

Immunophenotypical and functional heterogeneity of dendritic cells generated from murine bone marrow cultured with different cytokine combinations: implications for anti-tumoral cell therapy

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SUMMARY

Dendritic cells (DC) are professional antigen-presenting cells that can be used as immune adjuvant for anti-tumoural therapies. This approach requires the generation of large quantities of DC that are fully characterized on the immunophenotypical and functional levels. In a murine model, we analysed the *in vitro* effects of granulocyte-macrophage colony-stimulating factor (GM-CSF) alone or combined with interleukin-4 (IL-4) or Flt3 ligand (Flt3-L) on the number, immunophenotype and functions of bone marrow-derived DC. In GM-CSF cultures, we have identified two populations based on their level of expression of major histocompatibility complex (MHC) class II molecules: MHC-II^{hi} cells, exhibiting the typical morphology and immunophenotype of myeloid DC (CD11c⁺ 33D1⁺ DEC-205⁺ F4/80⁺), and MHC-II^{lo} cells, heterogeneous for DC markers (30% CD11c⁺; 50% 33D1⁺; DEC-205⁻; F4/80⁺). The addition of Flt3-L to GM-CSF induced a twofold increase in MHC-II^{hi} DC number; besides, the MHC-II^{lo} cells lost all DC markers. In contrast, after addition of IL-4 to GM-CSF, the two populations displayed a very similar phenotype (CD11c⁺ 33D1⁻ DEC-205⁺ F4/80⁻), differing only in their expression levels of MHC class II and costimulatory molecules, and showed similar stimulatory activity in mixed leucocyte reaction. We next analysed the migration of these cultured cells after fluorescent labelling. Twenty-four hours after injection into the footpads of mice, fluorescent cells were detected in the draining popliteal lymph nodes, with an enhanced migration when cells were cultured with GM-CSF + Flt3-L. Finally, we showed that MHC-II^{hi} were more efficient than MHC-II^{lo} cells in an anti-tumoral vaccination protocol. Altogether, our data highlight the importance of characterizing *in vitro*-generated DC before use in immunotherapy.

INTRODUCTION

Dendritic cells (DC) are the most potent antigen-presenting cells (APC) of the immune system.^{1,2} They are capable of initiating primary immune responses^{3,4} and are the major stimulators of the T-cell-mediated allogeneic responses in mixed leucocyte reaction (MLR).^{5,6} Thus, DC can be useful to reinforce an otherwise inefficient immune response, such as

that found in cancer patients. Studies carried out in murine models have shown that DC pulsed with tumour-associated antigens (TAA) in various forms induced protective and therapeutic anti-tumour immunity.^{7–9} Based on these results and on additional *in vitro* data obtained with human DC,^{10,11} clinical trials have now been initiated.^{12,13}

However, the clinical use of DC requires the *in vitro* generation of large quantities of cells. This is accomplished through the use of various combinations of cytokines,^{14–19} which could alter the functional properties of DC. Indeed, it is known that the capacity of DC to capture, process, migrate and present antigens, which varies according to their differentiation/maturation stage,^{1,20–22} is dependent on *in vitro* culture conditions.^{14,16,23,24}

To address these issues, we have used murine dendritic cells which can be easily generated from bone marrow-derived precursors under the effect of granulocyte-macrophage colony-stimulating factor (GM-CSF).¹⁸ In this work, we have analysed and compared the effects of GM-CSF alone or in combination with two cytokines which play an important role

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Abbreviations: APC, antigen-presenting cells; DC, dendritic cells; Flt3-L, Flt3 ligand; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-4, interleukin-4; LN, lymph nodes; L1210/hCD4, L1210 murine lymphocytic leukaemia cell line expressing the human CD4 molecule; MLR, mixed lymphocyte reactions; MHC-II^{hi}, high major histocompatibility complex class II expression; MHC-II^{lo}, low MHC class II expression.

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in DC differentiation: interleukin-4 (IL-4), which induces their functional maturation,^{7,19} and Flt3 ligand (Flt3-L), which dramatically increases their number after *in vivo* administration to mice.²⁵ In particular, the murine model allows evaluation of the effects of cytokines on DC functions such as homing capacity and efficiency in vaccination protocols. These data underline the heterogeneity of *in vitro*-generated DC and the importance of their characterization before use for immunotherapy.

MATERIALS AND METHODS

Animals

DBA/2 and C57BL/6 mice were purchased from IFFA-Credo (L'Arbresle, France). Male and female mice were 7–14 weeks of age at the time of the experiments.

Tumour cell line

L1210/hCD4 cells derived from the L1210 murine lymphocytic leukaemia cell line [American Type Culture Collection (ATCC, Rockville, MD) no. CCL219] have been genetically modified to express on the cell membrane the human CD4 molecule (hCD4) as an artificial TAA (B. M. Colombo, R. Lacave, C. Pioche-Durieu, C. Masurier, F. M. Lemoine, M. Guigon & D. Klatzmann, manuscript in preparation). Cells were maintained in RPMI-1640 (Gibco-BRL, Paisley, UK) supplemented with 10% decomplexed fetal calf serum (FCS; Dutscher, Brumath, France), 1% L-glutamine and 1% antibiotics.

Generation of DC from bone marrow

Bone marrow (BM) cells were obtained from DBA/2 mice as previously described²⁶ and cultured at 7×10^5 cells/ml in RPMI-1640 supplemented with 5% FCS, 1% L-glutamine, 20 µg/ml gentamicin, 50 µM 2β-mercaptoethanol, and 2000 U/ml recombinant murine (rm) GM-CSF (Genzyme, Cambridge, MA) with or without 1000 U/ml of rmIL-4 (PeproTech, London, UK) or with or without 250 ng/ml of human Flt3-L (Immunex, Seattle, WA) in 24-well plates at 37° in 5% CO₂. On days 2, 4 and 6, 90% of the medium was replaced by appropriate cytokine-containing medium. On day 6, cells in suspension were harvested, and loosely adherent aggregates of growing DC were dislodged by vigorous pipetting and were then counted. In some experiments, cells were cultured for another 3 days with appropriate cytokine-containing medium at a concentration of 5×10^5 – 7×10^5 cells/ml.

