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Role of hepatocyte growth factor in the development of dendritic cells from CD34+ bone marrow cells

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ABSTRACT

Background and Objectives. **Hepatocyte growth factor (HGF) is known to augment the effects of stem cell factor, interleukin-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), erythropoetin, and granulocyte colony-stimulating factor, all of which are involved in hematopoiesis. HGF is also known to have a role in immune responses. The aim of this study was to investigate whether HGF is involved in the development of dendritic cells (DC) from CD34+ bone marrow cells.**

Design and Methods**. CD34+ cells obtained from three healthy donors were incubated in various combinations of HGF, GM-CSF, and tumor necrosis factor (TNF) for 12 days. Developing cell populations were analyzed for surface markers, morphology and functional capacities by flow cytometry, light microscopy and mixed lymphocyte reaction, respectively.**

Results. **Incubation with HGF alone generated greater number of dendritic cells from CD34+ bone marrow cells than incubation with GM-CSF, or a combination of GM-CSF with TNF. HGF was also found to potentiate the effect of GM-CSF on DC and monocyte development. The effects of HGF were inhibited by the concurrent use of TNF.**

Interpretation and Conclusions. **HGF appears to be a significant factor in the development of dendritic cells from CD34+ bone marrow cells.**

Key words: dendritic cells, hepatocyte growth factor, granulocyte-macrophage colony stimulating factor, tumor necrosis factor, CD34, bone marrow

The dendritic cell (DC) family consists of specialized antigen presenting cells, which are present throughout the tissues with exception of the brain and central cornea.[1] Dendritiform morphology is not unique to the DC, and can be encountered in several cell types, including macrophages and B-lymphocytes; the distinguishing feature of the DC is its functional characteristics.[2,3] Dendritic cells, which are derived from the bone marrow, peripheral blood and cord blood CD34+ cells, and also DC of the skin show a number of characteristic features, including morphology, low phagocytic activity, high membrane density of HLA class II molecules and accessory molecules, expression of CD1a , and a strong capacity to stimulate allogeneic T-lymphocytes.[4-6] Granulocyte macrophage-colony stimulating factor (GM-CSF) was shown to be a critical cytokine for the generation of DCs from both murine and human precursor cells.[7] Other factors such as tumor necrosis factor-α (TNF-α), kit ligand, FLT-3 ligand, interleukin-4 (IL-4) and interleukin-2 (IL-2) were also shown to contribute to this process.[8-11]

Hematopoiesis takes place in the yolk sac in the embryo and fetus, but shifts to the liver and spleen, and then to the bone marrow after birth. The potential importance of hepatocyte growth factor (HGF) in fetal hematopoiesis was recognized when HGF, and its receptor, *c-met*, were detected in the yolk sac and the fetal liver.[12,13] They were then shown to be present in bone marrow cells and stroma in adults, suggesting that HGF may have a role in hematopoiesis throughout human life.[14,15] Furthermore, HGF is known to induce mitosis in hematopoietic stem cells,[15] and previous studies demonstrated that it induces proliferation and differentiation of stem cells by augmenting the effects of kit ligand, IL-3, GM-CSF and erythropoetin, and leads to a 5-8 times increase in BFU-E and CFU-GEM.[14-16] HGF also has a role in IL-6 and TNF-induced monocyte differentiation.[17] The HGF receptor, *c-met*, is upregulated in activated monoctyes, suggesting that it may have an important role in B and T-lymphocyte function.[18-20] HGF is likely to have other important interactions, which are yet to be discovered. The aim of this study was to investigate whether HGF on its own, or in combination with GM-CSF and TNF-α, is involved in the development of dendritic cells from CD34+ bone marrow cells.

Design and Methods

CD34+ cell isolation and culture

Mononuclear cells were isolated from heparinized bone marrow aspirates of three consenting steady state healthy adult subjects by Ficoll density gradient separation (Density 1077, Lymphoprep Nycomed Pharma, Oslo, cat. #608096). The subjects were peripheral blood stem cell donors for transplantation, and bone marrow examination was performed

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for routine examination. Stem cells were positively purified with CD34 MoAb 561-coated M 450 Dynabeads (Dynal, Oslo, Norway), incubating 4×10^7 beads with 2.5 to 5×10^7 cells/mL for 45 minutes at 4°C. Rosetted cells were separated with a magnet, and beads were detached from the cells with DETACHaBEAD CD34 (Dynal) according to the manufacturer's instructions. This yielded 91±6% pure viable CD34 cells.

