their maturation as driven by IFN$\gamma$, R848 and PGE2 (maturation cocktail C3A).

IFN$\gamma$ alone (maturation cocktail C3C) was sufficient to substantially increase the expression of the phenotypic markers CD83, CD86, and HLA-DR on the surface of CD137L-DCs (Fig. 3A). Furthermore, C3C also enhanced T-cell proliferation to levels resembling those induced by exogenous application of complete C3 (Fig. 3B). Nevertheless, the levels of IFN$\gamma$ detected in MLRs were not markedly different when untreated (C0) or C3C-treated CD137L-DCs were employed (Fig. 3C), suggesting that C3C only partially phenocopies C3. A salient observation was that the TLR7/8 ligand R848 alone (maturation cocktail C3D), was not able to increase the expression of the co-stimulatory molecules on the surface of CD137L-DCs, yet promoted their maturation to sufficient extent to enhance both T-cell proliferation and IFN$\gamma$ secretion (Fig. 3). As it stands, R848 plus IFN$\gamma$ (maturation cocktail C3B) were as potent as the complete C3 cocktail in its ability to drive the functional maturation of CD137L-DCs (Fig. 3). PGE$_2$ was dispensable for this effect, since C3A-treated CD137L-DCs were functionally identical to their C3B-treated counterparts. This demonstrates that R848 and IFN$\gamma$ are essential and sufficient for the complete functional maturation of CD137L-DCs. Additional optimization steps were undertaken to determine the minimum effective concentrations of these agents, revealing that 1 µg/mL R848 coupled to 50 ng/mL IFN$\gamma$ (maturation C3B.1 in Figure S1) exert optimal effects in this setting (Fig. S1).

**Phenotypic characterization of cDCs and CD137L-DCs matured in the presence of R848 and IFN$\gamma$**

cDCs and CD137L-DCs were generated from autologous monocytes for 6 d and matured for a further 18 h using either TNF$\alpha$, IL-1$\beta$, IL-6, and PGE$_2$ (to produce mature cDCs) or R848 and IFN$\gamma$ (in the case of CD137L-DCs). At this time point, these 2 types of DC already differed morphologically. Upon maturation, cDCs developed many small dendrites and remained non-adherent. In contrast, mature CD137L-DCs exhibited a predilection for a spindle-like shape and an increased adherence as compared with untreated CD137L-DCs, resulting in a distinctively flattened morphology (Fig. 4A). More importantly, the expression levels of surface maturation markers were significantly different in mature cDCs and CD137L-DCs as compared with their respective immature counterparts. Upon maturation, the levels of CD14 were decreased, while the expression of CD83,
Figure 3. Interferon-γ and R848 are sufficient to induce the functional maturation of CD137L-derived dendritic cells. (A–C) Monocytes were treated with immobilized CD137-Fc for 6 d followed by exposure to the indicated maturation cocktails (see also Table 2) for 18 h to obtain mature CD137L-derived dendritic cells (CD137L-DCs), which were then subjected to phenotypic and functional characterization. (A) Cells were immunostained for CD83, CD86, and HLA-DR expression and analyzed by fluorescence cytometry. Unshaded and gray histograms represent the unstained (control) and stained samples, respectively. Values associated with each histogram indicate the percentages of positive cells and mean fluorescence intensity (MFI). Bar charts on the right report the mean percentage of positive cells and mean MFI ± SD of data acquired from 2 different donors. (B) Upon 18 h of treatment with cocktails C3, C3A, C3B, C3C, and C3D the CD137L-DCs were co-cultured for additional 5 d with carboxyfluorescein succinimidyl ester (CFSE)-stained allogeneic T cells at a ratio of 1:10. T-cell proliferation was then quantified by fluorescence cytometry based on CFSE dilution. Numbers in the histograms and bar charts represent percentages of proliferating cells and CFSE MFI. (C) Supernatants from the co-cultures of mature CD137L-DCs and allogeneic T cells were harvested and the levels of interferon-γ (IFN-γ) were quantified by ELISA. Means ± SD of triplicate measurements are depicted. *P < 0.05, **P < 0.01 (two-tailed, unpaired Student t test). This experiment has been performed independently twice, yielding with comparable results. PHA, phytohemagglutinin; Rst, resting.
similar pattern was observed with regard to the priming of CD4+ T cells by these distinct DC subsets (Fig. 5A). A magnification of 63 ×. Scale bar = 20 µm. (B) DC subsets were harvested and the expression of CD14, CD83, CD86, and HLA-DR was determined by flow cytometry upon staining with specific antibodies. These data are representative of at least 3 independent experiments yielding comparable results. (C) Viable DC numbers in each group were quantified by trypan blue exclusion assays. Bar charts represent cell count per million cells. Means ± SD of triplicate counts are depicted. These data are representative of 2–3 independent experiments yielding comparable results.