Flow cytometric analysis

Cells (5×10^5 – 1×10^6) were suspended in phosphate-buffered saline (PBS)/3% FCS/0.02% sodium azide, centrifuged at 350 g for 5 min, resuspended in 100 µl of buffer, following by single or double labelling with saturating concentrations of monoclonal antibody (mAb).

Depending on the analysis performed, either fluorescein isothiocyanate (FITC)- or biotin-conjugated 14.4.4S mAb (PharMingen, San Diego, CA) was used against major histocompatibility complex (MHC) class II molecules. The latter was revealed with streptavidin Tri-color (Caltag Laboratories, San Francisco, CA). CD11c expression was analysed with the unlabelled N418 mAb (hamster IgG, ATCC HB224) revealed

by a phycoerythrin (PE)-conjugated F(ab')₂ goat anti-hamster immunoglobulin G (IgG) (Caltag Laboratories).

The following antibodies were selected: a panel of FITC-conjugated mAb against B220 (RA3-6B2), mCD3 (500-A2), mCD4 (CT-CD4), mCD8α (CT-CD8α), Mac-1 (M1-70.15), F4/80 (F4/80), from Caltag Laboratories; CD44 (IM7, PharMingen), a panel of biotin-conjugated mAb against CD54 (KAT-1, Caltag Laboratories) and a panel of uncoupled rat mAb against DEC-205 (NLDC 145, Serotec, Oxford UK), spleen DC (33D1, ATCC TIB 227), CD40 (3/23, Serotec), B7-1 (1G10, PharMingen) and B7-2 (GL-1, PharMingen). The biotin-conjugated mAb were revealed with streptavidin phycoerythrin (PharMingen) and the uncoupled rat mAb with an FITC-conjugated F(ab')₂ goat anti-rat IgG (Caltag Laboratories). In order to minimize non-specific binding, cells were preincubated with 2.4G2 (FCγII/IIIR, ATCC HB 197) mAb.

The following isotypic immunoglobulin controls were used: FITC-conjugated rat IgG2b and hamster IgG (Cedarlane, Hornby, Ontario, Canada), biotin-conjugated rat IgG2a, unconjugated hamster IgG, rat IgG2a, and rat IgG2b from Caltag Laboratories.

Cells were fixed in 1% paraformaldehyde, and analyses were performed on a FACScan (Becton Dickinson, Mountain View, CA).

In some experiments, MHC class II-stained DC were sorted on day 6 of the culture on a FACStar Plus (Becton Dickinson).

Allogeneic mixed leucocyte reaction (MLR)

Responder cells from inguinal, brachial and axillary lymph nodes (LN) from C57BL/6 mice and increasing numbers of 25 grey-irradiated BM-derived stimulator cells were cocultured in 0.2 ml of RPMI-1640 supplemented with 10% FCS, 2 mM glutamine, 5×10^{-5} M 2β-mercaptoethanol, and 20 µg/ml gentamicin in U-bottom 96-well microplates. Four days later, cells were pulsed with 1 µCi/well of [³H]TdR for an additional 12 hr before harvesting and scintillation counting.

Migration studies

Cells (5×10^6 – 10×10^6) were incubated with 2 µM fluorescent dye (PKH2) (Zynaxis, Malvern, PA) at room temperature for 5 min, then RPMI-1640/10% FCS was added to stop the reaction. After three washes, 5×10^5 cells/25 µl were injected subcutaneously (s.c.) into the footpad of anaesthetized (Avertin 2.5% tribromoethanol) DBA/2 mice. Homolateral popliteal and inguinal LN were removed 6, 14, 24, 48 hr and 5 days after injection.

LN cells were obtained after collagenase digestion as previously described²⁷ and suspended in staining buffer (PBS/3% FCS/0.02% sodium azide) for flow cytometric analyses.

Vaccination of mice with MHC-II^{hi} and MHC-II^{lo} cells pulsed with a model TAA

MHC-II^{hi} and MHC-II^{lo} cells were sorted on day 6 of a BM culture in the presence of GM-CSF. Unsorted and sorted cells were re-seeded separately in new wells, and pulsed with 10 µg/ml of the antigen (hCD4) in the form of soluble protein.²⁸ On day 7, cells in suspension were harvested and pooled with loosely adherent aggregates of growing DC, and used for vaccination. For this purpose, cells were irradiated

(25 grey) and injected intravenously (i.v.) into the retro-orbital sinus of groups of five DBA/2 mice (10^5 cells/100 μ l PBS/mouse). Controls were mice identically injected with unpulsed cells. Eighteen days later, mice were challenged by i.v. injection of 10^5 L1210/hCD4 tumour cells/100 μ l PBS/mouse. Animal survival was followed up to 70 days.

Statistical analysis

Results are presented as the mean \pm SD of data from individual cultures. The paired Student's *t*-test was used for statistical analysis of the observed differences, except for the comparison between day 6 and day 9, in which the unpaired Student's *t*-test was used. Statistical significance was defined as $P < 0.05$.

RESULTS

Analysis of DC generated in GM-CSF cultures: evidence for two populations

After 6 days of culture of BM cells with GM-CSF, two non-adherent populations with similar size and structural features were distinguished according to their level of expression of MHC class II molecules (Fig. 1): $51 \pm 12\%$ of cells had high MHC class II expression (MHC-II^{hi}), whereas $27 \pm 13\%$ of cells displayed weaker expression (MHC-II^{lo}) ($n=9$). The two

populations also differed morphologically: the MHC-II^{hi} cells had numerous and long cellular processes typical of DC, while in MHC-II^{lo} cells the processes were short or absent. The remaining MHC class II-negative cell population contained essentially granulocytes (not shown).

