Micro-environmental factors in the survival of human B-lymphoma cells

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Abstract

Burkitt lymphoma (BL) cells retain a high inherent propensity to undergo apoptosis indicating that net growth of the tumour population *in vivo* is likely to be influenced profoundly by its micro-environment. Here we investigate micro-environmental factors that affect BL-cell survival *in vitro*. We show that survival, and consequently net production, of tumour cells is enhanced by autocrine factors and, to a greater extent, by paracrine factors provided by relevant stromal elements of the tumour (fibroblasts and follicular dendritic cells) and by macrophages. Promotion of BL-cell survival by paracrine elements was mediated by cell/cell contact and by short-range soluble factor(s). IL-4, IL-10 and TNF-α promoted, whereas TGF-β1 inhibited, tumour-cell production. Macrophages engaged in phagocytosis of apoptotic BL cells were less effective than untreated macrophages in supporting net expansion of BL populations. These results suggest that the net production of tumour cells in BL is supported by multiple micro-environmental factors that modulate apoptosis. *Cell Death and Differentiation* (2000) **7**, 59 – 69.

Keywords: apoptosis; B-cell; Burkitt; lymphoma; micro-environment; macrophage

Abbreviations: bFGF, basic fibroblast growth factor; BL, Burkitt lymphoma; CD40L, CD40 ligand; EGF, epidermal growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HFF, human foetal fibroblast; IGF, insulin-like growth factor; IL, interleukin; LPS, lipopolysaccharide; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor

Introduction

Net growth in cell populations is governed by factors that regulate (1) cell-cycle entry, progression or exit and (2) apoptosis. Paradoxically, oncogene activity can lead not only to increased rates of cell-cycle entry and progression but also to increased susceptibility to apoptosis (reviewed in[1]). c-Myc, for example, appears to drive both proliferation and apoptosis through common properties that are required for its DNA-binding and transcriptional-regulatory activities, the proliferative response being dependent upon the presence of additional signals derived from receptors for cell-growth/survival factors.[2,3] Availability of apoptosis-inhibiting signals derived from the micro-environment of tumours is therefore potentially of great significance to net tumour growth. Little information, however, is currently available on the nature of such survival signals. Here we investigate this question in relation to Burkitt lymphoma (BL).

BL is a monoclonal B-cell malignancy characterised by translocations resulting in the juxtapositioning of c-*myc* to immunoglobulin heavy-chain or light-chain loci (t(8;14), t(2;8) or t(8;22) translocations) (reviewed in[4]). The lymphoma probably arises at a germinal-center stage of B-cell development[5,6] and vestiges of the phenotype of normal germinal-centre B cells are retained by BL tumour cells and by certain BL-derived cell lines. These features include (1) display of a specific pattern of cell-surface antigens (CD10+CD23−CD39−CD77+) that is restricted to germinal-center B cells,[5,7,8] (2) presence of little or none of the cytoplasmic inhibitor of apoptosis, Bcl-2,[9,10] and (3) a propensity to undergo apoptosis rapidly by default,[11] a necessary property underlying normal physiological selection of germinal-centre cells.[12] BL-derived cell lines that retain these features have been previously categorised as 'group I' lines.[7,8] These lines used in early passage have provided important models for studies of BL *in vitro* that are likely to have *in vivo* relevance. In this study we use a group I BL cell line to model *in vitro* the responses of BL cells to micro-environmental factors.

In situ, BL tumour-cell populations show evidence both of rapid proliferation and of frequent apoptosis, a likely result of the deregulation of c-Myc which drives not only proliferation but also apoptosis in BL tumour cells.[13] Thus, in the histological picture of BL, mitotic figures are readily apparent as is the pyknotic appearance of apoptotic cells and bodies and the apoptotic cell-derived debris contained within the numerous infiltrating 'starry sky' macrophages.[14–17] Phagocytic clearance of apoptotic cells by macrophages is a highly efficient process which confounds accurate assessments of the rates of apoptosis *in vivo*. Apoptotic cells are only visible using standard histological methods for short periods since they are rapidly engulfed by macrophages (which can occur prior to many of the characteristic morphological changes[18]) and, once engulfed, are rapidly degraded such that they

quickly become unrecognisable.[19] Therefore, ready histological observation of free apoptotic cells and of numerous macrophages containing apoptotic debris, as observed in BL, is evidence indeed of high-frequency apoptosis *in situ*. Freshly isolated BL cells and group I lines show a clear tendency to undergo apoptosis by default *in vitro*[10,11,13,20] and the histological picture of BL indicates that this inherent apoptosis-susceptibility is only incompletely blocked *in vivo*. This suggests that autocrine or paracrine factors of limited availability in the micro-environment of the tumour are required to provide survival signals necessary for expansion of the tumour-cell population. In addition, there may be a delicate balance between pro- and anti-apoptotic micro-environmental factors that favours net tumour growth.

In this study we investigate the effects on BL-cell apoptosis of micro-environmental factors that have possible relevance to BL *in vivo*. We demonstrate (1) autocrine and (2) paracrine survival pathways, the latter stimulated by fibroblasts, follicular dendritic cells (the antigen-capturing cells of germinal centres) and macrophages. In addition, we show that certain soluble factors that are produced by macrophages (IL-10 and TNF-α) or T cells (IL-4) can enhance BL-cell population expansion by promoting BL-cell survival. By contrast our observations indicate that TGF-β1, which is known to be produced by macrophages that have phagocytosed apoptotic cells,[21] inhibits BL-cell survival. Significantly, macrophages fed with apoptotic BL cells were less effective than their untreated counterparts in providing soluble factors that support BL-cell survival. Therefore, the tumour micro-environment in BL is likely to provide multiple signals that can contribute both positively and negatively to net tumour growth.

