

Membranes, molecules and biophysics: enhancing monocyte derived dendritic cell (MDDC) immunogenicity for improved anti-cancer therapy

Cyril Rauch, Hiba Ibrahim and Neil Foster*

*Correspondence: n.foster@nottingham.ac.uk

School of Veterinary Medicine and Science, University of Nottingham, UK.

Abstract

Despite great medical advancement in the treatment of cancer, cancer remains a disease of global significance. Chemotherapeutics can be very expensive and drain medical resources at a national level and in some cases the cost of treatment is so great that it prohibits their use by local health authorities. Drug resistance is also a major limiting factor to the successful treatment of cancer with many patients initially responding well but then becoming refractory to treatment with the same drug and in some case may become multi-drug resistant. The immune system is known to be important in the prevention of tumors by eliminating pre-cancerous or cancerous cells. This concept of immune surveillance has largely been superseded by the concept of immunoeediting whereby the immune system imposes a selective pressure on tumor cells which may either control tumor growth or inadvertently select for tumor cells which have evolved to escape the immune response and which may induce tumor development. Stimulation of the immune system by vaccination offers many benefits in the treatment of cancer. It is highly cost effective and vaccines can be manipulated to include multi-antigens which in some cases may overcome equilibrium (and selective pressure) while also preventing the establishment of reactivated cancer cells, since cancer antigen-specific memory would be induced following the initial vaccination/booster phase. To date studies using vaccination as a treatment for cancer have been a little disappointing, probably due to insufficient level of immunogenicity. In this review we will discuss methods of manipulation of the immune system to increase the anti-cancer activity of dendritic cells *in vivo* and how monocyte derived dendritic cells may be manipulated *ex vivo* to provide more robust, patient-specific treatments.

Keywords: Dendritic cell, MDDC, vaccine, immunogenicity

Review

The global impact of cancer

Although new chemotherapeutics and greater diagnostic modalities have been developed, cancer remains a disease of global significance. There are around 12.7 million cases of cancer globally per year and this is estimated to increase to 26 million per year by 2030 [1]. In 2010, 324,579 cases of Cancer were diagnosed in the UK and 157,275 deaths due to cancer were recorded [2]. The enormous cost of the disease suffered by governments and healthcare providers undoubtedly negatively impacts on other areas of health spending and in many cases newly developed drugs are too expensive for health authorities to purchase. The cost has been estimated to increase from £30000 to £40000 per patient per year between 2010 and 2021 [3]. Furthermore, there is significant emerging resistance in many cases to chemotherapy [2], and a wealth of research has now been published which has shown the various mechanisms, detection and amelioration of resistance [4-9] by tumours to chemotherapeutic drugs. In the developing world many cancers remain untreated, due simply to the financial burden of current treatments, even more significant when

considering that current global estimations of 12.7 million new cases of cancer per year are predicted to increase to 26 million per year by 2030 [1]. Less expensive and effective new treatments would, therefore, be of obvious benefit in both the developed and developing world.

The concept of 'immuno-surveillance' in cancer biology is not new, and suggests that the immune response largely keeps in check the development of tumours. The development of efficient cancer vaccines therefore would appear to be a rational and cost effective therapeutic approach to the treatment of cancer.

The role of dendritic cells in adaptive immunity and vaccination

Antigen presenting cells (APCs) bridge the gap between innate and adaptive immunity, since APCs are innate immune cells which present antigens to the adaptive immune system (T and B lymphocytes). All APCs express major histocompatibility complex II (MHCII) as well as MHCI on their cell membranes, either constitutively or when activated. Antigen presentation to lymphocytes also requires a second (co-stimulatory signal) provided by molecules such as CD40, CD80 and CD86 which

engage with their counterparts (CD40 ligand and CD28) on the surface of T lymphocytes. The extent to which these molecules are expressed on the surface of APCs is paramount to the induction of a robust lymphocyte response to antigen. Another factor which may influence the lymphocyte response is the number of lymphocytes, with cognate T cell receptors, which can engage antigen on the APC surface and the most effective APCs have large surface areas due to membrane folding or cellular extensions (pseudopodia).