Detailed immunophenotypical analysis of MHC-class II-expressing cells was first performed on unsorted cells ($n=3$) and then confirmed on sorted cells (Table 1 and Fig. 4). All these cells were negative for B and T lymphocyte (B220, CD3, CD4, CD8 α), and granulocyte (GR1) markers, but expressed the myeloid markers F4/80 and Mac-1. Dendritic markers were differently expressed on MHC-II^{hi} and MHC-II^{lo} cells: after 6 days of culture, CD11c was expressed by all MHC-II^{hi} cells and by 30% of MHC-II^{lo} cells; 50% of MHC-II^{hi} cells only expressed 33D1 and CD40 markers. Both populations expressed high levels of the CD44 and CD54 adhesion molecules and heterogeneous levels of B7-1 and B7-2 costimulatory molecules, which however, were very low for the MHC-II^{lo} population.

At day 9, the majority of both populations remained non-adherent, displaying the same morphological features as on day 6, but with spontaneous formation of aggregates in the MHC-II^{hi} population (Fig. 2a,b). Immunophenotypical changes occurred in the MHC-II^{hi} cell population with

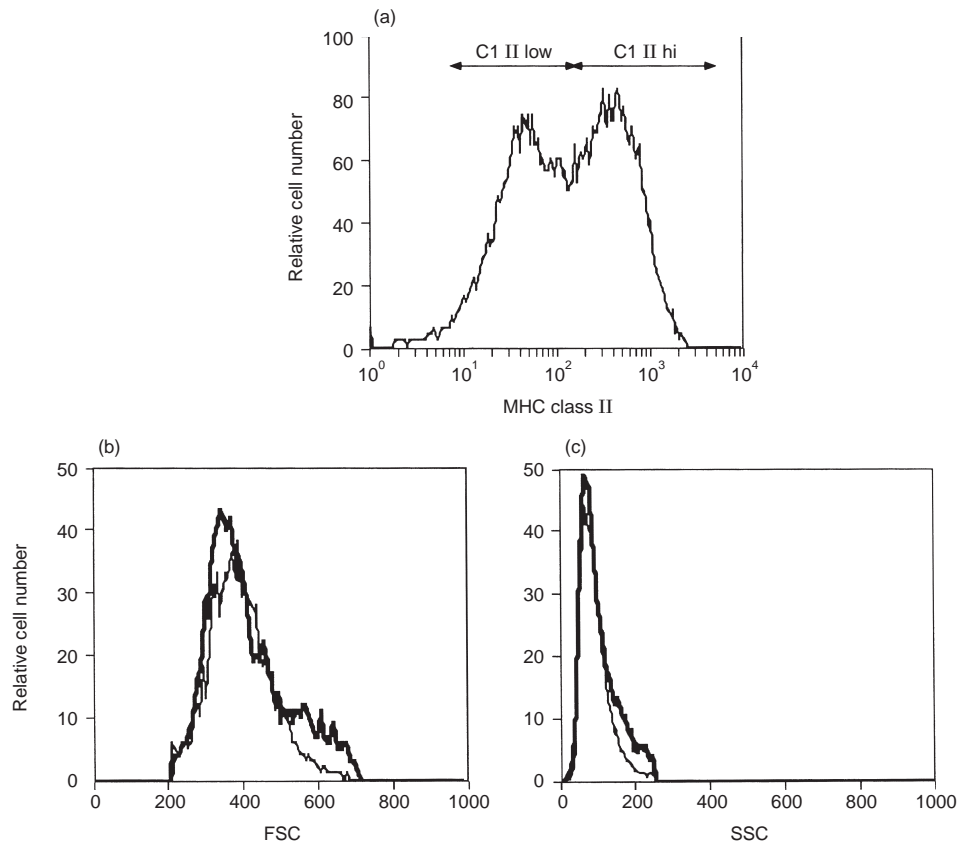


Figure 1. FACS analysis of bone marrow cells cultured in GM-CSF. Bone marrow cells cultured for 6 days in the presence of GM-CSF were stained with mAb for MHC class II molecules for flow cytometry analysis. (a) Histogram showing two populations expressing MHC class II molecules at high (MHC-II^{hi}) and low levels (MHC-II^{lo}). (b) and (c) Histograms representing the size (FSC) and the structural features (SSC) of gated MHC-II^{hi} (thick line) and MHC-II^{lo} (thin line) cells. Few MHC class II-negative cells were observed in this experiment.

Table 1. Immunophenotypical analysis of MHC-II^{hi} and MHC-II^{lo} cells cultured in GM-CSF for 6 and 9 days

| | Day 6 | | Day 9 | |
|--------------|----------------------|----------------------|----------------------|----------------------|
| | MHC-II ^{hi} | MHC-II ^{lo} | MHC-II ^{hi} | MHC-II ^{lo} |
| CD3 | - | - | - | - |
| CD4 | - | - | - | - |
| B220 | - | - | - | - |
| GR1 | - | - | - | - |
| CD11c | + | 30% +* | + | 30% +* |
| DEC-205 | - | - | + | - |
| 33D1 | 50% +* | - | + | 50% +* |
| F4/80 | + | + | + | + |
| MAC-1 | + | + | + | + |
| B7-1 | + | ±† | + | ±† |
| B7-2 | + | ±† | + | ±† |
| CD40 | 50% +* | - | + | - |
| CD55 | + | + | + | + |
| CD54 | + | + | + | + |
| CD8 α | - | - | - | - |

Cells obtained after 6 or 9 days of culture in GM-CSF were analysed by flow cytometry after double-staining with mAb against MHC class II molecules and additional indicated markers ($n=3$).