Results

Autocrine/juxtacrine effects on survival of BL cells

BL was modelled in these studies using a group I, biopsy-like line, L3055 in early passage. Initial experiments were undertaken to determine simply the extent to which homotypic interactions between BL cells modulated tumour cell survival. These effects of cell 'crowding' were studied both under optimal (in 10% serum) and suboptimal (1% serum) conditions (Figure 1). Figure 1A shows the levels of apoptosis in L3055 BL-cell populations cultured in 1% serum in vessels allowing settling of cells on flat plastic surfaces of relatively high (25 cm^2) *versus* relatively low (7.6 cm^2) area. As shown in Figure 1B, apoptosis was also monitored in L3055 cells cultured in 10% serum in round-bottomed *versus* flat-bottomed (i.e. less crowded) culture wells. Each of these experimental approaches demonstrated that BL-cell survival was moderately enhanced under conditions in which cells were brought into closer vicinity with their neighbours. Thus, as the seeded concentration of L3055 cells was increased the proportion of apoptotic cells at the end of the culture period fell. Concentration of cells on a smaller flat area (Figure 1A) or onto a curved culture surface (Figure 1B) resulted in reduction in apoptosis at lower cell numbers as compared with cells cultured under less crowded conditions. Expression of Bcl-2 in L3055 cells virtually abolished apoptosis in these cultures at all seeding concentrations tested (Figure 1A). These results show that BL-cell survival is supported by one or more autocrine/juxtacrine factors.

Paracrine effects on survival of BL cells

In order to determine possible effects of non-tumour cells of relevance to BL, we co-cultured L3055 cells with various lineages representative of tumour-infiltrating or stromal cell types, specifically human foetal fibroblasts, a follicular dendritic cell line HK,[22] human monocyte-derived macrophages and, to emulate an activated-T-cell-derived signal (since activated T cells are also known to infiltrate BL[23]), CD40-ligand (CD40L)-expressing L-cells.[24] As shown in Figures 2A and 3A, apoptosis in L3055 cells that were co-cultured with fibroblasts, HK cells, macrophages, or CD40L-expressing cells was substantially inhibited in all cases. In
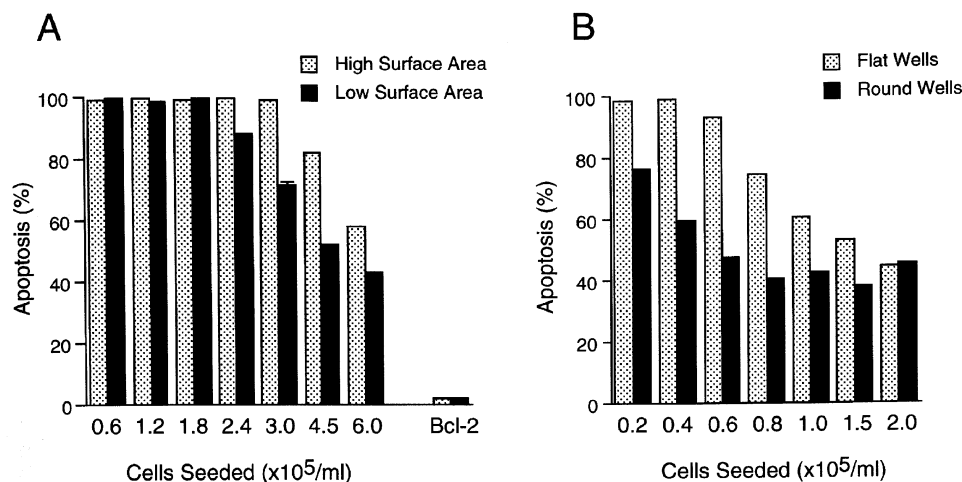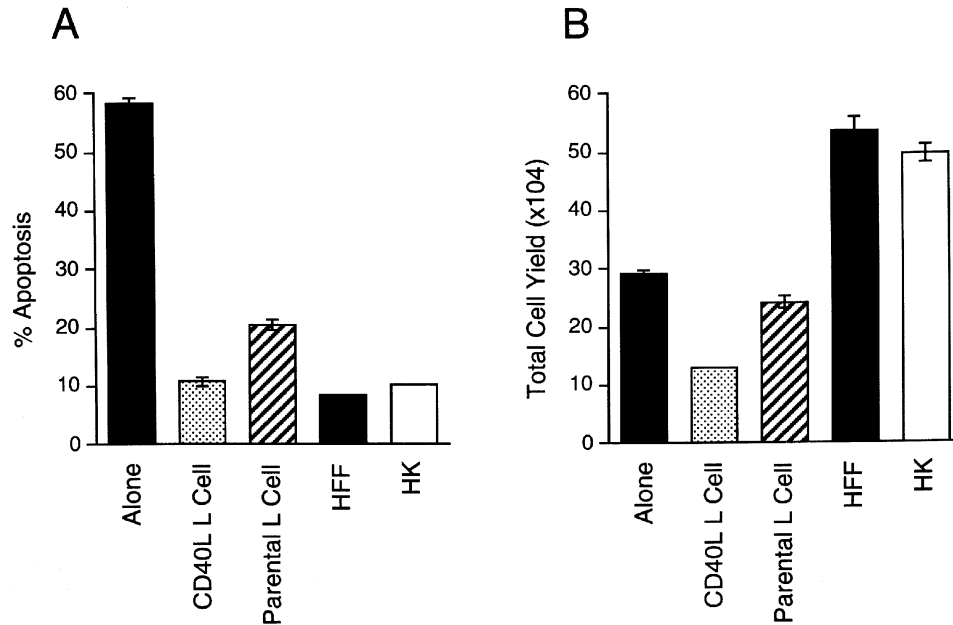