$CD34^+$ cells were incubated in culture medium in 8 separate groups, which were the medium with nothing else added as the control, HGF, GM-CSF, TNF, GM-CSF/HGF, TNF/HGF, GM-CSF/TNF, and GM-CSF/TNF/HGF groups.

Cell culture was performed in liquid culture medium by transferring 5×10^5 $CD34^+$ cells/mL into IMDM (Sigma cat. #95H46381), which consisted of inactivated 20% fetal bovine serum (Gibco, cat. #10109-064), 10 mmol/L HEPES, 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2-mercaptoethanol, 100 µg/mL of penicillin, and 100 µg/mL streptomycin. Two hundred µg/mL of GM-CSF (Schering-Plough, Leucomax), 50 u/mL of TNF (Sigma, cat. #M021265) and 20 ng/mL of HGF (Sigma cat. #H1404) were then added to the culture media of the study groups. Samples were distributed in 6-well plates for each of the experiment groups, and cells were incubated under 5% CO_2 atmosphere at 37°C. Suspensions within these plates were removed every 4 days, centrifuged at 800 g followed by separation of the cells, and transferred to new 6-well plates containing culture media with the corresponding growth factor mixture. The experiment was concluded following two passages and 12 days of incubation.

Flow cytometry cell surface marker analysis

Dual antibody staining was carried out on days 4, 8 and 12, in order to search for changes in antigen expression as the experiment progressed. The following antibodies were used in this study: IgG1-FITC/IgG1-PE (Immunotech cat. #1203-Isotype control); CD1a-FITC/HLA-DR-PE (Pharmingen cat. #34224X/Immunotech cat. #1295); CD1a-FITC/CD13-PE (Pharmingen cat. #34224X/Immunotech cat #0778); CD14-FITC (Immunotech cat. #1420).

After 10 µL of antibody had been added to 100 µL of the cells, they were incubated at 4°C for 20 minutes. The cells were then fixed by Q-prep (Coulter-Louton, England), and analyzed by flow cytometry (Coulter Epics Elite-EST), which was calibrated by using an immuno-check kit (Coulter) prior to analysis, and CVs were adjusted to 2%. The area of analysis was gated at forward scatter (FSC) and side scatter (SSC) axes and co-ordinates using isotypic controls, and fluorescent intensities of the reflections due to background and non-specific staining was adjusted to 2%. Logarithmic analyses were then performed on the reflections due to FITC (analyzed by PMT-2) and PE (analyzed by PMT-3).

Mixed leukocyte reaction

Responder cells in the mixed lymphocyte reaction were peripheral blood mononuclear cells obtained by Ficoll density gradient separation. These cells (5×10^4/100 µL) were cultured in RPMI 1640 medium containing 10% FCS, 1% glutamine, and 1% antibiotics, in 96-well U-bottomed culture plates (Costar Cambridge, MA, USA) for 3 days with 30 Gy irradiated stimulatory cells (1×10^4/100 µL). The stimulatory cells consisted of a control group of allogeneic monocytes, which were isolated from a healthy adult and the various cell populations generated from the $CD34^+$ cells in this study. $^{[3H]}$thymidine (Amersham, UK) incorporation was measured following 8 hours pulsing with 1 µCi/well. The mean counts per minute (cpm) of the three study samples were estimated for each group.

Staining and other assays

Cell counts in all suspensions were performed by Coulter Max-M, and morphologic analyses were performed by light microscopy following Wright staining. Viability was assessed by the trypan blue dye exclusion test.

Statistics

Statistical analysis was performed by paired-t test, and $p<0.05$ was accepted as being statistically significant. Results are presented as mean ± standard deviation.

Results

Three independent experiments were performed and the mean of the results was used for the final analysis. Cell counts and cell surface molecule expression, evaluated on the 4th day of the incubation, are summarized in Table 1.