*Percentage of positive cells for the indicated marker in the whole population. †Weak expression.

expression of 33D1 and CD40 by all cells and appearance of DEC-205 expression. In the MHC-II^{lo} population, the only change was 33D1 expression by 50% of the cells.

Therefore, BM cells cultured for 9 days with GM-CSF generated a MHC-II^{hi} myeloid DC population ($34 \pm 12\%$) expressing CD11c, F4/80, DEC-205 and 33D1 markers and a heterogeneous population of MHC-II^{lo} cells ($43 \pm 10\%$), containing both DC and cells rather displaying a monocyte-macrophage immunophenotype.

Addition of Flt3-L to GM-CSF cultures resulted in higher MHC-II^{hi} cell numbers after 9 days

The addition of Flt3-L to GM-CSF cultures slightly increased the total cell numbers after 6 and 9 days (1.2-fold; $n=9$; $P<0.02$ and 1.7-fold; $n=5$; $P<0.01$, respectively) as compared to GM-CSF alone. At day 9, the MHC-II^{hi} cell number was twofold higher than in the GM-CSF culture ($n=5$; $P<0.01$), whereas that of MHC-II^{lo} was not significantly different (Fig. 3).

In contrast, the addition of IL-4 to GM-CSF cultures slightly decreased the total cell number after 6 and 9 days of culture (0.8-fold; $n=6$; $P=0.03$ and 0.6-fold; $n=5$; $P<0.02$, respectively) as compared to GM-CSF alone. However, MHC-II^{hi} or MHC-II^{lo} cell numbers were not significantly different from GM-CSF culture.

The comparison of cell numbers between days 6 and 9 (unpaired *t*-test) showed that total cell as well as MHC-II^{hi} and MHC-II^{lo} cell numbers were maintained in GM-CSF + Flt3-L cultures, whereas in GM-CSF cultures, the MHC-II^{hi} cell number was slightly decreased ($P<0.05$). On the other hand, in GM-CSF + IL-4 cultures, the total cell number was decreased from day 6 to day 9 ($P<0.04$), mostly due to a decrease in MHC-II^{hi} cells ($P<0.02$).

Addition of Flt3-L or IL-4 to GM-CSF cultures induced immunophenotypical changes in both MHC-II^{hi} and MHC-II^{lo} populations

The addition of Flt3-L to GM-CSF cultures led to minor immunophenotypical changes in the MHC-II^{hi} population, which at day 6 did not express 33D1 (Fig. 4a). In contrast, marked changes were observed in the MHC-II^{lo} population at day 9, mainly involving a decrease in MHC class II molecules and disappearance of CD11c expression (Fig. 4b). Thus, the MHC-II^{lo} population no longer expressed characteristic immunophenotypical DC markers at day 9.

The addition of IL-4 induced changes in both populations as compared to cells generated in GM-CSF. After 6 days of culture, they showed weak expression of DEC-205 and no expression of 33D1 or F4/80 markers (Fig. 4a). After 9 days of culture, both populations strongly expressed DEC-205 (Fig. 4b), whereas CD40 was expressed by all MHC-II^{hi} and by 50% of MHC-II^{lo} cells (not shown). Therefore, after 9 days of culture with GM-CSF + IL-4, both populations showed a very similar immunophenotype except for the expression of MHC class II, B7-1 and B7-2 molecules which remained lower for the MHC-II^{lo} cells. Thus, these populations, and especially MHC-II^{lo} cells, appeared immunophenotypically different from cells generated in the presence of GM-CSF.

It should be pointed out that the addition of either cytokine to GM-CSF cultures did not modify the morphology of MHC-II^{hi} and MHC-II^{lo} populations.

Influence of the cytokines on the function of MHC-II^{hi} and MHC-II^{lo} populations in allogeneic MLR

The stimulating activity in MLR is a characteristic property of DC.^{18,19} MLR were first performed using, as stimulators,

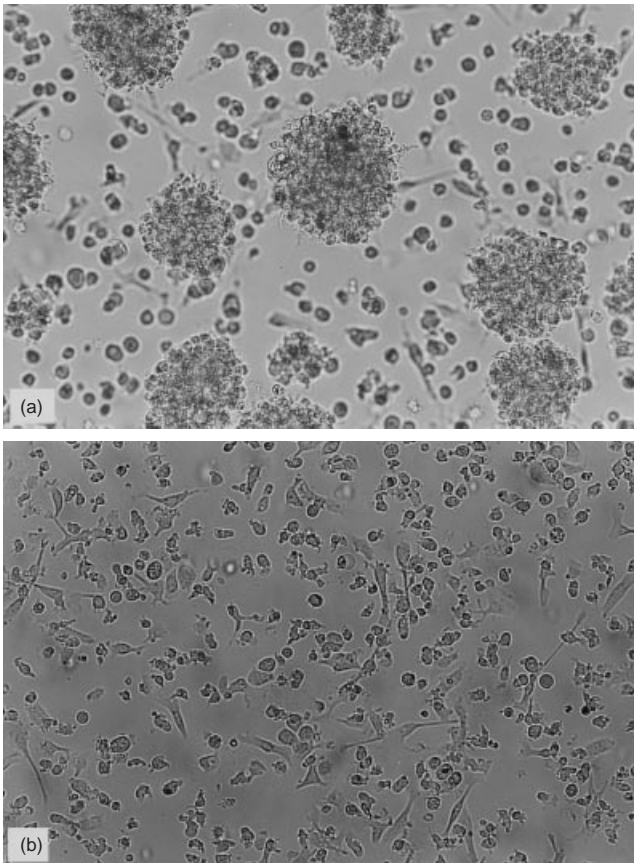


Figure 2. Morphological features of MHC-II^{hi} (a) and MHC-II^{lo} (b) cell populations after 9 days of culture. MHC-II^{hi} cells have long and numerous cellular processes and spontaneously form aggregates in culture; in MHC-II^{lo} cells the processes are short or absent. Magnification $\times 200$.

total BM-derived cells generated with different combinations of cytokines. Cells cultured in GM-CSF with either Flt3-L or IL-4 for 6 days were always more potent stimulators of MLR than cells cultured in GM-CSF alone ($n=3$). Cells cultured in GM-CSF or in GM-CSF + Flt3-L for 9 days had similar stimulatory activities, but were always less efficient than cells cultured with GM-CSF + IL-4 ($n=3$) (not shown).