**Figure 4.** Generation and maturation of classical and CD137L-derived dendritic cells. (A–C) Monocytes were treated with immobilized CD137-Fc for 7 d to generate CD137L-derived dendritic cells (CD137L-DCs), or with 80 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) plus 100 ng/mL interleukin (IL)-4 to generate classical dendritic cells (cDCs). For the last 18 h of culture, tumor necrosis factor α (TNFα) plus IL-1β plus IL-6 plus prostaglandin E_2 (PGE_2) were used to promote the maturation of cDCs, while 50 ng/mL interferon γ (IFNγ) plus 1 µg/mL R848 were used for the maturation of CD137L-DCs. (A) Brightfield microscopy images were taken on day 7 at a magnification of 63 × . Scale bar = 20 µm. (B) DC subsets were harvested and the expression of CD14, CD83, CD86, and HLA-DR was determined by flow cytometry upon staining with specific antibodies. These data are representative of at least 3 independent experiments yielding comparable results. (C) Viable DC numbers in each group were quantified by trypan blue exclusion assays. Bar charts represent cell count per million cells. Means ± SD of triplicate counts are depicted. These data are representative of 2–3 independent experiments yielding comparable results.

CD86, and HLA-DR was augmented on both cDCs and CD137L-DCs (Fig. 4B). However, we did not observe a complete absence of CD14 on the surface of mature DCs, a finding that is consistent with those from an independent study. Taken together, these data confirmed that the maturation cocktails employed for each DC type were effective.

Aside from the biological potency of DCs, another important consideration involves the amounts of DCs that can be generated with a given differentiation and maturation protocol. No significant difference could be detected between the absolute numbers of mature cDCs and mature CD137L-DCs produced as described above. In each case, between 55–70% of the monocytes differentiated into mature DCs (Fig. 4C). Hence, CD137L signaling in monocytes can promote the generation of DCs while maintaining a viability that is comparable to that ensured by classical differentiation protocols.

**Mature CD137L-DCs potently activate autologous, antigen-specific T cells**

So far, all the assessments involving CD137L-DCs had been performed in allo- or xenogeneic systems. Therefore, we next investigated the capability of CD137L-DCs, as compared with cDCs, to initiate an antigen-specific response in an autologous setting. To this aim, we utilized a pool of peptides derived from the cytomegalovirus (CMV)-encoded protein pp65 as antigenic stimulus, and generated a pp65-specific T-cell line from a pp65+/HLA-A2+ donor. After 9–10 d, peripheral blood mononuclear cell (PBMCs) treated with pp65-derived peptides were predominantly T cells, as determined by CD3 expression (98% purity). Within this population, 79% of T cells were CD8+ while 19% were CD4+ T cells (data not shown).

To comparatively assess the antigen-presentation capacity of the cDCs and CD137L-DCs, we first pulsed these cells with pp65-derived peptides, and then co-cultured them with autologous pp65-specific T cells. We next determined IFNγ expression among T cells as a measure of their activation status after 18 h. No T-cell activation occurred in the absence of pp65-derived peptides (data not shown). Conversely, when CD8+ T cells were exposed to mature CD137L-DCs pulsed with pp65-derived peptides, 48% of them were found to express IFNγ. All other DC preparations were less active than mature CD137L-DCs as only 24.1%, 28.6%, or 17.8% of the CD8+ T cells expressed IFNγ in response to priming by immature cDCs, mature cDCs or untreated CD137L-DCs, respectively (Fig. 5A, top panels). A similar pattern was observed with regard to the priming of CD4+ T cells by these distinct DC subsets (Fig. 5A, bottom panels).