Differences between the study groups began to appear on the 8th day of incubation (Table 2). Both cell surface molecule expression and cell counts were significantly greater at that time than on the 4th day

Table 1. Surface molecule expression and cell counts on the 4th day of incubation.

	Control	*HGF*	*GM-CSF/ TNF*	*GM-CSF TNF/HGF*	*GM-CSF*	*GM-CSF/HGF*	*TNF/HGF*	*TNF*
CD1a (%)	4±1	6±1.9	4.2±1.2	5.4±1.1	5.1±0.8	3.8±0.8	3.7±1.4	3.9±1
CD14 (%)	0.7±1	0.4±0.2	0.7±0.1	0.6±0.08	0.5±0.05	0.8±0.04	0.6±0.02	0.7±0.03
CD1a-DR (%)	3.2±0.6	3±0.8	3±0.2	3.1±1	3±0.5	2.5±0.8	3.1±0.8	3.2±0.08
CD1a-13 (%)	3.9±1.2	4.8±1	3.9±0.8	4.5±0.7	4.2±1	3.8±1.1	3.1±0.8	3±1
Cell count (x10^6/mL)	0.7±0.04	0.8±0.06	0.8±0.05	0.74±0.04	0.88±0.08	0.82±0.07	0.68±0.1	0.69±0.1

Values are the mean ± SD of 6 wells and three independent experiments. There was no statistical difference between the study groups on the 4th day of incubation.

Table 2. Surface molecule expression and cell counts on the 8th day of incubation.

	Control	*HGF*	*GM-CSF/ TNF*	*GM-CSF TNF/HGF*	*GM-CSF*	*GM-CSF/HGF*	*TNF/HGF*	*TNF*
CD1a (%)	12.8±2.8	16±2.1	13±2	16.1±2.8	15.3±2	15±1.4	13±2.4	14±1.9
CD14 (%)	20±2.7	16.2±3	8.4±1.6	10.4±1.5	25.4±1.9	28.5±2.6	8±1.4	10.4±2
CD1a-DR (%)	9.1±1.9	9.9±1	9.1±2	10.6±3	10.3±1.3	11.7±1.8	11.4±1	10.8±1.1
CD1a-13 (%)	11±2.8	12.4±2.6	11.6±2.1	12.8±0.9	13±1	12.7±1.1	11.8±1.7	12±1.5
Cell count (x10^6/mL)	1.2±0.2	1.6±0.25	1.5±0.1	1.5±0.2	1.5±0.3	1.7±0.15	0.9±0.1	0.85±0.1

The rise in cell number was similar in all groups with the exception of the TNF and TNF/HGF groups which had the lowest rises ($p<0.05$). There was no difference between the groups with respect to CD1a and CD1a/HLA-DR expression. However, GM-CSF/HGF and GM-CSF groups led to a greater level of CD14 expression than the other groups ($0.001<p<0.05$).

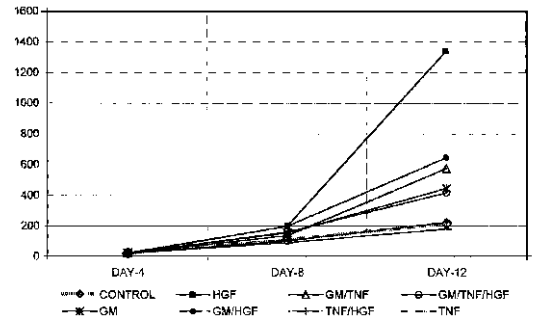

Figure 1. Level of CD1a/HLA-DR positive cell generation from CD34+ cells.

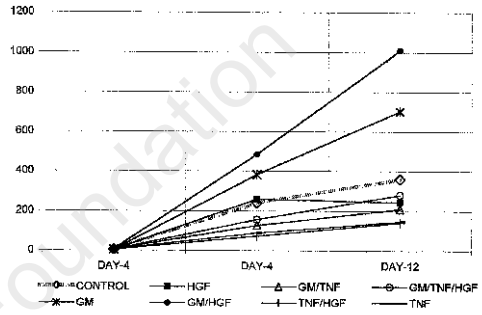

Figure 2. Level of CD14 positive cell generation from CD34+ cells.

of incubation. The rise in cell number was similar in all groups with the exception of TNF and TNF/HGF, which had the lowest rises ($p<0.05$). There was no difference between the groups with respect to CD1a and CD1a/HLA-DR expression (Figure 1). However, GM-CSF/HGF and GM-CSF groups led to a greater level of CD14 expression in comparison with the other groups ($0.001< p<0.05$) (Figure 2).