In order to compare the stimulatory activities of MHC-II^{hi} and MHC-II^{lo} cell populations, similar experiments were performed with sorted cells (Fig. 5). The MHC-II^{hi} cell population generated in GM-CSF with or without Flt3-L was always a more potent stimulator in MLR than the MHC-II^{lo} cell population at day 6 of culture and to a lesser extent at day 9. When cells were cultured with GM-CSF + IL-4, the difference in the stimulatory effect of MHC-II^{hi} and MHC-II^{lo} populations was smaller at day 6 and no longer observed at day 9.

Cells obtained after culture with different combinations of cytokines migrate differently to draining lymph nodes

Another specific property of DC is their capacity to home to the T-cell areas of peripheral lymphoid tissues.^{29–31}

We first determined the homing kinetics of total cells generated in 6-day cultures with the three combinations of

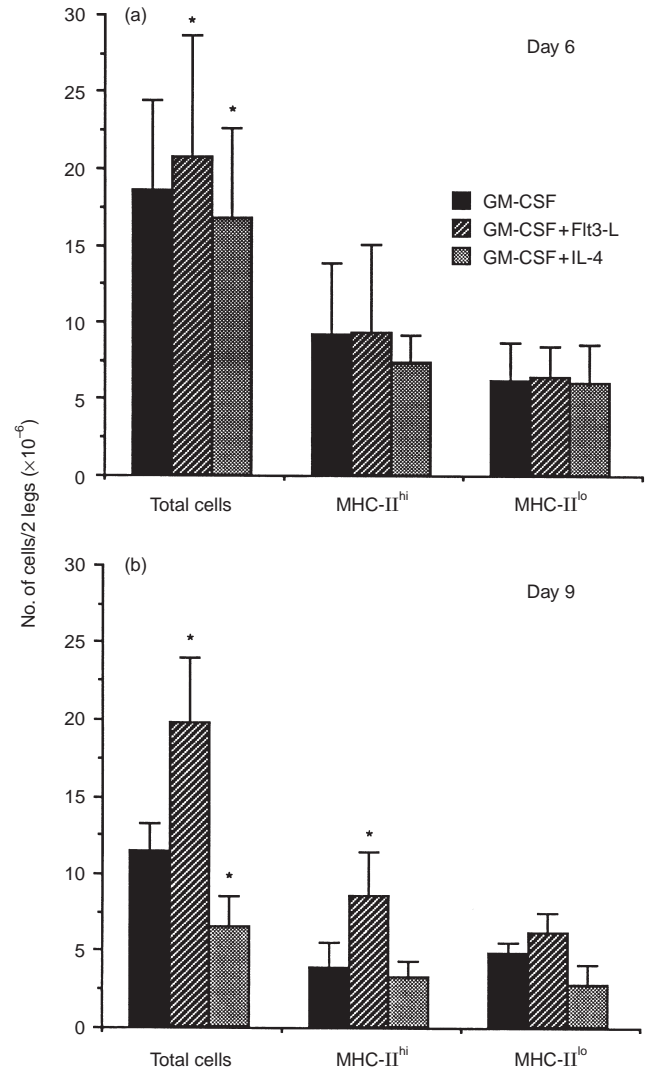


Figure 3. Cell numbers in culture with GM-CSF, GM-CSF + Flt3-L, GM-CSF + IL-4 after 6 (a) and 9 (b) days. The percentages of MHC-II^{hi} and MHC-II^{lo} cells were determined by flow cytometry analysis after double-staining for MHC class II molecules and CD11c markers. These percentages allowed calculation of the number of cells obtained per well for each population. The number of cells of each population is expressed/two legs (tibiae and femurs). Mean \pm SD of six and five independent experiments at day 6 and day 9, respectively. *Significantly different compared to GM-CSF alone.

cytokines. Cells stained with the fluorescent dye PKH2 were s.c. injected into the mouse footpad. Six, 14, 24, 48 hr and 5 days later, the draining popliteal LN were harvested and analysed by flow cytometry. Regardless of the culture conditions, the number of fluorescent cells recovered in the popliteal LN peaked at 24 hr (not shown).

Then, we compared the homing capacity of cells cultured for 6 and 9 days with the different combinations of cytokines by analysing the draining LN 24 hr after injection. Cells obtained after 6 days of culture were able to migrate to the draining popliteal LN regardless of the cytokine combination used (Fig. 6). Nevertheless, a significantly higher number of PKH2-stained cells was found in the LN after injection of cells cultured in GM-CSF + Flt3-L (577 ± 316 stained cells/ 10^6

