Pergamon

European Journal of Cancer, Vol. 35, Suppl. 3, pp. 36–38, 1999

Printed in Great Britain
0959-8049/99/$ - see front matter

PII: S0959-8049(99)00090-8

Original paper

Dendritic Cells or their Exosomes are Effective Biotherapies of Cancer

L. Zitvogel,[1] N. Fernandez,[1] A. Lozier,[1] J. Wolfers,[1] A. Regnault,[3] G. Raposo[2] and S. Amigorena[3]

[1]Unité d'Immunologie, Département de Biologie Clinique, Institut Gustave Roussy, Villejuif; [2]CJF 95-01 INSERM; and [3]UMR144 CNRS, Institut Curie, 12 rue Lhomond, 75006 Paris, France

DC DERIVED-EXOSOMES ARE IMMUNOGENIC *IN VIVO*

An increasing body of literature alludes to the effective antitumour immune responses mediated *in vivo* following passive transfer of antigen loaded splenic or bone marrow derived-dendritic cells (DCs) or administration of Flt3L [1–3] in mice. Indeed, DCs show the essential properties required for antigen presenting cells (APCs) as potent immunotherapeutic agents: migration and homing; antigen uptake; processing and presentation; as well as costimulation of lymphocytes. We aim to examine the possibility that in addition to direct cell-cell contacts and to cytokine production, DCs may also trigger T cell responses through the secretion of antigen presenting vesicles, called exosomes.

APCs contain specialised late endocytic compartment, MIIC (major histocompatibility complex (MHC) class II enriched compartments), that harbour newly synthesised MHC class II molecules in transit to the plasma membrane [4, 5]. MIICs have lysosomal characteristics (are acidic and bear lysosomal marker molecules, i.e. lysosome associated membrane protein (LAMP), tetraspanins) and are involved in antigen processing and peptide binding to class II molecules. However, functionally different subclasses of MIICs exist, encompassing membrane sheet and/or internal vesicle, multivesicular bodies (MVB)-containing compartments. Ultrastructural studies in Epstein–Barr virus (EBV)-transformed B cells have demonstrated that multivesicular bodies are exocytic compartments in that their limiting external membrane can fuse with the plasma membrane resulting in the release, in the extracellular milieu, of their internal vesicular content. The externalised vesicles, termed exosomes, carry in their membrane MHC class II molecules with their peptide binding domain oriented towards the extracellular milieu. During their formation, internal vesicles arise from the budding of a portion of the outer endosomal membrane toward the endosomal lumen [6].

We first examined secretory lysosomes from immature human monocyte-derived DCs cultured in IL-4 + GM-CSF. Ultrastructural studies revealed that these cells contain numerous internal vesicles (multivesicular MIICs) as well as MIICs displaying electron dense concentrically arranged membrane sheets (multilamellar MIICs). As described by Kleijmeer and colleagues [7] in EBV transformed B cells, we also found that in human monocyte derived (MD)-DCs generated in IL-4 + GM-CSF for 7–10 days, multivesicular MIICs express MHC class I molecules. Both markers were found in the external membrane of the endosomes and the intraluminal 60–90 nm vesicles. In contrast to CD63 or CD82, MHC class I molecules were also detected at the cell surface [8]. Multivesicular MHC class I- and class II-containing compartments were often observed in close apposition to the cell surface, suggesting their direct fusion with the plasma membrane [8]. Consistent with this possibility, 60–90 nm vesicles were often observed close to the outer side of the plasma membrane. These vesicles were abundantly labelled with anti-MHC class I and II, CD63 and CD82 specific antibodies. Therefore, 60–90 nm vesicles, bearing the same markers as the internal vesicles of multivesicular MIICs (class I, class II, CD63, CD82) are released by human DCs.

These vesicles were isolated from DCs' culture supernatants following differential ultracentrifugation [7] and analysed by whole mount immuno-electron microscopy. A homogeneous population of vesicles of 60–90 nm diameter was observed (Figure 1). Like the vesicles from the exocytic profiles, over 95% of these vesicles were labelled with the anti-CD63 and anti-CD82 antibodies, as well as with anti-MHC class I and class II antibodies.

Similar vesicles were observed in the supernatants of mouse DCs. We used the well characterised growth factor-dependent D1 DC line [9] and bone marrow-derived DCs (BM-DCs) to analyse the exosomal production by mouse DCs. As in the case of human DCs, immature murine DCs exhibit abundant multivesicular endosomal compartments, which were occasionally observed in close apposition to the plasma membrane. These LAMP-1 positive endosomal population stained for MHC class I and II molecules by confocal immunofluorescence. Exosomes, harvested from D1 or BM-DCs supernatants, expressed MHC class I, class II and costimulatory signal molecules as detected by whole mount electron microscopy and Western blot. MHC class I