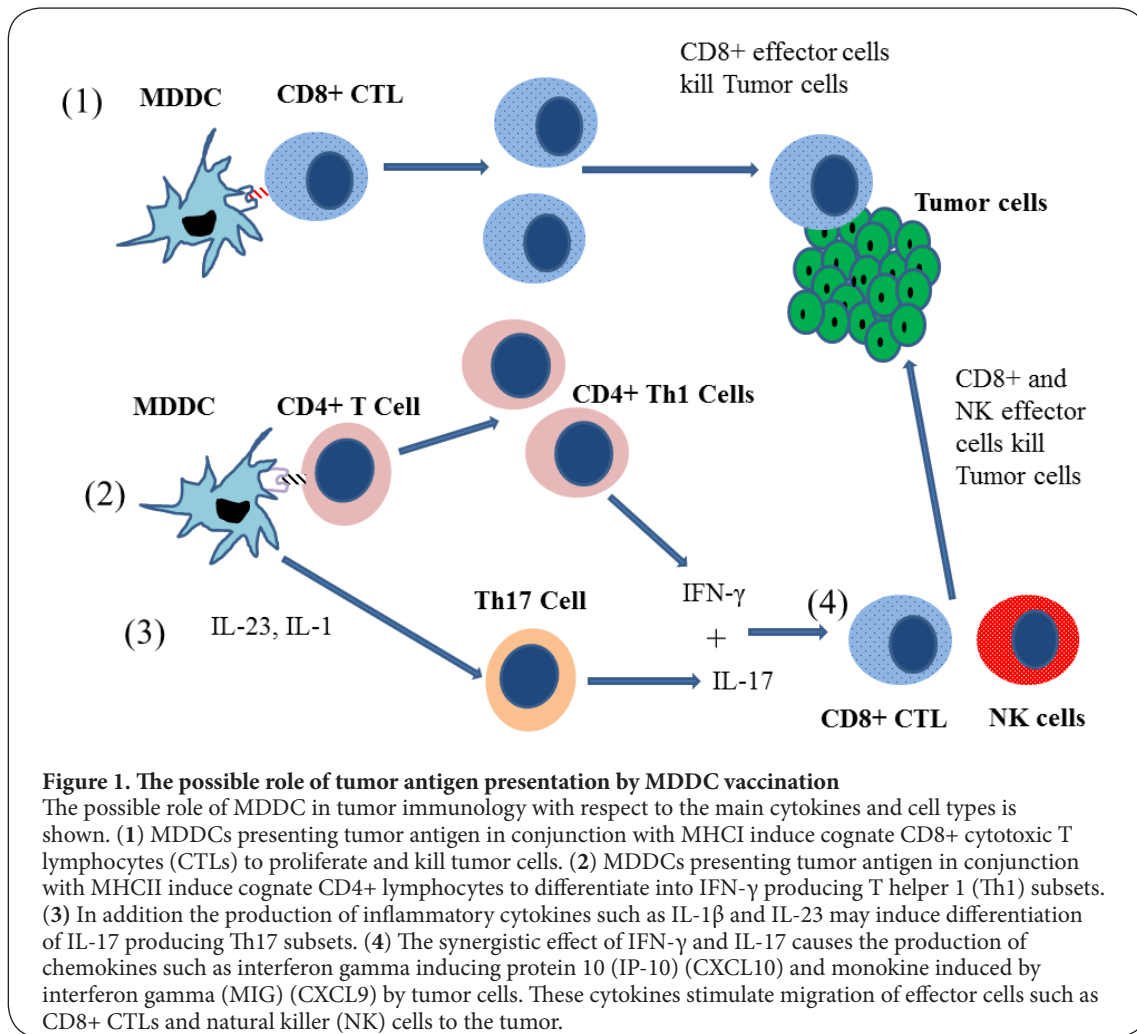
Amongst APCs, only dendritic cells (DCs) have the ability to present antigens to naïve T lymphocytes [10-11] and B lymphocytes [12] and the amount of antigen presented by DCs is orders of magnitude greater than can be presented by macrophages [13]. DCs are therefore essential in the development of adaptive immunity and as such are important targets for vaccine antigens and adjuvants. In humans, DCs exist as a number of different sub-populations. These include Langerhans cells (LCs) and interstitial DCs (iDCs) (found in peripheral tissues), conventional DC (cDCs) which are further subdivided into type 1 and type 2 subsets (mDC1/mDC2) and plasmacytoid DCs (pDCs). In addition to these subsets, monocytes can be differentiated into monocyte derived DCs (MDDCs). Previously, the DC activation model suggested that precursors of LCs and iDCs migrate from blood into tissues and remain in an immature form until activated by pathogens or cytokines. Once activated these cells mature as they migrate to the draining lymph node where they present antigens to cognate lymphocytes [14-15]. However, more recent studies have indicated that this model may be too simplistic and that activation of DCs is more tightly controlled than was previously believed. For example a recent study has suggested that migrated rat pseudo-afferent DCs do not mobilize CD40 stores to the cell membrane during interaction with steady state T lymphocytes but do mobilize CD40 to the immune synapse during allogeneic interaction but only for a limited time period [16] and a study by Geissmann [17] has also shown that MHCII stores in human LCs, migrating from inflamed skin into draining lymph nodes, remain cytoplasmic and only low levels of CD86 are detected on the cell membrane. While LCs may actually migrate into embryonic tissue and renew their population *in situ*, rather than from blood precursors [18] as was previously thought. However human DC populations are controlled in steady state conditions or activated and renewed *in vivo*, it is clear that much more of the biology of these DC subsets has yet to be revealed. Furthermore, the relative number of circulating DCs is very low with cDCs representing about <0.6% of the total peripheral blood mononuclear cells [19-20] and pDCs representing <0.4% [21]. It would not be a viable proposition to obtain DCs for *in vitro* manipulation (e.g., culturing with tumor antigen) since the numbers obtained would be much too low. With this in mind strategies have been developed which attempt to expand DC populations *in vivo* or manipulate MDDCs *in vitro* for replantation into the syngeneic patient (Figure 1).