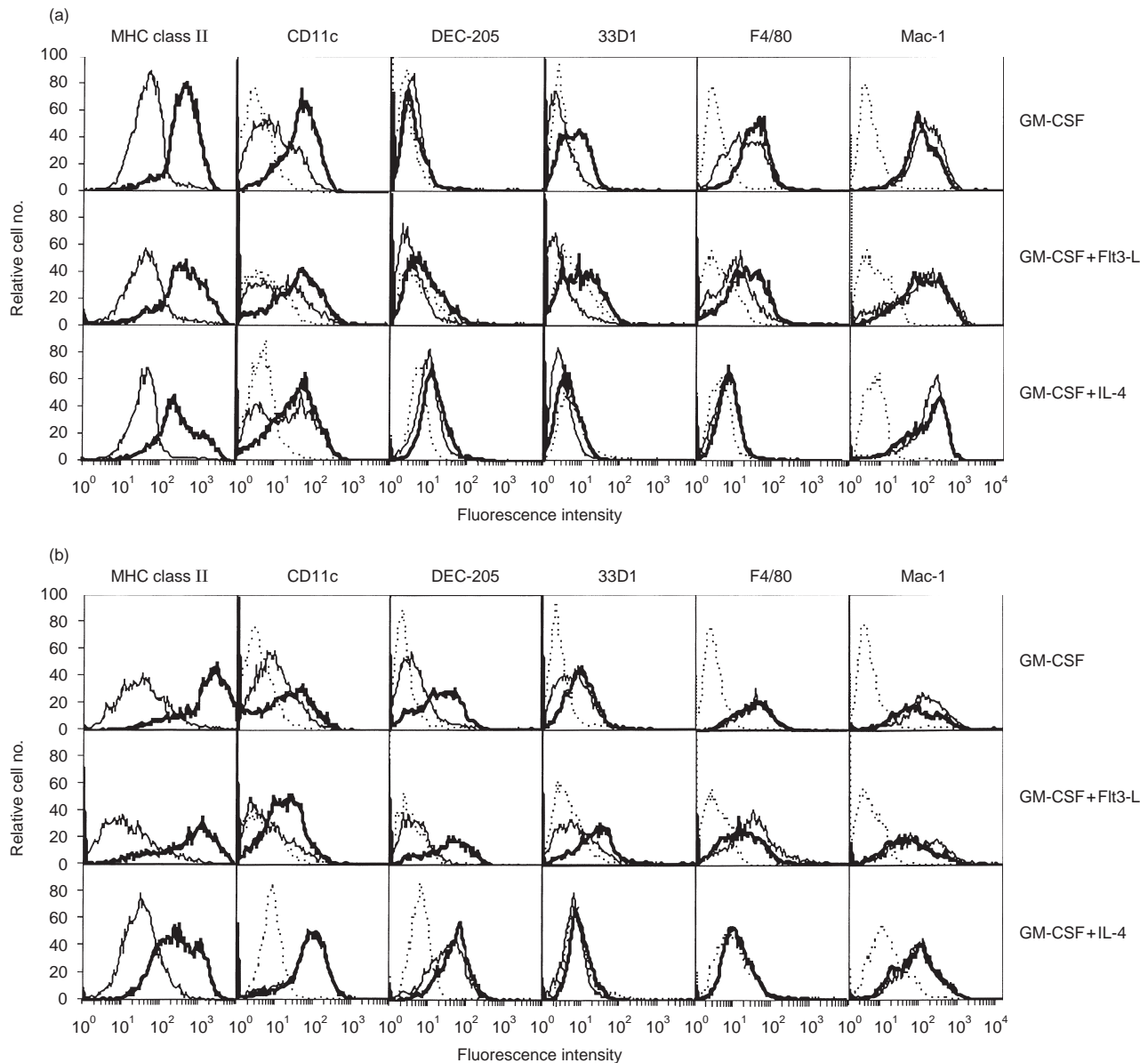


Figure 4. Cell surface immunophenotype of MHC class II-expressing cells obtained after 6 (a) and 9 days (b) of culture in GM-CSF alone (upper row) or in combination with Flt3-L (middle row) or IL-4 (lower row). At day 6 of culture, cells were sorted by flow cytometry according to their high or low expression of MHC class II molecules. Immediately and after 3 days of culture (day 9), cells were double-stained for MHC class II molecules and for additional indicated markers. Histograms: dotted line, negative controls labelled with the same amount of isotype-matched control mAb; thick line, MHC-II^{hi} cells; thin line, MHC-II^{lo} cells. Representative data from three independent experiments performed on unsorted cells are shown.

LN cells) as compared to GM-CSF alone (280 ± 86 stained cells/ 10^6 LN cells) ($P=0.02$). The number of fluorescent cells was also increased, albeit not significantly, after injection of GM-CSF + IL-4 cultured cells (587 ± 455 stained cells/ 10^6 LN cells; $P=0.08$, $n=3$).

After injection of cells cultured for 9 days in GM-CSF + Flt3-L, the number of fluorescent cells in the LN remained unchanged (529 ± 315 stained cells/ 10^6 LN cells), whereas it was strongly reduced after injection of cells cultured with GM-CSF or GM-CSF + IL-4 (i.e. 56 ± 17 and 32 ± 5 stained cells/ 10^6 LN cells, respectively).

MHC-II^{hi} cells are more efficient than MHC-II^{lo} cells in an anti-tumoural vaccination protocol

BM-derived DC pulsed with tumour antigens have been used for immunotherapy of murine tumours.³² In the following experiment, a vaccination protocol was used to analyse the respective protective effects of MHC-II^{hi} and MHC-II^{lo} cells generated in GM-CSF culture and pulsed with the soluble antigen hCD4 in the form of protein. As shown in Fig. 7, only hCD4-pulsed MHC-II^{hi} cells resulted in 100% tumour protection 70 days after the challenge with L1210/hCD4 tumour cells. In contrast, the percentage of animal survival was only