Figure 1 Autocrine survival of BL cells. L3055 cells were cultured at the indicated seeding cell concentrations and apoptosis assessed by light scatter analysis after the incubation period. (**A**) Incubation in flasks for 72 h allowing cells to settle on the surface of high (25 cm^2 stippled bars) or low (7.6 cm^2 filled bars) relative area. Transfectants expressing high levels of Bcl-2 are shown for comparison. Means $\pm$ S.D. of triplicate samples. (**B**) Incubation in flat- (stippled bars) or round- (filled bars) bottomed wells for 96 h. Both experiments representative of three similar

parallel, total yields of L3055 cells were markedly enhanced by co-culture with fibroblasts, HK cells or macrophages (Figures 2B and 3B), indicating that enhanced cell survival was accompanied by expansion of the surviving cell population. By contrast, co-culture with CD40L-expressing L cells inhibited BL-cell expansion (Figure 2B). This resulted from a change in BL-cell phenotype that includes cell-growth arrest (Figures 4 and 5).

Under these conditions in which paracrine survival effects were substantial, no autocrine/juxtacrine effects on BL-cell survival were demonstrable. Furthermore, the results of both Figures 2 and 3 show that apoptosis-susceptibility of L3055 cells cultured on supporting cells of fibroblast, follicular dendritic cell, and macrophage origin is markedly lower than that of L3055 cells cultured alone. It appears, therefore that these paracrine/juxtacrine survival signals are more potent than the autocrine/juxtacrine signals described above.

Transwell cultures were set up to determine whether the pro-survival effects on L3055 cells of fibroblasts, HK cells

Figure 2 Paracrine survival and proliferation of BL cells. L3055 cells (10 ml, 2×10^5 ml) were cultured in 25 cm^2 flasks either alone or in direct contact with the indicated cells for 72 h after which % apoptosis (**A**) and total cell yield (**B**) were assessed. Means $\pm$ S.D. of duplicate samples. CD40L: CD40-ligand, HFF: human foetal fibroblasts, HK: follicular dendritic cell line. Experiment representative of three similar
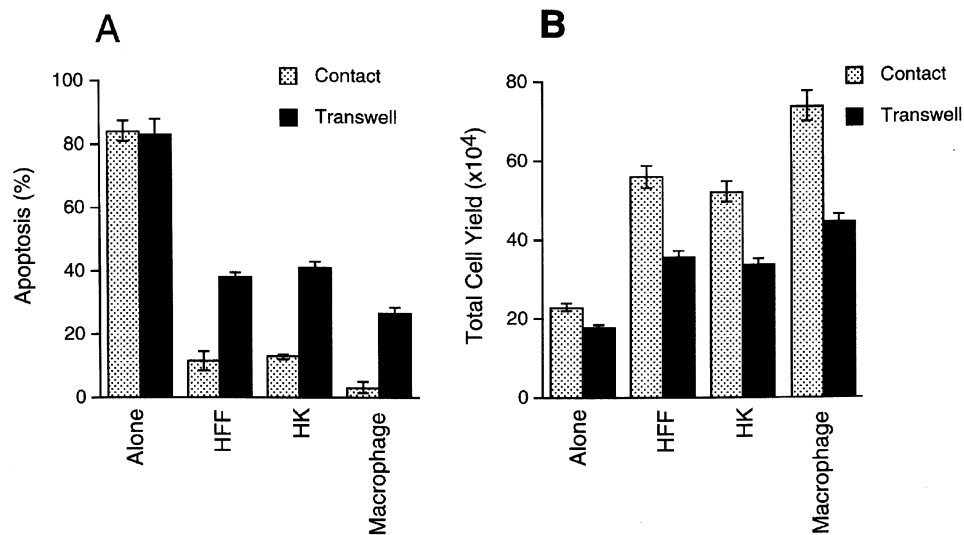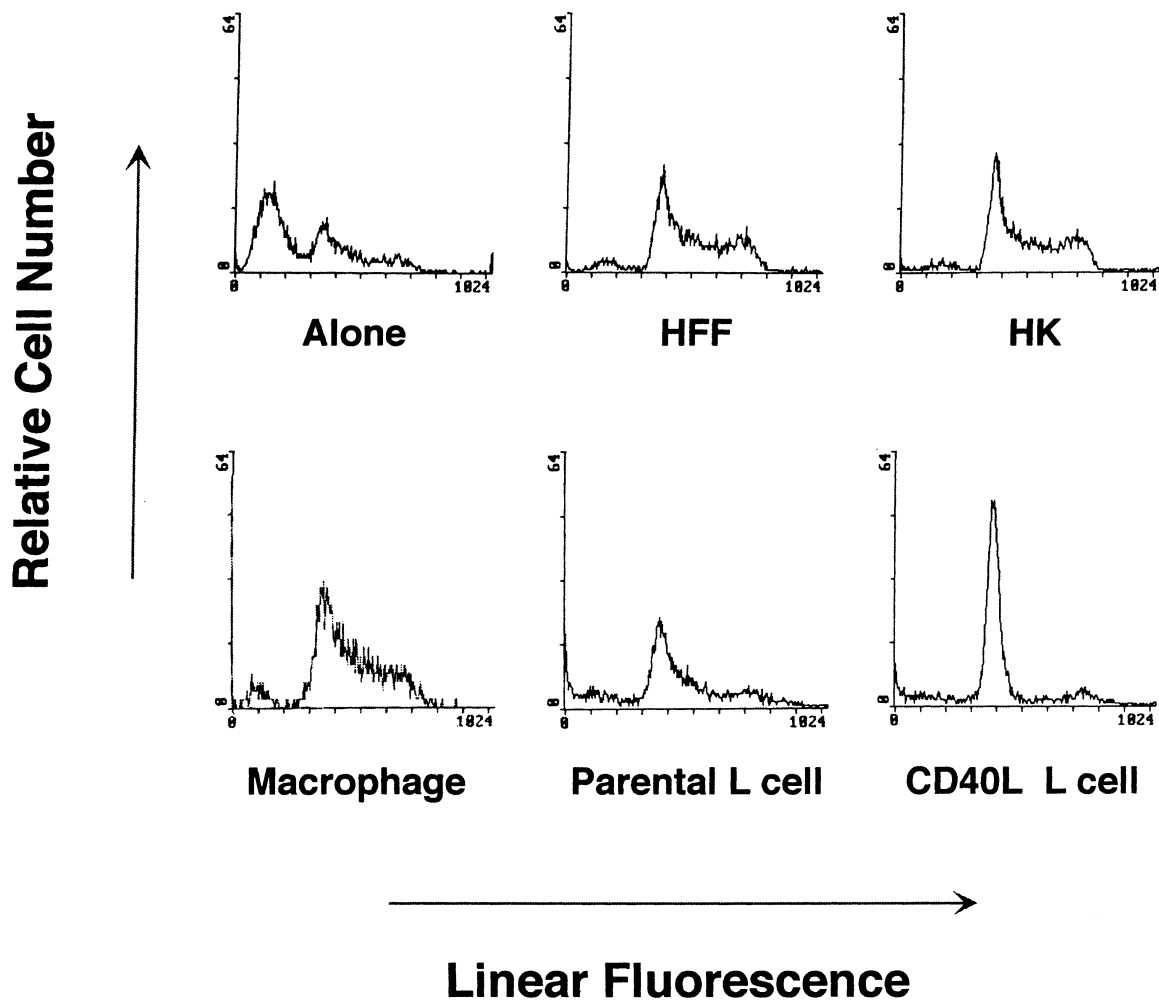

