MAINTAINING DENDRITIC CELL VIABILITY

David Vremec[1], Jacinta Hansen[1], Andreas Strasser[1,2], Philippe Bouillet[1,2], Hans Acha-Orbea[3], Yifan Zhan[1], Meredith O'Keeffe[4] and Ken Shortman[1,2,4].

[1] The Walter and Eliza Hall Institute, 1G Royal Parade, Parkville, Vic. 3052, Australia

[2.]Department of Medical Biology, University of Melbourne, Melbourne, Vic. 3010, Australia.

[3.]Department of Biochemistry, University of Lausanne, Epilanges, Switzerland.

[4.]Burnet Institute, Melbourne, Vic. 3004, Australia

ABSTRACT

When dendritic cells (DCs) are isolated from tissues, purified and placed in a nutritive culture they die more rapidly than their normal turnover *in vivo*. This can distort culture assays of DC function. We therefore tested several approaches to prolonging DC survival in culture. Of the cytokines tested GM-CSF was most effective at preserving the viability of conventional DCs (cDCs) but was ineffective for plasmacytoid DCs (pDCs). Surprisingly, fms-like tyrosine kinase 3 ligand (FL) produced only a marginal improvement in DC survival and IL7 improved survival of $CD8^-$ cDCs, but not of other DCs. Genetic manipulation of cell death pathways was tested, to avoid activation effects of cytokine signalling. The isolation of DCs from mice transgenic for bcl-2 under the vav promotor was especially effective in maintaining pDC viability but gave only partial improvement in cDC viability. In contrast, $CD8^-$ cDC isolated from $bim^{-/-}noxa^{-/-}$ mice showed excellent culture survival, whereas pDC and $CD8^+$ cDC from these mice showed only marginal improvement in viability.

INTRODUCTION

To determine the role of individual cells of the immune system, particular cell types are often isolated and purified from lymphoid tissues, then their functional capacity analysed in culture. This reductionist approach can yield valuable information, but removal from the tissue environment may also distort the behaviour of the cells away from their normal *in vivo* functions. This has been a significant problem in the study of dendritic cells (DCs). One culture artefact we have previously studied is the "spontaneous" activation of mouse spleen DCs when purified then placed in culture; we found this was largely due to the artificially close proximity of the pure DCs to each other, resulting in a form of self activation[1]. A more serious problem is the fast rate of death of certain DC subtypes in culture; this can distort *in vitro* assays of such functions as antigen processing and presentation. This problem is especially troublesome with mouse plasmacytoid DCs (pDCs) that survive for weeks *in vivo*[2] but may die very rapidly in culture[3]. It is also a major problem with the $CD8^+$ subset of conventional DCs

(cDCs) isolated from mouse spleen that already have a rapid turnover *in vivo*[4], but this is increased *in vitro* to the point that even overnight culture produces a marked loss of cell viability.

We now test several cytokines involved in DC development and function *in vivo* to determine their effectiveness in maintaining viability in culture. We also test some mice with genetic modifications of the cell death pathways[5], to determine if such mice could provide a convenient source of DCs with an extended culture survival time.

RESULTS AND DISCUSSION.

DC death in culture

When individual DC subtypes are extracted from mouse spleen, purified by sorting then placed in a nutritive culture medium that supports the development of these DC types in culture[3,6,7], but without the usual cytokines or activating agents, they do not expand in numbers but show a rapid loss of viability (Figures 1 and 2). Note that these Figures depict the drop in the total recovery of viable cells compared to the original count, not the percentage of viable cells amongst those cells recovered; the latter can give false concept of survival if cells that die disintegrate or otherwise disappear from the count. Only around 25% of the pDC survived overnight (18hr) culture, although they show a half-life of around a week *in vivo*[2]. Almost no pDC survived by 2 days of culture. The $CD8^+$ cDCs also showed very poor survival, only 15% remaining after overnight culture, in contrast to an apparent half-life of 1.5 days in the spleen[4]; this turnover rate in spleen is now known to be due to limited DC division as well as loss by death[8], so the actual death rate *in vivo* may be slower. The $CD8^-$ cDC survived much better in culture, with only 30% loss overnight, although this rate of death is still beyond the apparent half-life in spleen of 2.5 days[4].

Culture survival in the presence of cytokines

We tested if some cytokines suspected of enhancing DC survival *in vivo* could prolong DC survival in culture (Figure 1). Granulocyte-macrophage colony stimulating factor (GM-CSF), although not the major cytokine driving differentiation of these DC types, has been shown to contribute to their development[3,9], possibly by reducing cell death. It has been used to sustain DC viability during culture assays of antigen presentation, although it has additional effects including the induction of CD103 and the promotion of antigen cross-presentation[3]. Addition of GM-CSF did indeed promote the survival of cDCs, with over half the $CD8^+$ cDCs and most $CD8^-$ cDCs now surviving overnight culture. However GM-CSF gave no improvement in survival of pDCs.