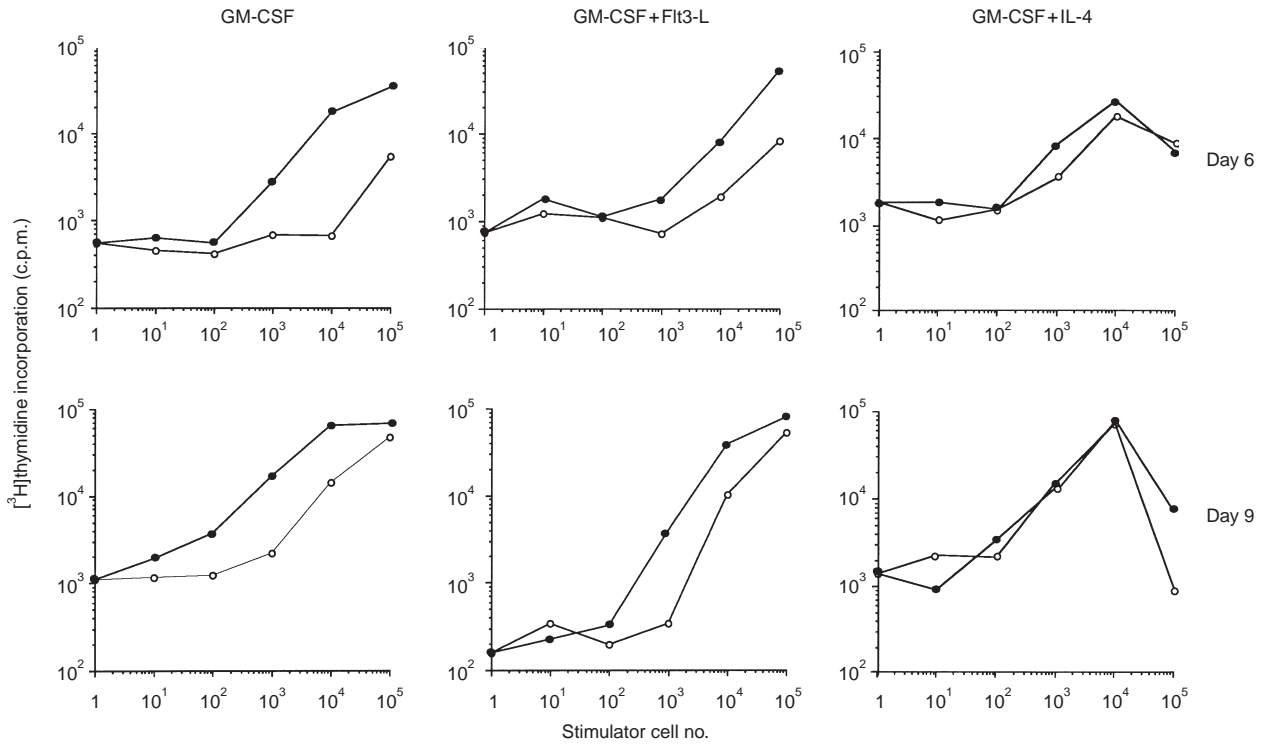


Figure 5. MLR with sorted MHC-II^{hi} (●) and MHC-II^{lo} (○) cells after a 6 (upper panel) or 9-day (lower panel) culture in GM-CSF alone (left panel) or in combination with Flt3-L (middle panel) or IL-4 (right panel). Cells from DBA/2 mice were sorted at day 6 and subcultured until day 9 as described in Fig. 2. Graded numbers of cells were irradiated and cocultured with 10^5 responding LN cells from C57BL/6 mice. Proliferative responses were measured (c.p.m.) on day 4, which corresponds to the peak of response under our experimental conditions.

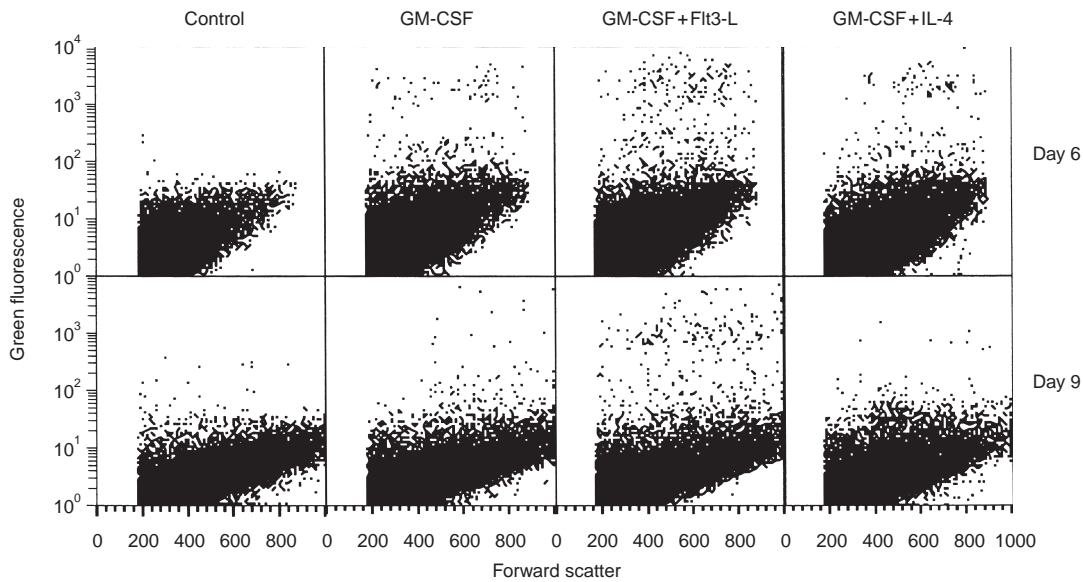


Figure 6. FACS analysis of popliteal LN cells 24 hr after injection of cells cultured in GM-CSF, GM-CSF+Flt3-L, or GM-CSF+IL-4 for 6 (upper panel) or 9 days (lower panel). Cells were stained with PKH2 and injected s.c into the footpad of syngeneic mice. Twenty-four hours later, the popliteal draining LN were harvested and digested by collagenase treatment. LN cells (1.5×10^5) were analysed by flow cytometry for their size (forward scatter) and PKH2 green fluorescence. Controls were popliteal LN cells from non-injected mice. Representative data from one of three independent experiments performed with three or four mice per group.