Correspondence to L. Zitvogel, e-mail: zitvogel@igr.fr

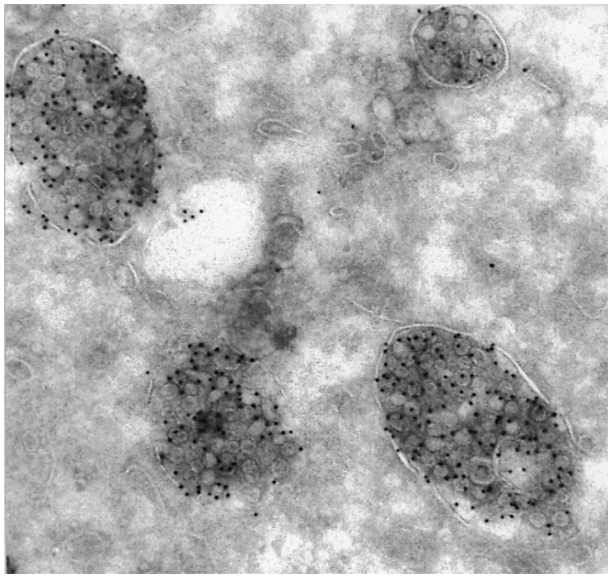

Fig. 1. MHC class II compartments of mouse D1 DCs. Ultrathin cryosections of D1 cells were immunogold labelled for MHC class II (protein A-10 nm gold). MHC class II are localised in compartments displaying internal membrane vesicles (MVB).

and class II as well as CD86 and transferrin receptors (TfRs) were found on exosomes; the three latter markers were enriched in exosomes as compared with the cell lysates. In contrast, although detected in the cell lysates, H2-M, Ii chain, and calnexin (an endoplasmic reticulum specific marker) were undetectable in the exosomal preparations. The size and morphology of immature mouse DC-derived exosomes were similar to that derived from human DCs.

Exosomes have allostimulatory capacities and are MHC class I-restricted antigen presenting vesicles *in vitro*.

Tumour peptide pulsed DCs-derived exosomes induce tumour growth suppression in tumour bearing mice. We tested the capacity of these vesicles to induce T cell-mediated immune responses *in vivo*. Bone marrow derived-DCs cultured in IL-4 + GM-CSF (BM-DCs) [1, 2] loaded with acid eluted tumour peptides were previously shown to mediate specific anti-tumour immune responses. P815 is an immunogenic but agressive mastocytoma, syngeneic of DBA/2 (H-2^d), for which very few effective immunotherapies on day 10 established tumours have been reported. Acid eluted tumour (P815) peptides were pulsed onto syngeneic mouse BM-DCs as previously described [2]. Exosomes were prepared from the DC supernatants by differential ultracentrifugation and utilised for *in vivo* immunisation. Therapy of day 10-established P815 tumours (50–90 mm^2 in size) was carried out using a single intradermal (i.d.) administration of 3–5 μg of exosomes/mouse. Within a week, tumour growth stopped in the groups receiving exosomes derived from autologous tumour peptide pulsed DC and 40–60% mice were tumour free at day 60. These animals had a long-lasting immune response and rejected a lethal tumour challenge with P815 but not with the syngeneic leukaemia L1210. Groups of mice immunised with exosomes derived from self splenic peptide pulsed DC showed no effect on tumour growth as compared with control mice groups. Therefore, P815 peptide pulsed DC derived-exosomes promoted tumour regressions. Similar antitumour effects were achieved in the day 3–4 established TS/A tumour model. These antitumour effects were not found in athymic Nu/Nu counterparts, indicating that T cells are required for the exosome-induced antitumour immune responses. In addition, exosomes directly prime tumour specific CTL responses in P815 bearing hosts. Importantly, intravenous i.v. or i.d. adoptive therapy using $5–10\times10^5$ immature BM-DCs pulsed with acid eluted tumour peptides was not as efficient as i.d. administration of exosomes derived from the same cells to cure mice. Similar results were achieved on established TS/A tumours in BALB/c animals and on prophylaxis studies using the P1A tumour peptide.

The physiological role of DC derived-exosomes remains unclear. It is conceivable that T helper cytokines be delivered to the DC upon arrival in the lymph node T cell enriched areas. Antigen presenting vesicles would then be released to amplify specific T cell clonal expansion. Alternatively, other host APCs could take up these exosomes to transport these antigenic vesicles to specific sites where priming of naïve T cells and/or B cell crosstalk could be elicited.

These data support the implementation of DC-derived exosomes for cancer immunotherapy as a novel dendritic cell-free therapeutic cancer vaccine and suggest that exosomes may represent a physiological means of communication between DCs and T lymphocytes.