Cell counts on the 12th day of incubation were significantly greater than those on the 8th day of incubation for all groups (Table 3). Maximum rises were encountered in HGF, GM-CSF, and HGF/GM-CSF groups, and the rises in cell counts on the 12th day of incubation were again lowest in the TNF-containing groups ($0.001< p<0.05$).

The proportion of cells with CD1a and CD1a/HLA-DR positivity on the 12th day of incubation was also significantly greater than that on the 8th day of incubation for all groups (Figure 1). CD1a and CD1a-HLA-DR expression were highest in the HGF group, being nearly 2 to 3 times the levels encountered with GM-CSF/TNF and GM-CSF/HGF groups, which had the next highest levels of expression ($0.0001<p<0.05$). CD1A-HLA-DR expression in the GM-CSF, TNF, TNF/HGF and HGF/GM-CSF/TNF groups was similar. CD13 expression of the CD1a positive cells decreased significantly between the 8th and 12th days of incubation in all groups.

CD14 expression on the 12th day of incubation decreased in the HGF group whilst it significantly increased in the HGF/GM-CSF group in comparison with the 8th day of incubation (Figure 2). CD14 expression was greatest with the GM-CSF/HGF group next greatest with the GM-CSF group, with HGF only while TNF-containing groups had the lowest levels of expression ($0.0001<p<0.05$). The GM-CSF/TNF group, despite leading to a similar level of CD1a expression as the GM-CSF/HGF group, suppressed CD14 expression. It, therefore, resulted in a high level of dendritic cells, but only a low level of monocyte development.

Mixed leukocyte cultures were performed on the 12th day of incubation in order to investigate the ability of the cultured cells to induce T-lymphocyte proliferation (Table 4). The cell suspension prepared from the HGF group led to the highest levels of T-lymphocyte proliferation. This proliferation was more than double that caused by the next effective groups, GM-CSF/TNF and GM-CSF/HGF, and was 13.5 times greater than the proliferative effect of the monocyte suspension ($0.0001<p<0.05$). These findings match the CD1a and CD1a/HLA-DR expression profiles induced by these particular groups.

Cells were also evaluated microscopically on the

Table 3. Surface molecule expression and cell counts on the 12th day of incubation

	Control	*GF*	*GM-CSF/ TNF*	*GM-CSF TNF/HGF*	*GM-CSF*	*GM-CSF/HGF*	*TNF/HGF*	*TNF*
CD1a (%)	16±1	65.9±6.7	30±3.1	20.1±3.1	21±2.4	28±2.2	20±1.9	19±1.6
CD14 (%)	20±3.8	9±2.6	9.1±2	10.8±1.8	26±2.9	36±2.8	10.1±1.1	12.1±1.2
CD1a-DR (%)	12±1.1	49.6±4.5	25±2.5	16±1.9	16.5±1.5	23±2.1	16±1.3	15.±2
CD1a-13 (%)	11.4±1	10.1±1.8	11.8±2.1	13±1.2	12.7±2	13.1±2.2	10.7±1.4	10.5±1.3
Cell counts	1.8±0.4	2.7±0.35	2.3±0.2	2.6±0.2	2.7±0.3	2.8±0.25	1.4±0.15	1.2±0.2

Maximum rise in cell counts was found in the HGF, GM-CSF, and HGF/GM-CSF groups. The rise was lowest in the TNF-containing groups (0.001<p<0.05). CD1a and CD1a-HLA-DR expression was highest in the HGF group, being nearly 2 to 3 times that found in the GM-CSF/TNF and GM-CSF/HGF groups, which had the next highest levels of expression (0.0001<p<0.05). CD1A-HLA-DR expression in the GM-CSF, TNF, TNF/HGF and HGF/GM-CSF/TNF groups was similar. CD14 expression was greatest in the GM-CSF/HGF group, next greatest in the GM-CSF group, with HGF only and TNF-containing groups having the lowest levels of expression (0.0001<p<0.05).