Expanding DC populations *in vivo* as a mechanism for increased tumor immunogenicity

Cytokines

Expanding the tumor antigen expressing DC populations would appear to be a rational approach to increasing the immunogenicity of cancer vaccines. One cytokine known to increase DC populations in lymphoid organs is fms-like tyrosine-kinase 3 ligand (Flt-3 ligand) [22-24] and daily administration of Flt-3 ligand has been shown to induce tumor regression and decrease tumor growth in mice with fibrosarcoma [25]. Flt-3 ligand and CD40 ligand were also shown to synergise to further expand DC numbers in mice with B10.2 or B10.5 tumors and this treatment led to a reduction in tumor growth or, in some cases, complete cure [26]. Flt-3 ligand delivered via particle mediated transfection was also reported to inhibit the growth of MCA205-induced Sarcomas in mice and in this study increased CD11c+ cDCs were shown in tumor tissues as well as increased CD80 and MHCII expression [27]. Intra-nodal administration of Flt-3 ligand with antigen-encoding RNA has also been reported to expand both cDC and pDC populations in a murine melanoma model [28]. In this study Flt-3 ligand and RNA induced expansion of nodal Th1 cells and increased tumor-specific CD8+ lymphocytes via antigen presentation by pDCs. The study also showed that 70% of mice were cured of tumors following Flt-3 ligand/RNA inoculation compared with only 11% cured following inoculation with RNA alone. This suggests that Flt-3 ligand may act as a DC adjuvant and that its inclusion in vaccine formulations may significantly enhance the immunogenicity of cancer vaccines.

Conversely, a study by Taylor et al., [29] has reported that progression of murine myeloproliferative disease (MPD) (induced by inactivating knock-in mutation in the RING finger domain of the c-Cbl E3 ubiquitin) is prevented by treatment with the Flt-3 kinase inhibitor AC220 and continued for as long as AC220 was administered. A study by Greystoke et al., [30] has also indicated that elevated Flt-3 ligand can be used to predict patients who develop neutropenic sepsis following chemotherapy against lymphoma. It is difficult to say whether there was a true causal link in this case but increases in plasma Flt-3 ligand in irradiated non-human primates was shown to be inversely correlated with neutropenia and when peak concentrations subsided this was correlated with a return of total blood cells counts to normal [31]. The treatment of neutropenia usually requires administration of granulocyte-colony stimulating factor (G-CSF) and a more stable form of recombinant G-CSF (Pegfilgrastim) has been developed [32]. Interestingly, administration of Pegfilgrastim to patients with gynaecological malignancies was shown to significantly increase circulating numbers of both cDCs and pDCs and as well as preventing neutropenic sepsis, caused by chemotherapy or possible future Flt-3 therapy, Pegfilgrastim appears to also expand DC populations *in vivo* in its own right. Therefore, studies to date have certainly highlighted the



immune-therapeutic potential of Flt-3 ligand in cancer but the use of Flt-3 to increase DC populations *in vivo* may have some draw-backs which need to be studied more thoroughly.

Although Flt-3 ligand is probably the best studied of molecules which expand DC populations *in vivo* others cytokines have also been shown to have therapeutic potential. Macrophage inflammatory protein 3 alpha (MIP-3α) is an inflammatory chemokine which binds to CC-chemokine receptor 6 [33]. CCR6 is involved in the peripheral recruitment of immature Langerhans cells but it is CCR7 expression by mature DCs which induces their migration into the T cell areas of lymph nodes. Vaccination of mice with another cytokine granulocyte macrophage colony stimulating factor (GM-CSF) has been shown to have a very potent anti-tumor effect [34] and more recently Choi and Kim, [35] reported that plasmids encoding MIP-3 α and GM-CSF synergistically induced DC recruitment and increased T helper (Th) cell and cytotoxic T lymphocyte (CTL) activity at the site of murine EML/Muc1 tumors and draining lymph. However, there is also evidence to suggest that tumours may suppress the host immune response

via CCR7 expression and that expression of CCR7 may also be involved in metastasis [36], via ligation of the cytokine CCL21 [37]. It is clear therefore that the exact relationship between CCR7 expression, DCs and tumor regression, or indeed progression, needs further clarification.