Collated data from 4 independent experiments confirmed that mature CD137L-DCs are indeed significantly more potent activators of CD8+ and CD4+ T cells than the other types of DCs tested in this setting (Fig. 5B).

The same conclusion was reached by measuring IFNγ secretion after 5 days of co-culture. Although pp65-pulsed immature and mature cDCs were able to induce considerable IFNγ secretion by T cells (5.6 ± 0.2 ng/mL and 7.2 ± 0.2 ng/mL, respectively), untreated CD137L-DCs were significantly more effective, inducing the release of more than twice as much IFNγ (14.6 ± 0.6 ng/mL). Mature CD137L-DCs were comparatively the most efficient, stimulating T cells to secrete the highest amount of IFNγ (25.7 ± 1 ng/mL) (Fig. 6A).

Often the levels of Th1 cytokines inversely correlate with those of Th2 cytokines. Therefore, it was surprising to detect a profile of IL-13 secretion in response to the various DC subsets that was similar to that observed for IFNγ. In general, the concentrations IL-13 were below 2000 pg/mL in the co-cultures involving unpulsed DC subsets, with the exception of those containing unpulsed mature CD137L-DCs, in which we found 2997 pg/mL of IL-13 (Fig. 6B).
Upon exposure to immature or mature cDCs pulsed with pp65-derived peptides, T cells released 2612 ± 3 pg/mL and 2684 ± 24 pg/mL IL-13, respectively. The levels of IL-13 were even higher in the supernatants of T cells co-cultured with peptide-pulsed, untreated CD137L-DCs (4438 ± 24 pg/mL), and peptide-pulsed, mature CD137L-DCs (5428 ± 35 pg/mL) (Fig. 6B).

CD137L-DCs promote a superior killing activity in antigen-specific autologous T cells

In order to assess the cytotoxicity of T cells re-stimulated by distinct DC subsets, we next sought to determine their lytic activity against peptide-pulsed target cells. Donor monocytes and a target lymphoblastoid B-cell line (clone CM371) were confirmed to be HLA-matched, as they both expressed HLA-A2 (Fig. 7A).

A dose-dependent killing of target cells was observed at increasing effector/target (E:T) cell ratios in all conditions (Fig. 7B). Surprisingly, the maturation of cDCs and CD137L-DCs had no or little effect, respectively, on their ability to trigger the cytotoxic functions of T cells (Fig. 7B). In the absence of pp65-derived peptides, T cells re-stimulated by either immature or mature cDCs had no cytotoxic activity, whereas T cells re-stimulated by CD137L-DCs, be them mature or not, killed 4–20% of target cells. Furthermore, regardless of the nature of re-stimulating DCs, pulsing target cells with pp65-derived peptides enhanced their capacity to induce T-cell killing.

Of particular importance, the T cells re-stimulated by CD137L-DCs were at least twice as effective as T cells re-stimulated by cDCs, as measured by the specific lysis of target cells at various E:T cell ratios. T cells re-stimulated by untreated CD137L-DCs killed 49%, 63%, and 75%, of target cells at E:T cell ratios of 5:1, 10:1, and 20:1, respectively, while 16%, 23%, and 35% of target cells were killed by T cells primed by mature cDCs. The efficacy of mature CD137L-DCs was quite similar to that of untreated CD137L-DCs (Fig. 7B). These data demonstrate that autologous, antigen-specific T cells that have been re-stimulated by CD137L-DCs, regardless of their maturation status, are functionally superior to T cells re-stimulated by cDCs.

Discussion

DCs are the key orchestrators of antigen-specific T-cell responses and thus DC-based vaccines are attractive tools for the implementation of anticancer immunotherapy. However, DC vaccination has been ineffective to date, calling for technical improvements in the protocols whereby DCs are prepared, loaded with antigens and endowed with immunostimulatory properties.

It has previously been shown that DCs differentiated upon CD137L engagement are more effective as compared with classically-derived DCs in stimulating the proliferation of allogeneic T cells.14,15 Moreover, the high expression levels of perforin and IFNγ exhibited by CD8+ T cells exposed to CD137L-DCs suggest that these DCs may be more potent than cDCs in the induction of cytotoxic T-cell activity.14 These promising activities of CD137L-DCs prompted us to investigate whether these APCs may also be functionally superior in a more challenging and clinically-relevant, antigen-specific autologous setting.