Figure 3 Paracrine support of BL-cell survival and proliferation by soluble factors. L3055 cells were cultured in the presence or absence of the indicated 'feeder' cells either in direct contact (stippled bars) or in transwells containing 0.4 μm pore membranes. After 96 h, % apoptosis (**A**) and total cell yield (**B**) were assessed. Means $\pm$ S.D. of quadruplicate samples. Experiment representative of four similar
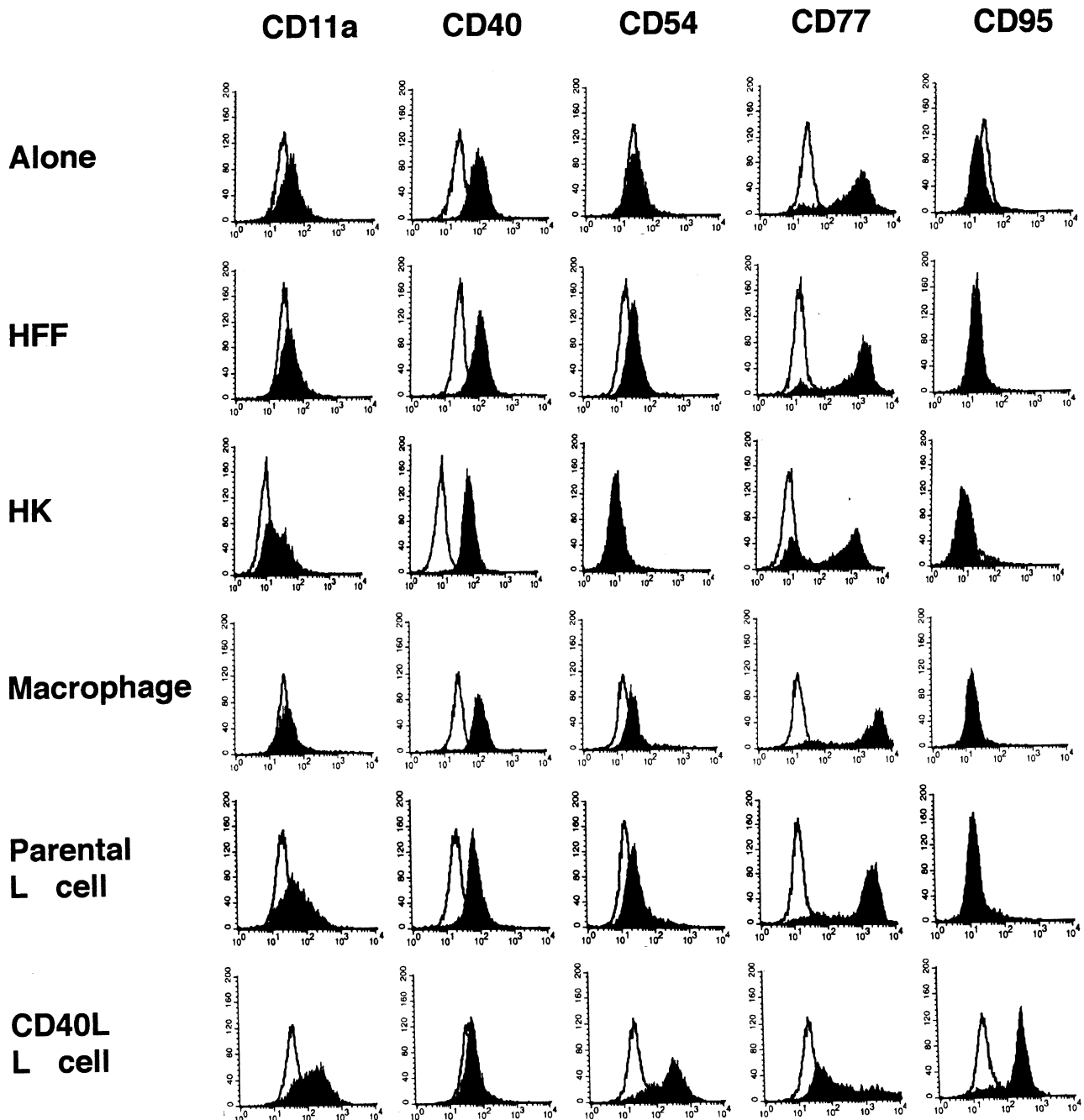
Figure 4 Changes in surface phenotype of BL cells cultured in the presence of CD40-ligand. L3055 cells were cultured for 72 h either alone or in direct contact with the indicated 'feeder' cells. Expression of the indicated cell-surface antigens was subsequently assessed by flow cytometry after indirect immunofluorescence staining. Histograms show relative cell number (ordinate) *versus* log fluorescence intensity (abscissa). Open histograms represent background staining (isotype control)