Hohman et al., [38] were the first to identify a population of murine cells with both Natural killer cell (NK) cell and DC-like properties. These cells were termed NKDCs and later Chan et al., [39] reported that these cells produce IFNγ and kill cells expressing tumor antigen. Recently it has been shown that murine splenic NKDCc will proliferate *in vitro* when cultured with IL-21 and when replaced back into B16F10 (melanoma) tumor bearing C57BL/6 mice, the IL-21-cultured NKDCs were associated with a reduction in tumor size [40]. This may suggest that administration of IL-21 with tumor antigen could increase immunogenicity of cancer vaccines *in vivo*. However, one study has suggested that NKDCs may actually be a population of activated NK cells [41] and to date a similar NKDC population has not been reported in humans. IL-24 is another cytokine which has come into prominence recently

and is being investigated for its effect on many different cancers. IL-24 was first shown to be a protein product encoded by the melanoma differentiation-associated gene 7 (mda-7) [42] and was shown to have an anti-cancer effect in a number of human cancerous cell lines, including colorectal, prostate and cervical cancers [43]. Low expression of mda/IL-24 has been associated with increased nodal involvement in human breast cancer [44] and reduced survival of colorectal cancer patients [45]. One mechanism by which mda/IL-24 has anti-cancer effects is by the promotion of cell death, such as the generation of ceramide, the production of reactive oxygen species and increased ER stress and mda-7 gene transfer via vectors such as adenovirus is currently being investigated [46]. High expression of mda-7 has also been shown in DCs within germinal centres of melanoma patients [47] which may suggest a different mechanism by which DCs suppress tumors via direct suppression rather than presentation of tumor antigens. However, increased expression of molecules essential for antigen presentation (HLA-DR, CD40 and CD80) was also measured in human DCs transfected with IL-24 gene containing adenovirus and pulsed with lysates from SMMC-7721 (human hepatocellular carcinoma cells) [48]. This was also associated with increased production of IL-24, IL-12 and TNF- α and when these DCs were cultured with cytokines induced killer cells (ICK), they stimulated greater lytic activity by ICKs on SMMC-7721 cells. Once again these latter experiments suggest that IL-24 could be an important adjuvant if used in DC vaccines.