Indeed, our findings reveal that CD137L-DC-stimulated T cells exert a superior cytotoxicity toward target cells. In contrast to what is observed for cDCs, the maturation of CD137L-DCs upon the exogenous application of LPS and IFNγ did not further enhance their ability to promote T-cell cytotoxicity. This suggests that either CD137L-DCs are already fully mature, or that a different cytokine cocktail may be required to enhance their biological activity. Of note, low levels of MHC class II molecules are expressed on the surface of CD137L-DCs, suggesting that they have not yet
reached their full potential as APCs, especially relative to the capacity to activate CD4+ T cells. The treatment of CD137L-DCs with R848 and IFNγ was sufficient to promote the maturation of these cells, as evidenced by the increased expression of typical DC maturation markers such as CD83, CD86, and HLA-DR, coupled and the reduction of CD14. Importantly, T cells activated by CD137L-DCs exposed to R848 and IFNγ underwent a greater proliferative response than T cells stimulated by untreated CD137L-DCs.

In spite of the fact that CD137L-DCs exposed to LPS plus IFNγ and R848 plus IFNγ expressed similar levels of maturation markers, the latter treatment induced a comparatively more intense T-cell stimulation. Such an enhanced co-stimulatory activity of CD137L-DCs treated with R848 and IFNγ is presumably due to the TLR7/8 agonist. Chamberlain et al. reported that the ligation of TLR7 by single-stranded RNA leads to TNFα secretion by monocytes upon the induction of the NF-kB and phosphoinositide-3-kinase (PI3K) pathways. Although the engagement of CD137L on the surface of monocytes also induces basal TNFα production, the combination of CD137L, R848, and IFNγ may lead to synergistic effects on TNFα secretion. Extracellular TNFα can interact with CD137L-DCs in an autocrine or paracrine manner, thereby favoring their maturation. An alternative mechanism underlying the synergism between R848 and IFNγ might relate to the fact that IFNγ increases TLR8 expression in human cDCs. Considering that R848 is a TLR7 agonist, the upregulation of TLR8 by IFNγ would naturally increase the sensitivity of CD137L-DCs to R848.

In order to further evaluate the immunotherapeutic potential of CD137L-DCs, we characterized their immunostimulatory function in an autologous system based on the CMV-derived antigen pp65. In this setting, mature CD137L-DCs robustly stimulated the activation of pp65-specific CD8+ and CD4+ T cells (as measured in terms of IFNγ production) even within a brief period (18 h) of re-stimulation. In contrast, the other types of DC tested in this study were not able to induce comparable levels of IFNγ expression detected in both T-cell populations. More recently, the discovery of cytotoxic CD4+ T cells has cast doubt on the dogma that CD4+ T cells merely operate as helper for CD8+ cytotoxic T lymphocytes. Cytotoxic CD4+ T cells were observed in response to infection or vaccination against viruses such as herpes virus, West-Nile virus, and dengue virus. Likewise, melanoma-reactive CD4+ T cells have been shown to exert a cytotoxic activity, leading to tumor rejection via MHC class II-restricted interactions.
IL-13, a prototypic TH2 cytokine, was found to be secreted by DCs. CD137L, TNFR1, and TLR4 upon binding to CD137 utilizes TNFR1 to transduce a reverse signaling complex that is activated by recombinant CD137 or anti-CD137L (and perhaps other TLRs) might form a large multimeric signaling complex. These data are representative of at least 2 independent experiments yielding comparable results.

In conclusion, this study confirms and extends promising data from earlier studies by demonstrating the superior immunological potency of CD137L-DCs in an antigen-specific, autologous human system based on a viral model antigen. A further advance and a logical next step to develop CD137L-DCs for clinical use would be testing whether CD137L-DCs can also induce potent immune responses against human tumors.