Since the cytokine fms-like tyrosine kinase 3 ligand (FL) is the major cytokine driving development of these spleen DC types *in vivo*[9,10] as well as *in vitro*[3,6,7] and since it also promotes some expansion of the $CD8^+$ cDCs in the spleen[8], it seemed a likely candidate for promoting DC survival in culture. However addition of FL gave only marginal improvement in the survival of all DC types, and was less effective than GM-CSF in maintaining survival of the cDCs.

Some pDCs are believed to arise from lymphoid precursors[3] and this is promoted by the "lymphoid" cytokine IL7[11]. In addition pDCs have been reported to express mRNA for the IL7 receptor α chain[12]. To determine if IL7 would therefore promote the survival of pDCs, it was added to the cultures. Surprisingly IL7 gave no improvement in culture survival of pDCs, nor of $CD8^+$ cDCs, but did promote the survival of the $CD8^-$ cDCs.

Improved DC survival by genetic manipulation of apoptosis pathways

Although improved DC survival can be obtained by addition of certain cytokines, these activate a range of other DC functions that will obscure the interpretation of many culture-based experiments. Accordingly we tested if DCs derived from certain genetically engineered mice with blockages to cell death pathways[5] would show improved survival in culture (Figure 2).

Apoptosis in some cells may also be blocked by increased expression of bcl-2[16,17]. Introduction of a bcl-2 transgene has been shown to protect pDC from activation-induced death[18]. Accordingly we tested the DCs isolated from transgenic mice expressing bcl2 under the vav promoter[16]. Strikingly the pDCs now showed almost complete survival over the culture period. This seemed similar to the original results with cells of the B lymphocyte lineage, where high expression of bcl-2 blocked apoptosis and allowed prolonged culture survival[16,17]. Both the $CD8^+$ and the $CD8^-$ cDC subsets from these mice also showed improved culture survival, but much less than for pDCs.

The $CD8^+$ cDC subset is particularly prone to rapid activation induced cell death, via a type 1 interferon mediated apoptotic process[13]. In the absence of the pro-apoptotic molecules bim[14] and noxa[15], this activation-induced death is blocked[13]. Accordingly we tested if, even in the absence of activation, spleen $CD8^+$ cDCs from $bim^{-/-}noxa^{-/-}$ mice would show improved survival in culture. An improved

but still partial survival of $CD8^+$ cDCs and also of pDCs was obtained. However the $CD8^-$ cDCs now showed full survival and possibly some expansion in culture.

Conclusions

We have documented several approaches that can lead to extended survival of DCs in culture. The response of pDCs differed from that of cDCs in these experiments; in this as in other respects pDCs resembled B lineage cells as much as cDCs. In contrast to the $CD8^-$ cDCs, where near full survival could be maintained for 2 days, the $CD8^+$ cDC subtype displayed significant cell death under all culture conditions tested; however this correlates with their rapid turnover in the spleen[4]. It was surprising that FL, the key cytokine for DC development, had only a minimal effect on DC culture survival and equally surprising the IL7 did promote survival, not of pDCs but only of $CD8^-$ cDCs. However the addition of GM-CSF was a simple way of extending the culture survival of the $CD8^+$ cDCs, as well as the $CD8^-$ cDCs, to almost as long as *in vivo*, although GM-CSF did not improve the culture survival of pDCs. However GM-CSF has multiple effects on cDCs, so this approach is not always useful. Using mice genetically modified to block cell death pathways can prolong DC survival without triggering cytokine responses. Although survival, particularly of $CD8^-$ cDCs, was prolonged using $bim^{-/-}noxa^{-/-}$ mice, we found breeding these mice was difficult. The bcl2-vav transgenic mice, easier to breed, provided a practical source of spleen DCs with some improvement in cDC survival and with remarkable pDC survival in culture.

METHODS

Mice

All mice were bred at the Walter and Eliza Hall Institute (WEHI). Most experiments used C57BL/6J Wehi mice, 6-8 weeks old. The $bim^{-/-}$ $noxa^{-/-}$ mice[13] were selecting after crossing $bim^{+/-}$ $noxa^{-/-}$ mice with $bim^{+/-}$ $noxa^{-/-}$ or $bim^{+/-}$ $noxa^{+/-}$ mice. The bcl2-vav transgenic mice[17] were selected after crossing male

transgenics with wild-type females. All procedures were approved by the WEHI Animal Ethics Committee.