and macrophages resulted from factors that are diffusible through membranes containing 0.4 μm pores. As shown in Figure 3A, survival of L3055 cells that were cultured either in direct contact with supporting cells or separated from them by cell-impermeable membranes was significantly better than that of BL cells cultured alone. BL-cell yields (Figure 3B) reflected these observations. However, both survival and yield of L3055 cells separated from supporting

cells by membranes were significantly lower than that of L3055 cells cultured in direct contact with supporting cells. Supernatants isolated from cultures of fibroblasts, HK cells and macrophages failed to promote survival of L3055 cells (data not shown) indicating that the soluble factors which are functional in the transwell system either are active only over short-range (high local concentration) or are highly labile. These results demonstrate that multiple lineages of

Relative Cell Number → **Linear Fluorescence**

Alone   HFF   HK

Macrophage   Parental L cell   CD40L L cell

Figure 5 Cell-cycle change in BL cells cultured in the presence of CD40-ligand. L3055 cells were cultured for 72 h either alone or in direct contact with the indicated 'feeder' cells. Permeabilised BL cells were stained with propidium iodide and analyzed by flow cytometry. Proportions of cells in Sub G0, G0/G1, S and G2/M respectively were: Alone: 54.4, 25.2, 7.9, 12.5; HFF: 12.8, 42.7, 17.6, 26.9; HK: 12.0, 49.2, 16.1, 22.7; Macrophage: 9.5, 48.8, 21.2, 20.5; Parental L cell: 24.2, 41.8, 14.2, 19.8; CD40L L cell: 17.6, 65.3, 5.9, 11.2

supporting cells can provide paracrine survival signals, at least some of which are provided by soluble factor(s).

Phenotypic stability of surviving BL cells

We reasoned that paracrine survival pathways of potential significance to BL *in vivo* should promote survival and proliferation without change in group I phenotype, this being the phenotype of the tumour cells *in vivo*. Figure 4 shows immunophenotyping data comparing untreated L3055 cells with cells surviving co-cultivation with fibroblasts, HK cells, macrophages or CD40-ligand-expressing L cells (together with parental L cells). With the exception of those co-cultivated with CD40-ligand, no phenotypic changes in L3055 cells were noted. Cells exposed to CD40-ligand (both cell-bound (Figure 4) and soluble (data not shown) forms), showed several changes in cell-surface phenotype indicating substantial deviation from group I. Thus L3055 cells surviving co-culture with