Other molecules

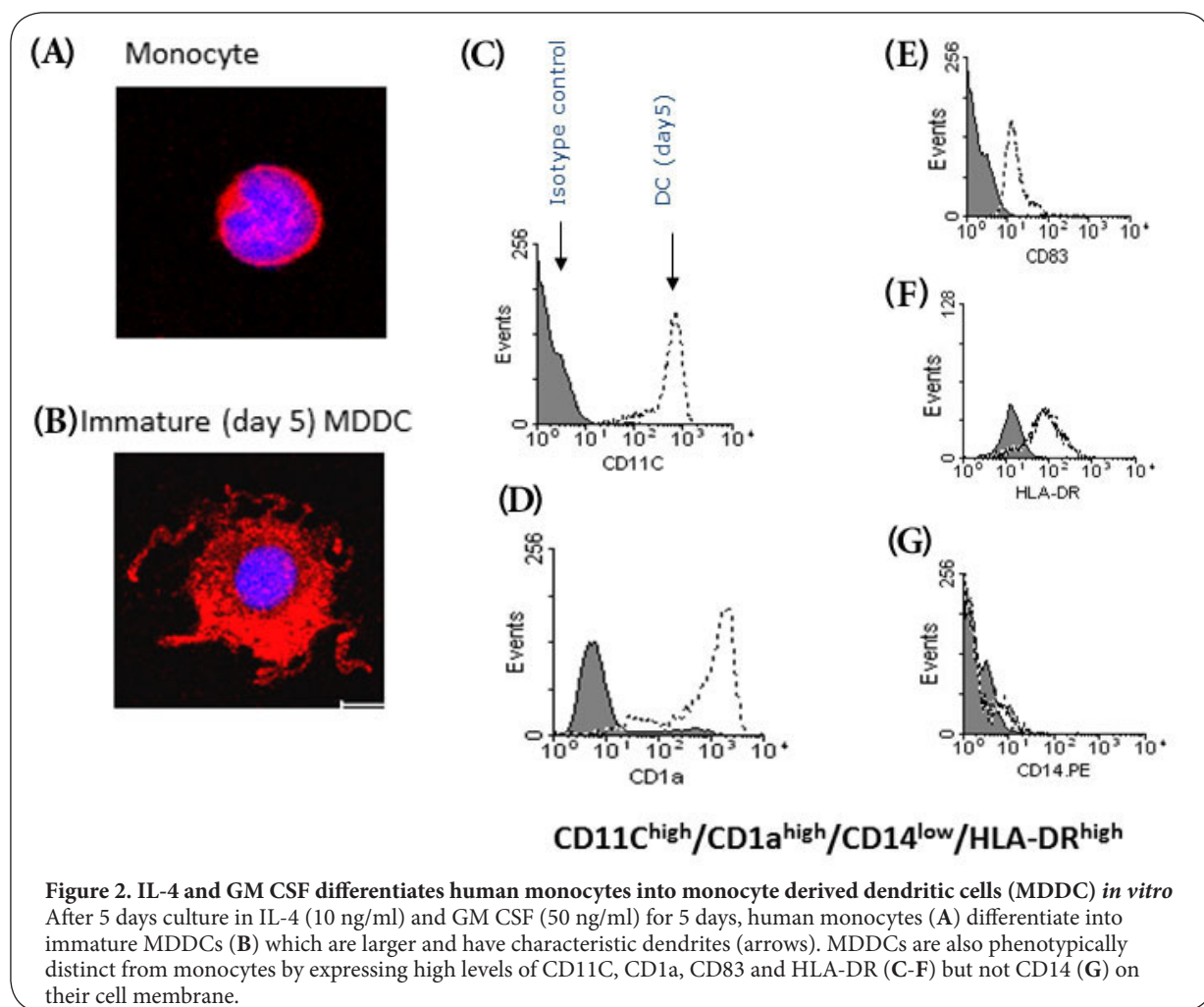
Carthamus tinctorius (CT), or safflower, is used in Chinese traditional medicine to improve blood circulation. A study by Chang et al., [49] has reported that when murine bone marrow derived DCs were cultured with CT extract and pulsed with a JC cell (murine mammary adenocarcinoma) lysate, prior to administration into tumor bearing mice, they reduced tumor mass by >15%. This was also associated with a skewed Th response towards IFN- γ -producing Th1 cells and *in vivo* the CT-DC combination induced proliferation of CD8+ lymphocytes. An earlier study also reported that when CT polysaccharide was administered into mice bearing either S180 Sarcoma or LA795 lung tumors it was associated with a respective tumor inhibition or shrinkage and increased activity of CD8+ lymphocytes and natural killer (NK) cells, although DC activity was not studied [50]. Polyinosinic-cytidylic acid (poly (I:C)) has also been reported to increase DC populations removed from cervical cancer patients and when pulsed with E7 human papilloma virus antigen they increased surface expression of CD11C and CD86 [51]. It should be noted that although CD11C is a classic marker for myeloid DCs it is also has very important immunological properties. For example CD11C is a receptor for compliment protein iC3b and increases phagocytosis of iC3b-opsonized particles [52], while antibody blockade of CD11C on the surface of LCs ameliorates proliferation of

allogeneic T lymphocytes [53], thus indicating a key role in the induction of innate and adaptive immunity. In another study, Poly: IC has also been shown to inhibit metastasis in a B16-F10 murine lung cancer model and was associated with increased DC maturation, increased CD8+ (CTL) activity and skewing of the immune response towards Th1/Th17 [54], while administration of a combination of the synthetic lipopeptide Pam3Cys with Poly: IC induced greater maturation of DCs and conferred tumor protection in mice [55].

The therapeutic potential for Monocyte derived DCs (MDDCs) in patient-specific cancer therapy

DCs can be derived *ex vivo* from blood monocytes (MDDCs) and since monocytes represent a large population of blood cells, numerous MDDCs can be derived in culture media containing granulocyte-monocyte colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) [56]. Compared with their autologous monocytes, these MDDCs have increased surface area due to the development of dendrites (Figures 2A and 2B) and express a characteristic surface phenotype in which they express high levels of CD11C, CD83 and CD1a (which are not expressed by monocytes) and increased HLA-DR but very low levels of CD14, which is highly expressed by monocytes (Figures 2C-2G). These immature MDDCs can then be matured using various other cytokines including, TNF- α and IL-1 β [57] while we have recently shown that human MDDCs mature in response to IFN- γ , IL-1 β and novel cytokines, such as IL36 β or IL36 γ , allowing easy measurement of immunological parameters such as further increases in HLA-DR and CD83 with a concurrent decrease in CD1a [58] (Figures 3E-3G). Furthermore, in humans and murine models, MDDCs appear to be a physiological population generated *in vivo* during inflammation [59,60], possibly serving to replenish spent peripheral DC populations. Autologous MDDCs can be safely injected back into recipients without inducing a host versus DC reaction (as long as utmost sterility can be achieved). This approach could allow patient specific vaccines to be developed according to tumor type and antigen expression. As such, a number of studies have now been reported to show that autologous monocyte derived DCs (MDDCs) pre-loaded with relevant tumour antigen may be of some benefit in certain cancers [61-63].