Isolation of spleen DCs

Spleens were chopped, digested with collagenase-DNase, light density cells isolated by a density cut, non-DCs depleted using immunomagnetic beads, then the individual DC subtypes sorted, as described in full detail elsewhere[19]. Cells were labelled with antibodies against CD11c (N418, used as a PE conjugate) CD45RA (14.8, used as an APC conjugate), CD8 (YTS169.4, used as a PerCp.Cy5.5 conjugate) and CD172a (P84, used as an FITC conjugate). Any residual NK cells, T cells or B cells were identified using biotinylated antibodies against CD49b (DX5), TCRβ (H57-597) and CD19 (1D3) respectively, and stained with a PE.Cy7-Streptavidin secondary reagent. Propidium iodide (PI, 0.5ug/mL) was added in the final wash to label dead cells. Sorting was performed on a FACSAriaII instrument (BD, San Jose, CA, USA) gating out dead cells, autofluorescent cells and doublets. The pDCs were sorted as $CD11c^{int}$ $CD45RA^{hi}$ $CD49b^{-}$ $CD19^{-}$ $TCR\beta^{-}$, the $CD8^{+}$ cDCs as $CD11c^{hi}$ $CD45RA^{-}$ $CD49b^{-}$ $CD19^{-}$ $TCR\beta^{-}$ $CD8^{+}$ $CD172a^{-}$ and the $CD8^{-}$cDCs as $CD11c^{hi}$ $CD45RA^{-}$ $CD49b^{-}$ $CD19^{-}$ $TCR\beta^{-}$ $CD8^{-}$ $CD172a^{+}$. Sorted cells were recovered, counted and suspended in culture medium. Purity of sorted DC subtypes was 98 to 99%.

Culture survival assays

The incubation medium[6,7] was a modified RPMI-1640 medium with additional HEPES buffering, iso-osmotic with mouse serum (308mOs), containing 10% foetal calf serum (FCS). Incubation was for periods between 30 min (at which point samples were taken as the zero time control) and 42 hours at 37°C in a 10% CO_2-in-air incubator. Cells were cultured at 5 x 10^5 cells/mL, in a total volume of 200uL, in flat-bottom wells of a 96-well plate (BD). When required, Flt3L was added at 200ng/mL and GM-CSF at 1ng/mL and IL7 at 5 ng/mL. After the culture period, 25uL of a 10^6 particles/mL solution of blank 6-6.4 um Sphero calibration beads (BD) was added to each well and the plate centrifuged. The supernatant was removed and the pellets resuspended in 50uL EDTA-BSS-2%FCS containing 0.5ug/mL PI. Samples were analysed on an LSRII instrument

(BD) and files containing 5000 beads collected. The recovery of PI-excluding viable cells was calculated relative to the calibration bead count.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

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FIGURE LEGENDS

FIGURE 1. The effect of cytokines on DC survival in culture. DCs were isolated and purified from mouse spleen, then incubated in a nutritive culture medium with or without the addition of optimal concentrations of cytokines. The total recovery of viable (propidium iodine excluding) cells is presented. Open circles indicate controls without cytokine addition, closed circles cultures with cytokine added. Results are means +/- SEM of pooled data from 3 experiments, each with 3 cultures per point. The significance of differences in survival at 42 hr is shown as *** when $p<.001$.

FIGURE 2. The effect of genetic manipulation of cell apoptosis pathways on DC survival in culture. DCs were isolated and purified from the spleen of wildtype or genetically engineered mice and incubated in a nutritive culture medium without the addition of cytokines. The total recovery of viable (propidium iodide excluding) cells is presented. Open circles represent DCs from wildtype mice, closed circles DCs from genetically engineered mice. Results are the means +/- SEM of pooled data from 2 experiments, each with 3 cultures per point. The significance of differences in survival at 42 hr is shown as *** when $p<.001$.