fibroblasts, HK cells or macrophages remained unchanged in their expression of CD10, CD11a, CD23, CD39, CD40, CD49d, CD54, CD77, CD95 and Bcl-2 compared with untreated cells (Figure 4 and data not shown). Furthermore BL cells did not acquire adhesive activity to fibroblasts, HK cells or macrophages during co-culture. By contrast, BL cells exposed to CD40-ligand displayed increases in CD11a (LFA-1α), CD54 (ICAM-1) and CD95 (Fas) together with a decrease in CD40 (Figure 4 and data not shown). The changes in cell-surface phenotype of CD40-ligand-exposed BL cells were also accompanied by cell-cycle arrest in G1 (Figures 2B and 5), whereas BL cells co-cultured with fibroblasts, HK cells or macrophages remained in cycle (Figures 2B, 3B and 5), reflecting the status of the tumour cells *in situ*. Reduction in the sub-G0/G1 population in the cell-cycle histograms (Figure 5) confirmed the pro-survival effects of the 'feeder' cells. These results suggest that fibroblasts, HK cells and macrophages may promote BL-cell survival by mechan-
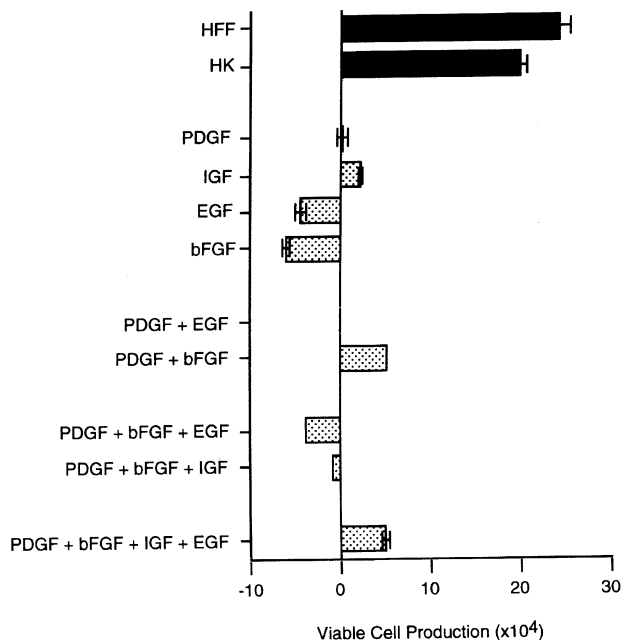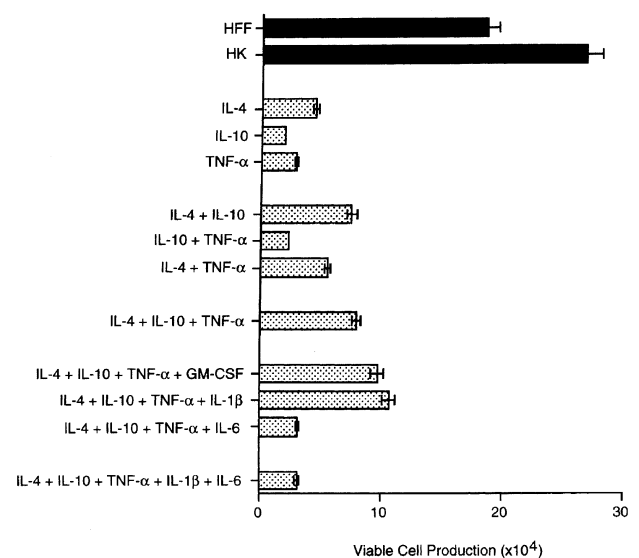
Figure 6 Effects of PDGF BB, IGF-I, EGF and bFGF on BL-cell population growth. L3055 cells were cultured for 72 h either alone, in direct contact with HFF or HK cells, or with the indicated cytokines (PDGF: 10 ng/ml; IGF-I: 100 ng/ml; bFGF: 100 ng/ml; EGF: 10 ng/ml). Net viable cell production was calculated as described in Materials and Methods. Means ± S.D. of duplicate samples. Experiment representative of two similar

isms of relevance to BL *in vivo*. CD40-ligand-expressing cells, on the other hand, fail to promote BL-cell survival by pathways relevant to the natural biology of the tumour since CD40-ligand provides additional signals that promote BL-cell differentiation.

Differential effects of exogenous cytokines on BL-cell survival

In order to gain further information of possible relevance to the mechanisms of paracrine survival of BL cells *in situ*, L3055 cells were tested for their responses to a range of exogenous cytokines, either alone or in various combinations. PDGF, IGF-I, bFGF and EGF were chosen in the first instance since they have been tested by others for their capacity to inhibit c-Myc-induced apoptosis (in fibroblasts).[3] As shown in Figure 6, none of these cytokines was found to be capable of promoting substantial expansion in viable BL cell numbers, although small increases in tumour-cell productivity were observed with IGF-I alone, and with combinations of PDGF BB+bFGF with, or without, IGF-I and EGF. However, EGF and bFGF either alone or combined in the presence of PDGF BB appeared to promote apoptosis of BL cells (Figure 6).

A further panel of cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α and GM-CSF (members of which may be considered relevant to germinal-centre B-cell survival or may be produced by tumour-infiltrating macrophages) was also tested for effects on BL-cell survival. The results, summarised in Figure 7, indicate that while IL-4, IL-10 and TNF-α each promoted low-level enhancement of viable

Figure 7 Effects of interleukins, TNF-α and GM-CSF on BL-cell population growth. L3055 cells were cultured for 72 h either alone, in direct contact with HFF or HK cells, or with the indicated cytokines (IL-1β: 0.1 ng/ml; IL-4: 20 ng/ml; IL-6: 10 ng/ml; IL-10: 5 ng/ml; TNF-α: 5 ng/ml; GM-CSF: 0.16 ng/ml). Net viable cell production was calculated as described in Materials and Methods. Means ± S.D. of duplicate samples. Experiment representative of three similar

tumour-cell productivity, the effect was doubled when the cytokines were combined. Inclusion of GM-CSF or IL-1β marginally improved yield of viable tumour cells, whereas inclusion of IL-6 markedly reduced the effect (Figure 7). In all cases, soluble factors that supported BL-cell survival failed to alter cell-surface phenotype or inhibit cell-cycle activity (data not shown).