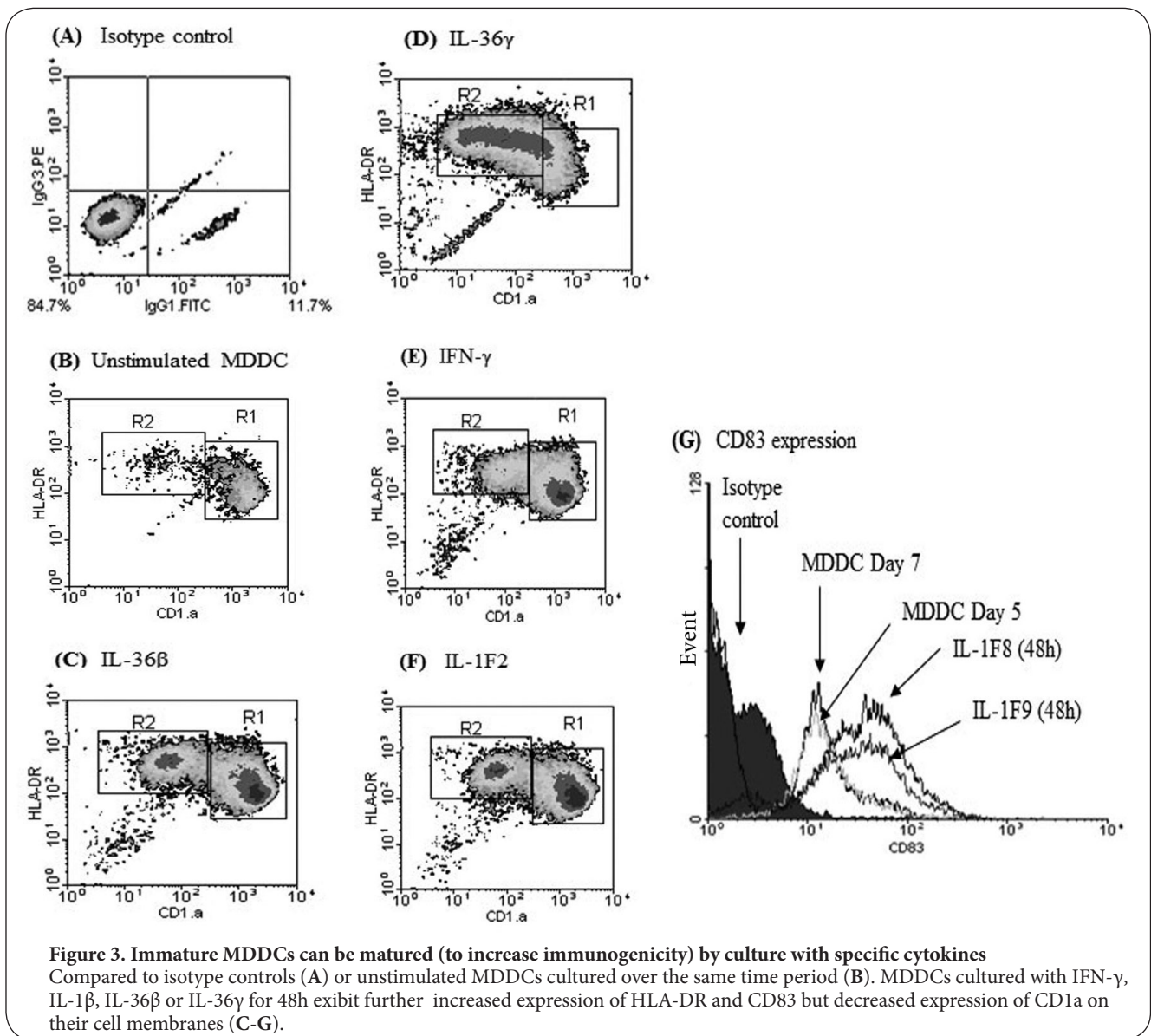
Meta-analysis of the first 1000 MDDC vaccinees, with different tumours, demonstrated that about half exhibited a clinical response, and that MDDC vaccination was associated with virtually no adverse effects [64]. In another study, survival of patients with advanced melanoma was increased from 5 months to 24 months following injection of autologous MDDCs loaded with melanoma antigen, and was significantly correlated with increased numbers of IFN- γ producing Th1 lymphocytes [65]. It has also been reported [66] that MDDCs pulsed with carcinoembryonic antigen (CEA) induced antigen-specific T cell response in 8 out of 11 patients with colorectal cancer, and that this technique was far superior