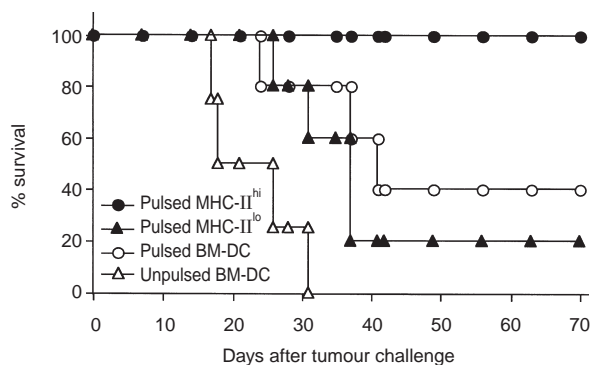


Figure 7. Effect of vaccination with MHC-II^{hi} and MHC-II^{lo} cell populations generated in GM-CSF culture pulsed with hCD4 soluble antigen. Survival of mice vaccinated with sorted hCD4-pulsed MHC-II^{hi} and MHC-II^{lo} cell populations, unsorted, pulsed or unpulsed BM-derived cells, after challenge with L1210/hCD4 tumour cells (five mice/group).

20% in the group of mice vaccinated with hCD4-pulsed MHC-II^{lo} cells. When vaccination was performed with unsorted hCD4-pulsed BM-DC, the percentage of mouse survival 70 days after L1210/hCD4 tumour challenge was 40%, but it may vary in relation to the amount of MHC-II^{hi} and MHC-II^{lo} cell populations present in the culture (data not shown). All control mice vaccinated with unpulsed BM-DC died before 31 days after tumour challenge.

Altogether, these data indicate that vaccination with either unpulsed BM-DC or with pulsed MHC-II^{lo} sorted cells did not protect, or only weakly protected, mice against tumour challenge. In contrast, vaccinations with pulsed-unsorted BM-DC and with pulsed MHC-II^{hi} sorted cells efficiently protected mice against tumour challenge.

DISCUSSION

Bone marrow was chosen for this study, because it is a potential source of DC in both mice and humans. Our data confirm the heterogeneity of DC generated from murine bone marrow as already noted by others³³ and provide further information on the different cell populations generated in GM-CSF cultures. Indeed, one cell population of typical DC coexists with cells characterized by a lower expression of MHC class II and costimulatory molecules and immunophenotypical heterogeneity.

The nature of the MHC-II^{lo} cell population, which represents about 40% of total cells, is not yet known. Several points should be emphasized. First, the level of MHC class II expression remained low in these cells throughout the duration of the culture, even after cell sorting and after addition of Flt3-L or IL-4 to GM-CSF. Immunohistochemical analysis of MHC-II distribution showed that its expression was both membrane and cytoplasmic whereas MHC-II^{hi} cells showed a preferential membrane expression (data not shown), indicating differences in maturation level.³⁴ Second, MHC-II^{lo} cells were heterogeneous and their immunophenotype appeared to be dependent on the cytokines present in the culture. In cultures with GM-CSF, some MHC-II^{lo} cells were CD11c⁻, probably committed to the monocyte/macrophage lineage. In cultures with GM-CSF and IL-4, they displayed DC markers similar to MHC-II^{hi} cells although still expressing low levels of MHC

class II and costimulatory molecules and thus, their relationship with other DC is still unclear. In cultures with GM-CSF + Flt3-L, MHC-II^{lo} cells did not display characteristic markers of DC, but appeared more committed to the macrophage-monocyte lineage.

We did not find differences in phagocytosis of latex beads which was low for both MHC-II^{lo} and MHC-II^{hi} cells as compared to macrophages (data not shown). However, MHC-II^{lo} cells were functionally different from MHC-II^{hi} cells. Indeed, they were generally less potent stimulators in MLR than MHC-II^{hi} cells, with the exception of cells generated in GM-CSF + IL-4. Furthermore, tumour-pulsed MHC-II^{lo} cells were inefficient in an anti-leukaemic vaccination protocol. This was shown using the non-immunogenic L1210 B-lymphocytic leukaemia expressing on the cell surface a candidate exogenous TAA, the hCD4. This antigen has been previously used as TAA in an anti-tumour vaccination approach in mice based on DNA immunization.³⁵ The results showed that only pulsed MHC-II^{hi} cells were efficient in the *in vivo* anti-leukaemic protection, indicating that the protective effect of pulsed cells may be correlated with the immunophenotype of the injected population.

Immunotherapy also required the generation of large numbers of DC. In mice, DC numbers can be dramatically increased in lymphoid and non-lymphoid organs by *in vivo* administration of Flt3-L.^{25,36} Under our *in vitro* conditions, Flt3-L combined with GM-CSF for 9 days resulted in a twofold increase in MHC-II^{hi} DC number, but at the same time, since there were no MHC-II^{lo} cells displaying the DC phenotype, only half of the total population was typical myeloid-related DC. This slight increase in the DC number is comparable to the *in vivo* effect obtained on BM-derived DC.³⁶ Interestingly, cells generated in GM-CSF and Flt3-L showed an increased capacity to migrate to draining LN. Further studies with purified populations are necessary to determine the population involved in this phenomenon.

Another important parameter is the functional maturation of DC, which can be induced *in vitro* by IL-4.^{7,8,19} Indeed, after 9 days of culture with GM-CSF + IL-4, both MHC-II^{hi} and MHC-II^{lo} cells displayed a similar DC phenotype but did not express 33D1 and F4/80 markers, and were thus phenotypically different from cells generated in GM-CSF. In line with their maturation state, cells obtained in GM-CSF + IL-4 cultures were more potent stimulators in MLR than cells generated with GM-CSF with or without Flt3-L.

Altogether, our results demonstrate the heterogeneity of DC generated *in vitro* from murine bone marrow. This is in agreement with the heterogeneity of DC previously reported *in vivo* and *in vitro*.^{27,37-39} Furthermore, we show that cytokines influence both the immunophenotype and the *in vivo* functions of *in vitro*-generated DC. Our data highlight the need to characterize the type of DC to be used in order to induce an efficient *in vivo* anti-tumoral immune response.

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