## Macrophages affect BL-cell survival by multiple mechanisms

It seems logical to predict that the removal of apoptotic BL cells by tumour-infiltrating macrophages is likely to be beneficial to the emerging tumour-cell population since apoptotic cells that become secondarily necrotic may have detrimental effects on their neighbours. Since monocyte-derived macrophages are known to phagocytose apoptotic group I BL cells, including L3055 cells,[25–27] such activity may contribute significantly to the low level of apoptosis and high level of viable-cell productivity observed in L3055 populations co-cultured in contact with macrophages (Figure 3). However, macrophage support of BL cells is also provided by the activity of soluble factor(s) that function in the absence of any clearance effects (Figure 3). Neutralising mAbs to IL-10 and TNF-α fail to inhibit the soluble supportive activity of these macrophages (Figure 8) demonstrating that, while the macrophage-derived soluble factors IL-10 and TNF-α can protect BL cells from apoptosis, additional macrophage-derived molecules are active in the present model. Indeed, the slight enhancement of viable-cell productivity in the presence of both anti-IL-10 and anti-TNF-α over that provided by macrophages alone (Figure 8) suggests that, in the context of additional macrophage-derived factors, IL-10 and TNF-α could display a combined inhibitory activity against BL cells.

To determine whether macrophages that are actively engaged in phagocytosis of apoptotic BL cells also produce soluble factors that affect BL-cell survival, we compared the ability of macrophages cultured alone or with viable, apoptotic or necrotic BL cells to support L3055 cells in transwells. The results, shown in Figure 9, indicate that macrophages that have phagocytosed apoptotic cells are less effective in supporting L3055 cells than macrophages alone, or macrophages that have been co-cultured with mainly viable or necrotic cells. L3055 cells themselves failed to provide substantial support for L3055 cells in the transwells, although low-level support provided by viable BL cells was significantly greater than that provided by apoptotic or necrotic BL cells (Figure 9). Macrophages responding to LPS were at least as effective as untreated

macrophages in producing soluble factors supporting L3055 cells.