to MDDC transfection using CEA mRNA. Phase I clinical trials using the CEA loaded MDDCs in colorectal cancer patients indicated that they induced both a CD4+ and CD8+ effector response, but that T suppressor activity may have impacted on the full effect of the vaccine [67]. MDDCs loaded with CEA and melanoma associated antigen 3 (MAGE-3) induced significant increases in the killing of target cells by CTLs [68]. In a murine model of Melanoma therapy, MAGE-3 pulsed MDDC induced tumour specific CTL responses and inhibited tumour growth [69]. Recent studies have also shown that when mature MDDCs (designed to initiate Th1 cells) were pulsed with HER-2 antigen, they induced a robust IFN- γ response in addition to enhanced tumour cell-killing capacity in breast cancer patients. Furthermore, a recall response was measured after 52 months [70]. The data therefore, indicates that tumour antigen loaded MDDCs are a realistic strategy for future cancer therapy. Although DCs have been shown to be important in anti-melanoma immune responses [71] advanced melanoma is normally associated with immune suppression. However, one study has shown that human

melanoma-conditioned media had no effect on the maturation of MDDCs, cytokine production (including Th1-inducing IL-12) or T cell proliferation [72]. This may suggest then that MDDC vaccines may be an ideally suited therapy for melanoma. Conversely, expression of Galectin-1 by lung cancer cell lines (A549 and NCI-H460) induces IL-10 producing MDDCs and IL-10-producing CD11C+ cells (DCs) have been shown to populate human lung tumors [73]. Since production of IL-10 by DCs is associated with the differentiation of tolerogenic T cells, the study discussed above may suggest that MDDCs vaccines may have detrimental effects on therapy, although the immune status of MDDCs loaded with lung tumor antigen has not been reported.

By using tumour cell lysates [74], it has been demonstrated that a maximum of 5 mg/ml lysate can be loaded into 1×10^6 MDDCs, and that a minimum of 1 mg/ml over a 24h culture period is required to produce measurable changes in DC immunogenicity. Such studies are critical to the future success of this strategy since the ability to deliver 1 effective vaccine dose may be necessary, as booster vaccines using tumour



loaded MDDCs will be killed by memory CTLs. Although Dhodapkar et al., [75] have reported that a single injection of MDDCs is enough to induce a rapid, antigen specific and broad T cell response in humans and it is possible that that techniques which can boost the immunogenicity of a single injection vaccine will undoubtedly produce a greater clinical effect, and could contribute to overcoming the effect of suppressor activity.

Enhancing MDDC vaccination by adequately engineering the cell membrane

The membrane is composed of two leaflets which are themselves composed chiefly of lipids and other amphipathic molecules. For a long time these were considered to be neutral, with regard to the complex biochemical processes taking

place within cells, but this view has now changed and it is well acknowledged that the lipid phase forming the boundary of cells is at least as important as the biochemistry taking place within cells. How the cell membrane is used by cells has become a much studied field of research and from these studies one can say that the lipid phase of the membrane has two main properties as it: (i) permits the stability required to allow transmembrane proteins to function properly and, (ii) allows exchanges with the extracellular medium via the mechanism of membrane recycling i.e., endocytosis and exocytosis. Both points (i) and (ii) have been shown to rely deeply on the biophysical properties of the cell membrane as well as the ability of cells to biologically control these processes.

What matters in the case of antigen presentation is the potential ability to control membrane exocytosis. Membrane