Materials and Methods

Recombinant proteins, peptides, and antibodies

The human CD137-Fc chimera was purchased from R&D Systems. The human IgG Fc was purchased from Millipore. GM-CSF, IL-4, TNFα, IFNγ, IL-1β, IL-6, IL-7, and IL-15 were purchased from Peprotech while IL-2 was from R&D Systems. GM-CSF, IL-4, IL-6, IL-7, and IL-15 were purchased from Peprotech while IL-2 was from R&D Systems. PGE2 and LPS were purchased from Sigma. R848, polyI:C, TNFα, IL-1β, IL-6, IL-7, and IL-15 were purchased from Peprotech while IL-2 was from R&D Systems. PGE2 and LPS were purchased from Sigma. R848, polyI:C, and recombinant flagellin were purchased from Invitrogen. The CMV pp65 PepTivator peptide pool was purchased from Miltenyi Biotec. Peptide pools were composed of 15-mer peptides with 11 amino acid overlaps, covering the complete sequence of the pp65 protein encoded by the CMV strain AD169 (Swiss-Prot Acc. no. P06725). Antibodies against human HLA-DR (clone
HLA-DR and their ability to induce T-cell activation, as measured for the expression of CD14, CD83, CD86, and IFNγ (clone 4S.B3), were purchased from eBioscience. Antibodies against human CD8 (clone RPA-T8) and HLA-A2 (clone BB7.2) were purchased from BD PharMingen.

**Cells and cell culture**

Human PBMCs were prepared by Ficoll-Paque (GE Healthcare) density gradient centrifugation. Monocytes were isolated from PBMCs by negative selection using the Monocyte Isolation Kit II (Miltenyi Biotec) and MACS magnetic separation, according to manufacturer’s instructions. The isolation of total T cells was performed via positive selection with the MACS system and anti-CD3 microbeads (Miltenyi Biotec). To acquire the CD8+ T-cell fraction, anti-CD8 microbeads and MACS (Miltenyi Biotec) were used for positive selection. Isolated monocytes and T cells exhibited a purity > 95% based on antigenic phenotyping by CD14, CD3 and CD8 staining, respectively. To generate CD137L-DCs, monocytes were seeded onto polystyrene dishes (Becton Dickinson) pre-coated with 10 µg/mL CD137-Fc, in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 µg/mL streptomycin and 50 µg/mL CD137-Fc, in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 50 µg/mL streptomycin and 50 µg/mL penicillin for 7 d (R10 PS medium). Maturation stimuli were administered during the last 18 h of incubation. Classical DCs were generated by culturing monocytes in R10 PS medium in the presence of 80 ng/mL GM-CSF and 100 ng/mL IL-4. Fresh media was added on day 3. For the last 18 h, cells were matured with either 1 µg/mL LPS + 50 ng/mL IFNγ or with 10 ng/mL TNFα, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 1 µg/mL PGE2. HLA-A2* Epstein-barr virus (EBV)-transformed B lymphoblasts (clone CM 371) were a gift from Dr Laura Rivino (National University of Singapore) and were maintained in R10 PS media. K562 cells were obtained from ATCC and were maintained in Iscove’s modified dultbecco’s medium (IMDM) supplemented with 10% FBS.

**Maturation of CD137L-DCs and cDCs**

Monocytes were seeded onto CD137-Fc-coated plates and cultured until day 6, when various cytokine cocktails (Table 1) were added to the culture for 18 h to induce maturation. Cells were then analyzed for the expression of CD14, CD83, CD86, and IFNγ production. For autologous experiments, CD137L-DCs were generated using 1 µg/mL R848 plus 50 ng/mL IFNγ or with 10 ng/mL TNFα, 10 ng/mL IL-1β, 10 ng/mL IL-6, and 1 µg/mL PGE2, for 18 h.

**Allogeneic MLRs**

Allogeneic T cells were isolated and stained with carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes, Invitrogen) followed by co-culture with CD137L-DCs or cDCs at a ratio of 10:1. Cultures were maintained in R10 PS media for 5 d and cell proliferation was determined by CFSE dilution on fluorescence cytometry. Supernatants from co-cultures were collected and IFNγ production was determined by ELISA.

**Cell quantification by trypan blue staining**

DCs were harvested upon trypsinization, washed and resuspended in PBS. Cells were mixed at 1:1 ratio with 0.4% trypan blue solution (Sigma T8154) and counted on a hemocytometer using brightfield microscopy.