Figure 1

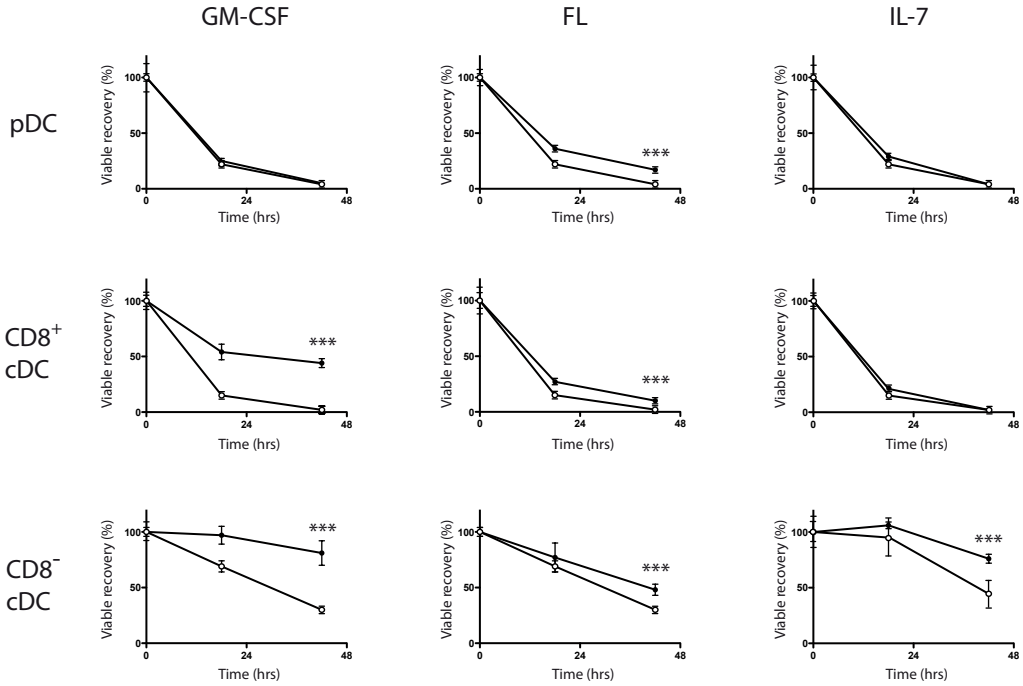
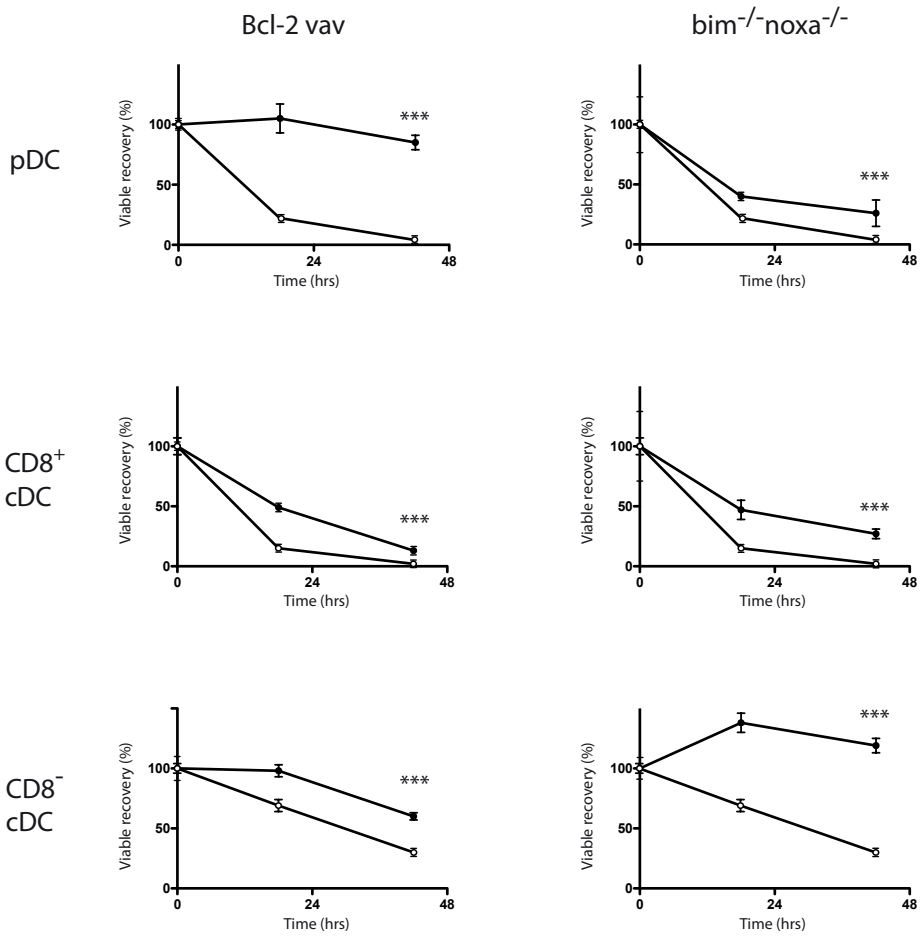

GM-CSF
FL
IL-7
pDC
CD8+ cDC
CD8- cDC
Viable recovery (%)
Time (hrs)
0
24
48
50
100
***

Figure 2


Bcl-2 vav
bim-/-noxa-/-
pDC
CD8+ cDC
CD8- cDC
Viable recovery (%)
Time (hrs)
***
0
24
48
50
100