Table 4. Mixed leukocyte reaction results.

Control	*HGF*	*GM-CSF TNF*	*GM-CSF TNF/HGF*	*GM-CSF*	*GM-CSF HGF*	*TNF/ HGF*	*TNF*	*Monocyte*
348±55	2440±520	1080±340	530±98	580±110	1000±270	595±68	550±49	180±40

Values are the mean counts per minute (cpm) ± SD of 8 wells and three separate study samples. The cell suspension prepared from the HGF group led to the highest level of T-lymphocyte proliferation which was more than double the level of proliferation caused by the next effective groups, GM-CSF/TNF and GM-CSF/HGF, and 13.5 times greater than the proliferative effect of the monocyte suspension (0.0001<p<0.05).

4th, 8th, and 12th days of incubation, and the increase in the dendritic cell counts with time was also confirmed morphologically. Cell viability was 94±3% in all groups on the 12th day of culture.

Discussion

Dendritic cells have been generated from CD34 positive cells by various methods and under various culture conditions. Incubation of CD34 positive cells from peripheral blood, bone marrow, or cord blood with a combination of GM-CSF and TNF is an efficient method of isolating DCs. Better yields have been reported when *leukocyte conditioning medium*, IL-3, kit ligand, FLT-3 ligand, and TGF-β, has been added to this combination.[2,3,9,21-23] A combination of kit ligand and FLT-3 ligand was shown to lead to a five-fold increase of $CD1a^+/CD14^+$ cells,[24] and Bykovskaja *et al.* recently reported that IL-2 on its own, or TNF-α in combination with kit ligand, may also lead to DC development.[11]

There have been no reports in the literature analyzing the role of HGF in DC development. We investigated this in our study by incubating CD34 positive bone marrow cells in various combinations of HGF, GM-CSF and TNF-α for 12 days. An increase in CD1a expression on $CD34^+$ cells was observed in all groups on the 8th day, and this reached a maximum on the 12th day of incubation. Cell counts decreased from the 12th day onwards, and a measurable level of apoptosis was detected in all culture media (unpublished results). This is consistent with other studies, which report an increase in CD1a expression from the 4th to 5th days of culture, reaching a maximum on the 10th to 12th days, followed by a decrease in cell numbers during the ensuing days.[21-23] CD13 expression of CD1a positive cells decreased significantly between the 8th and 12th days of incubation in all groups in this study. This finding is consistent with other reports, in which dendritic cell precursors were reported to express CD13, which disappeared with maturation.[25,26]

The most important finding of this study was that HGF, on its own, enhanced CD1a and CD1a-HLA DR expression to nearly double the levels produced by the GM-CSF/TNF combination. Previous studies showed that HGF with GM-CSF and/or IL-3 increases granulocyte-monocyte colony formation from stem cells by a factor of 5 to 8,[15] and that it has a major role in the development of monocytes and macrophages,[17,20] but the effect of HGF on CD1a positive cell development was not analyzed.

The combination of HGF with GM-CSF failed to achieve the same amount of CD1a positive cell development as that with HGF alone. GM-CSF is known to potentiate the development of CD14 positive as well as CD1a positive cells, and HGF is known to augment the effect of GM-CSF in the development of CD14 positive cells from CD34 positive cells. Therefore, the effect of the combination of GM-CSF and HGF could have been split into generating both CD1a and CD14 positive cells, leading to a lower level of CD1a positive cell development than expected in the light of the effect of HGF alone.

The combination of HGF with TNF led to a similar level of CD1a positive cell development as that produced by the TNF only group, which was nearly one third of the HGF only group. Therefore, TNF appears to block the effect of HGF in this context. This was also supported by the finding that the level of CD1a positive cells of the GM-CSF/TNF/HGF group was similar to that of the GM-CSF only group, but lower than that of the GM-CSF/TNF group. Furthermore, CD14 positive cell development of the GM-CSF/TNF/HGF group was lower than that of the GM-