DC ARE ASSOCIATED WITH NK CELL DEPENDENT-ANTITUMOUR EFFECTS *IN VIVO*

The feasability of using DCs as an immunotherapeutic vector has been hampered by the scarcity of these cells in the peripheral lymphoid tissue and by the fact that only limited numbers can be expanded *ex vivo* using current protocols with a cocktail of cytokines. Flt3L (FL) is a growth factor of primitive multipotent haematopoietic progenitor cells and myeloid/lymphoid committed progenitors. Maraskovsky and colleagues have demonstrated that daily subcutaneous s.c. injections of mice with human Chinese hamster ovary (CHO)-derived FL induces the production of large numbers of DCs in lymph nodes, spleen and other tissues. Indeed, mice treated for 9 days develop a 17-fold increase in the absolute numbers of class II+, CD11c+ DCs in spleen, a 4-fold increase in lymph nodes and a 6-fold increase in peripheral blood. Higher numbers of these cells are also detected in bone marrow, Peyer's patches, mesenteric lymph nodes, thymus, liver, lungs and in the peritoneal cavity. Organomegaly due to the hematopoietic effects of FL is only seen in spleen and lymph nodes. These organs revert to normal size and cellularity within 7–10 days upon cessation of FL treatment. DC produced by FL injection are equally efficacious for inducing antigen (Ag)-specific T cell responses both *in vitro* and *in vivo* as the rare DCs that can be purified from spleens of untreated mice.

Lynch and colleagues subsequently tested the efficacy of FL to elicit specific antitumour immune responses in tumour bearing mice and to eradicate tumours *in vivo* [3]. In an immunogenic methylcholanthrene-induced fibrosarcoma, profound dose–dependent antitumour effects were achieved following treatment with FL that were T cell-mediated. In addition to cognate immune responses, an unexpected finding was that in poorly immunogenic tumours or in nude and severe combined immunodeficiency (SCID) mice, significant antitumour effects were also found, suggesting a role for CD11b + cells or for the natural killer (NK) cells in the

tumoricidy. Shaw and colleagues [10] pointed out expansion of functional NK cells in Flt3L-treated mice.

In a class I negative tumour model (mesothelioma) syngeneic of C57BL6 mice, systemic administration of FL induced significant antitumour effects not only in immunocompetent but also in nude and Rag-/-mice [11]. In contrast, FL did not promote tumour growth retardation in beige mice nor in littermates treated with anti-NK1.1 depleting monodored antibody (MAb), stressing a critical role of NK cells in Flt3L-mediated antitumour effects. Interestingly, administration of anti-CD8α antibodies in mesothelioma bearing nude mice targeting lymphoid related-DC resulted in a significant but partial abrogation of the FL mediated-NK cell dependent, antitumour effects [11]. In contrast, targeting the myeloid CD4+ subset of DC was not associated with such effects. Passive transfer [11] of the D1 DC line into the tumour site was also associated with tumour growth retardation, unless anti-NK1.1 depleting antibody (Ab) were coadministrated with D1 cells *in vivo*. In this class I negative mesothelioma model, immunotherapy with DCs promotes NK cell dependent-antitumour immunity. Therefore, DC could be at the interplay between innate and cognate immune responses in the tumour microenvironment and could be implemented for cancer immunotherapy.

1. Mayordomo JI, Zorina T, Storkus WJ, *et al.* Bone marrow-derived dendritic cells pulsed with synthetic tumour peptides elicit protective and therapeutic antitumour immunity. *Nature Med* 1995, **1**, 1297–1302.
2. Zitvogel L, Mayordomo JI, Tjandrawan T, *et al.* Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. *J Exp Med* 1996, **183**, 87–97.
3. Lynch DH, Andreasen A, Maraskovsky E, Whitmore J, Miller RE, Schuh JCL. Flt3 ligand induces tumor regression and anti-tumor immune responses *in vivo*. *Nature Med* 1997, **3**, 625–631.
4. Nijman H, Kleijmeer M, Ossevoort M, *et al.* Antigen capture and MHC class II compartments in freshly isolated and cultured blood dendritic cells. *J Exp Med* 1995, **182**, 163–174.
5. Kleijmeer M, Ossevoort M, Veen CV, *et al.* MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells. *J Immunol* 1995, **154**, 5715–5724.
6. Raposo G, Nijman H, Stoorvogel W, *et al.* B lymphocytes secrete antigen presenting vesicles. *J Exp Med* 1996, **183**, 1161–1172.
7. Kleijmeer M, Escola JM, Griffith J, Geuze H. MHC class I molecules are present in MHC class II compartments from dendritic cells and B lymphocytes. *Cellular and Molecular Biology of Dendritic Cells*. Keystone Symposia. 1998, Abst. 220, 64.
8. Zitvogel L, Regnault A, Lozier A, *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nature Med* 1998, **4**, 594–600.
9. Winzler C, Rovere P, Rescigno M, *et al.* Maturation stages of mouse dendritic cells in growth factor-dependent long term cultures. *J Exp Med* 1997, **185**, 317–328.
10. Shaw SG, Maung AA, Steptoe RJ, Thomson AW, Vujanovic NL. Expansion of functional NK cells in multiple tissue compartments of mice treated with Flt3L: implications for anticancer and antiviral therapy. *J Immunol* 1998, **161**, 2817–2824.
11. Fernandez NC, Lozier A, Flament C, *et al.* Dendritic cells directly trigger NK cell functions: a cross-talk relevant in innate antitumor immune responses *in vivo*. *Nature Med* 1999, **5**, 405–411.