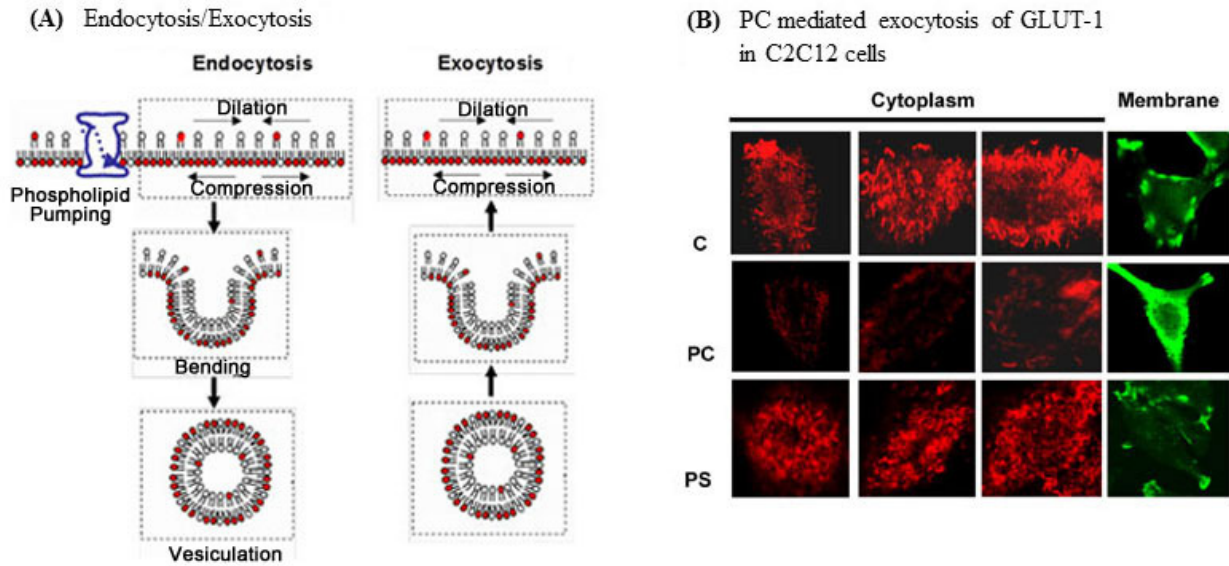


Figure 4. How membrane lipids influence endocytosis and exocytosis

The lipid number asymmetry induced fluid phase endocytosis : Sketch representing the current model that has been applied to living cells, links fluid phase endocytosis (A) and the membrane phospholipid number asymmetry maintained by a lipid flippase (the aminophospholipid translocase). In the left figure, the translocation of dark-head lipids into the inner leaflet induces a differential lipid packing between leaflets (namely a difference in surface pressures) leading to membrane bending and vesiculation. Note that it is assumed that the membrane recycling that occurs in cells, i.e., the exocytosis of vesicles of a size similar to endocytic vesicles (B), also allows the maintenance of the lipid asymmetry at the level of the plasmalemma. The relationship between the lipid number asymmetry and the vesicle radius can be determined fully considering the physical biology of the cell membrane see ref (75). A fundamental consequence resulting from the theory is that if the lipid asymmetry is inverted (i.e., if the outer leaflet contains more lipid than the inner leaflet) exocytosis should be promoted to balance the membrane stress. This was demonstrated by Rauch and Loughna (2005) using phosphatidylcholine (PC) as a phospholipid that is not translocated by the aminophospholipid translocase (i.e., flippase), the ability of PC to promote exocytosis was demonstrated in C2C12 myoblasts expressing the glucose transporter GLUT-1 (B). Upon incubation with cells, PC switched the balance of GLUT-1 from intracellular stores (in red) compartment to the membrane (in green). Note that phosphatidylserine (PS) that accumulates into the inner leaflet thanks to the aminophospholipid translocase activity does not generate exocytosis (C: control; PC: phosphatidylcholine; PS: phosphatidylserine).

recycling is driven by the ability of cells to form intracellular vesicles. The motor force behind the creation of vesicles resides, initially, in the membrane. The membrane is composed of two leaflets but these leaflets are not randomly composed, some lipids are preferentially located within the inner leaflet (e.g., phosphatidylserine or phosphatidylethanolamine) whereas others remain in the outer leaflets (sphingomyelin). The non random composition of the membrane is attributed to ATP-dependant lipid flippases ordering the membrane [76]. The creation of such an asymmetry in the type of lipids between the two leaflets induces a change in the physical properties of the membrane with an inner leaflet being more compacted than the outer one [77] (Figure 4). This compaction cannot be stored in the membrane, requiring the cell to release this energy and one way to do this is via the creation of intracellular vesicles. Naturally, one could argue that the model suggests that too much endocytosis should deplete the plasmalemma of the cell. However this never happens as exocytosis and endocytosis are two faces of the same coin and as a result

will balance each other [78]. In essence the lipid asymmetry between the two membrane leaflets permits a low cost in ATP usage and a constant and smooth recycling of the membrane.

How can this process be altered is down to how the physical properties of the membrane are affected. Let us assume that the lipid asymmetry is annihilated, endocytosis will stop. Now, if we reverse the lipid asymmetry – i.e., compact more the outer leaflet via the addition of exogenous lipid species compared to the inner leaflet; endocytosis will stop and exocytosis should be promoted [79-81]. In cells, not all receptors are displayed on the membrane, some stay within cells in endosomes or vesicles some may even recycle constantly between the membrane and the intracellular vesicles. By physically triggering membrane exocytosis it should be possible to empty all the intracellular compartments of their content leading to higher “expression” of antigens and relevant immunological molecules such as MHC and CD40. Experimentally, exocytosis can be promoted by simple incubation of phospholipids with cells such as phosphatidylcholine (PC) that is not translocated

by the lipid flippase from the outer into the inner leaflet. In these conditions, PC can promote exocytosis by reversing the endogenous lipid asymmetry and this has already been demonstrated in myocytes by Rauch and Loughna (2005) (Figure 3).

Conclusion

Manipulation of MDDCs may lead to a more robust presentation of cancer antigens which may increase the impact of current (and very promising) syngeneic MDDC cancer vaccines.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	CR	HI	NF
Research concept and design	✓	--	✓
Collection and/or assembly of data	--	✓	--
Data analysis and interpretation	✓	✓	✓
Writing the article	✓	✓	✓
Critical revision of the article	✓	--	✓
Final approval of article	✓	✓	✓
Statistical analysis	--	--	--

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