CSF/HGF group, which implies that TNF blocks the effects of HGF in this context too. Previous studies reported that HGF could stimulate stem cell proliferation and differentiation directly or indirectly,[14-16] and that TNF slowed stem cell development, leading to the termination of the stem cell cycle in the G0/G1 phase.[27,28] These findings were further supported by the results of this study, which showed that HGF alone, or HGF in combination with GM-CSF led to higher cell counts, and TNF and TNF-containing groups led to lower cell counts. There have been reports of antagonism between TNF and HGF in other cell lineages. For instance, HGF stimulates and TNF inhibits the development of melanocytes, and hepatocyte cytotoxicity during viral hepatitis is potentiated by TNF and inhibited by HGF.[29,30] It is, therefore, possible that TNF does oppose the effects of HGF. However, this view is not firmly established, as a number of reports which showed that TNF stimulates *c-met*, the receptor of HGF and induces stromal cell HGF synthesis, present contrasting views.[14]

The combination of TNF with GM-CSF led to a significant inhibition of the CD14 proliferative effect of the latter, possibly by forcing the cell into a CD1a positive lineage. This finding is in agreement with previous studies reporting the effect of combining GM-CSF with TNF on stem cell development.[8,21,22]

All cytokine combinations utilized in this study resulted only in a 4-fold increase of cell number. It will be necessary to test the combination of HGF with cytokines displaying a relevant proliferative potential such as FLT-3 or kit ligand.

Results of the mixed lymphocyte reaction, which is a measure of the functional capacity of dendritic cells, provided further evidence that HGF has a strong influence on dendritic cell development from $CD34^+$ bone marrow cells, as the HGF group led to 2 to 13.5 the level of T-lymphocyte proliferation caused by other groups. Dendritic cell suspensions were previously reported to lead to 100 times greater T-lymphocyte proliferation in mixed lymphocyte reactions than other cell lineages.[1-3] The lower levels of T-lymphocyte proliferation in response to dendritic cells in this study could be the result of using less pure dendritic cell samples (the suspension of highest dendritic cell purity in this study was approximately 50%), or due to differences between this study and previous reports with respect to the culture conditions and the dendritic cell/T-lymphocyte ratio.

Another finding of interest was the development of CD1a and CD1a/HLA-DR positive cells in the control group, which had no cytokine content. This finding was paralled by the results of the mixed lymphocyte reactions, in which the control group led to a greater level of T-lymphocyte proliferation than the monocyte group. Reid *et al.* reported 20% or more dendritic cell development in culture media which contained only serum.[8] The cytokine content of the serum, TGF-β in particular, is thought to be responsible for this development.[23] Additional experiments will be essential in order to achieve the same results with clinical grade materials such as human serum or serum-free medium. In fact, Rosenzwaig *et al.* obtained a dramatic increase of $CD1a^+/CD14^+$ cells by supplementing $CD34^+$ cell culture with IL-13 or IL-4, but a comparable increase of DCs was not confirmed when human serum was used instead of FCS.[31] Furthermore, a study should be performed to demonstrate that DCs possess HGF receptors, and the effect of HGF on the expression of DC maturation-activation antigens CD80, CD83 and CD86 should be demonstrated.

In conclusion, this study shows that HGF alone induces a greater level of dendritic cell development from CD34 positive bone marrow cells than GM-CSF, TNF, or the combination of GM-CSF with TNF. HGF also augments GM-CSF associated development of both dendritic cells and monocytes, but its effects are apparently blocked by the concurrent use of TNF.

Contributions and Acknowledgments

EO: conception, design, CD34 selection, cell cultures, drafting, critical revision for intellectual content. SR: design, critical revision for intellectual content, drafting, final approval. AAK: design, mixed lymphocyte cultures, data analysis. YT: design, flow cytometric analysis and interpretation. MC: design, microscopic analysis and staining, preparations of the graphics. SK: design and statistical analysis. FA: design and statistical analysis. TA: design, supervision of the work, final approval. The order of the authorship in this study was a joint decision by the co-authors and was based on the relative amount of effort and time spent in contributions described by the Vancouver criteria with all authors participating in a) conception and design, or analysis and interpretation of data; b) drafting the article or revising it critically for important intellectual content and c) final approval of the version to be submitted.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential Implications for clinical practice

- Hepatocyte growth factor may be utilized as a component of a cocktail of cytokines to generate dendritic cells for the purpose of tumor vaccination.[32-34]

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