**Generation of a pp65-specific T-cell line**

PBMCs were suspended at 10⁶ cells/mL in AIM V medium (Invitrogen) supplemented with 2% human AB serum (Sigma-Aldrich), 50 µg/mL streptomycin sulfate and 10 µg/mL gentamicin sulfate (AIM V 2Ab medium). PBMCs were then stimulated with the PepTivator peptide pool (0.1 µg/mL per peptide; Miltenyi Biotec) for 9–10 d in the presence of 10 U/mL IL-2 added after 24 h. On day 9–10, > 95% of cells were CD3 positive.

**Re-stimulation of pp65-specific T cells**

CD137L-DCs or cDCs were harvested and co-cultured with autologous pp65-specific T cells at a ratio of 1:10 in the presence of pp65-derived peptides (at either 1 µg/mL per peptide, for 18 h, or 0.1 µg/mL per peptide, for 5 d). As a negative control, no peptides were added to the culture.

**Cytotoxicity assays**

CD8+ T cells were pre-activated in an allogeneic MLR with pre-treated monocytes or DCs at a ratio of 10:1. After 5 d of co-culture, these CD8+ T cells were used as effector cells against K562 target cells. CD8+ T cells were thus harvested and co-cultured with K562 cells labeled with CFSE at E:T cell ratios of 1:1, 10:1, and 20:1. Upon overnight co-culture, total cells were harvested and the Annexin V Apoptosis Detection Kit (BD Biosciences) was used to determine the percentage of apoptotic cells among the CFSE-positive population by flow cytometry. For pp65-specific cytotoxicity assays, pp65-specific T cells were re-stimulated with DCs pulsed with pp65-derived peptides (0.1 µg/mL per peptide) at a ratio of 10:1 for 5 d. Cells were then harvested and stained for CD3 expression, and found to be > 95% positive for this T-cell marker. EBV-transformed B lymphoblasts were loaded with the DELFIA® BATDA Reagent (Perkin Elmer) and cytotoxicity assays were performed according to manufacturer’s instruction. In brief, DELFIA® BATDA-loaded target cells were pulsed with pp65-derived peptides (1 µg/mL per peptide) for 1 h at 37° C. Unpulsed target cells were employed as a control. Cells were washed and resuspended in AIM V medium supplemented with 2% human serum, 50 µg/mL streptomycin sulfate, 10 µg/mL gentamicin sulfate, 2 mM probenecid and 50 µM β-mercaptoethanol and then seeded into a 96-well plate at 10⁴ cells per well. pp65-specific T cells were then co-cultured with target cells at E:T ratios of 5:1, 10:1, and 20:1 for 3 h at 37° C. Europium solution was added to the harvested supernatants for 15 min and fluorescence was measured using a time-resolved fluorometer.

**Fluorescence cytometry**

In order to determine the expression of surface markers, cells were blocked with FcR blocking reagent (Miltenyi Biotec) and were then stained with specific antibodies in PBS containing 0.5% bovine serum albumin (BSA) and 0.1% sodium azide (staining buffer) for 45 min at 4° C in the dark. Cells were washed twice and resuspended in staining buffer. Unstained samples were used as a control. For intracellular cytokine staining, cells were cultured in the presence of 2 µg/mL brefeldin A (eBioscience) overnight and stained with anti-CD8 or anti-CD4 antibody followed by cell fixation using BD Cytofix/Cytoperm.
fixation/permeabilization kit (BD Biosciences). Intracellular staining of IFNγ was then performed following the manufacturer’s instruction. Fluorescence cytomtery was performed on a CyAn ADP Analyzer (Dako) or BD LSR Fortessa cell analyzer (BD Bioscience). Data were analyzed using Summit or FlowJo data acquisition and analysis software.

ELISA

The concentrations of IFNγ and IL-13 in cell supernatants were determined by the Human IFNγ and Human IL-13 ELISA Duoset (R&D Systems), respectively, according to manufacturer’s instructions. All measurements were performed in triplicates.

Photographs

Cell morphology was photographed using a Zeiss Axiovert 40 inverted microscope (Zeiss) and a Canon PowerShot G6 digital camera.

Statistical analyses

Statistical significance was determined by two-tailed, unequal Student t tests unless otherwise specified.

Disclosue of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This study was supported by grant (T1–2012 Oct-14) from the Academic Research Foundation, Singapore.

Supplemental Materials

Supplemental materials may be found here: http://www.landesbioscience.com/journals/oncoimmunology/article/26859/
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