Macrophages that have engaged in phagocytosis of apoptotic cells have been reported to produce TGF-β which may function in an anti-inflammatory negative feedback loop to suppress macrophage pro-inflammatory cytokine production.[21] We sought, therefore, to determine the effect of TGF-β on BL-cell survival and productivity. As shown in Figure 10, TGF-β was found to promote apoptosis (Figure 10A), and to abolish expansion (Figure 10B), of L3055 cells in a dose-dependent fashion (maximal at around 1 ng/ml). Significantly the pro-apoptotic action of TGF-β was dominant over the effect of the anti-apoptotic cytokines,
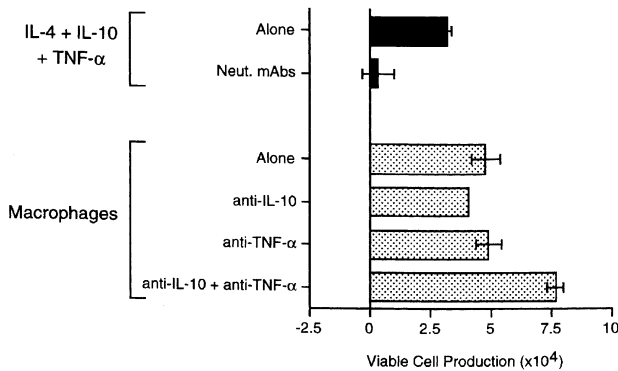
Figure 8 Effects of neutralising antibodies on macrophage-mediated support of BL-cell population growth. L3055 cells were cultured either in the presence of a mixture of IL-4, IL-10 and TNF-α, or in direct contact with macrophages. The indicated individual neutralising antibodies, or a mixture of neutralising antibodies against IL-4, IL-10 and TNF-α ('Neut. mAbs'), were tested for their ability to affect net viable BL-cell production. Means ± S.D. of duplicate samples. Experiment representative of two similar
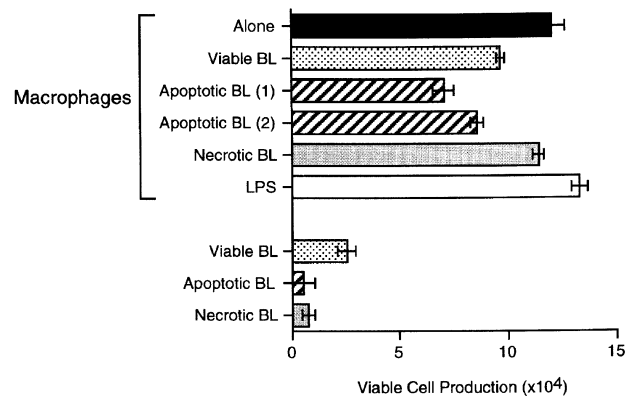
Figure 9 Support of BL cells by soluble factors released by macrophages co-cultured with viable, apoptotic or necrotic BL cells, or LPS. L3055 cells were cultured for 48 h in transwells either over viable, apoptotic or necrotic L3055 cells, or over macrophages which had been untreated ('Alone') or pretreated with viable, apoptotic ('Apoptotic BL (2)'), or necrotic L3055 cells or LPS for 16 h prior to transwell addition, or with apoptotic L3055 cells ('Apoptotic BL (1)') immediately prior to transwell addition. Net viable cell production was calculated as described. Means ± S.D. of duplicate samples. Experiment representative of three similar
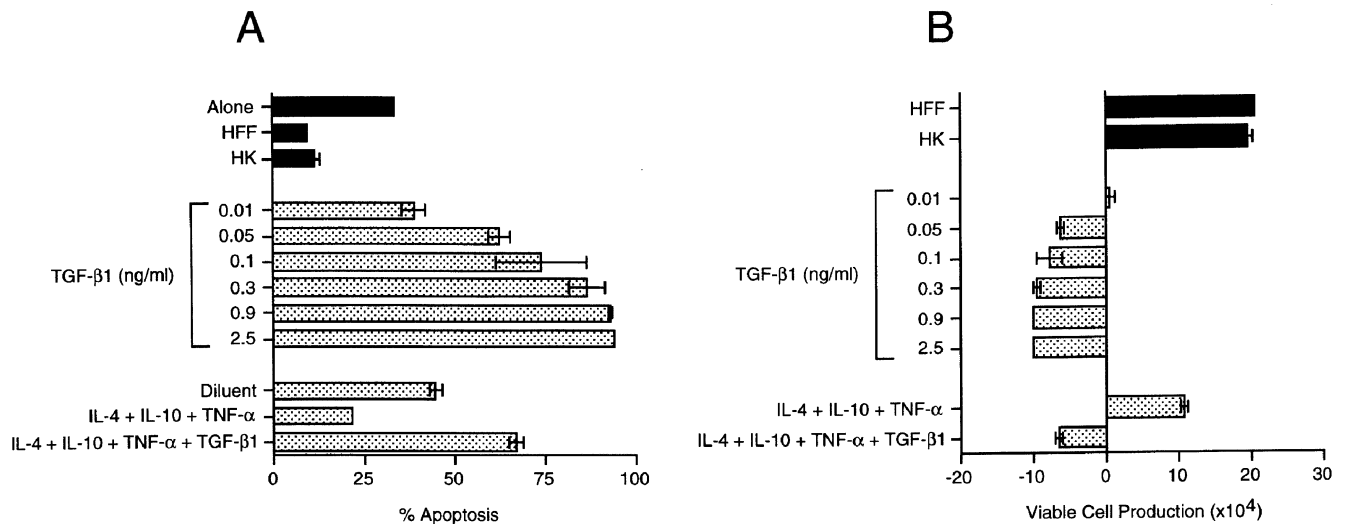
Figure 10 Effect of TGF-β1 on BL-cell survival and growth. L3055 cells were cultured either alone, in direct contact with HFF or HK cells, or with the indicated concentrations of TGF-β1, or with the combination of IL-4, IL-10 and TNF-α in the presence or absence of TGF-β1 (0.1 ng/ml). After 72 h % apoptosis (**A**) and net viable cell production (**B**) were assessed. Means ± S.D. of duplicate samples. Experiment representative of two similar

IL-4, IL-10 and TNF-α (Figure 10). Taken together, these results indicate that tumour-infiltrating macrophages can interact with BL cells both to promote, and to inhibit, tumour-cell survival.

Discussion

Little is known of the micro-environmental factors that may influence tumour-cell survival in BL, a B-cell malignancy in which tumour-cell numbers expand in the face of constitutive, high-rate apoptosis. In this study we have investigated the effects of a number of candidate micro-environmental factors in an established *in vitro* model of BL, the 'group I' cell line, the constituent cells of which, by many criteria, retain the phenotype of the tumour cells as they reside *in vivo*. We show that apoptosis in a group I BL line, L3055, which retains a high propensity to undergo 'spontaneous' apoptosis *in vitro*, can be modulated by multiple factors of potential relevance to the micro-environment of the tumour *in vivo*. We conclude that BL-cell survival and net expansion of the tumour-cell population are promoted both by autocrine and by paracrine mechanisms. We identify soluble factors that function either to promote or to inhibit BL-cell survival and we show that one survival mechanism, that supported by CD40-ligation, probably has no *in vivo* relevance. Finally, we provide evidence to suggest that the tumour-infiltrating ('starry sky') macrophages of BL could influence the tumour-cell population by a combination of pro-apoptosis and anti-apoptosis mechanisms.

Autocrine/juxtacrine modes of B-cell survival and proliferation are well established[28] and some of the underlying molecular mechanisms have been identified. For example, B-cell-derived CD23 acts to promote survival and growth of certain B-cell populations through binding to B-cell surface CD21 as well as other, as yet uncharacterised, receptors.[29–31] In addition, B-cell population survival may be enhanced through ligation of B-cell CD40 by autocrine CD40L.[32] These mechanisms are unlikely to apply to the present model since (1) group I BL cells, while they express CD21, produce little or no CD23, and (2) ligation of CD40 on group I BL cells alters their phenotype. Further work will be required to identify the additional autocrine mechanisms observed here which support survival of BL cells with retention of the group I phenotype. One candidate mechanism may involve interferon-α, a cytokine produced by BL cells that promotes BL-cell survival without appearing to alter the group I phenotype.[33] Whatever their nature, it is obvious that without these mechanisms it would not be possible to maintain group I BL lines *in vitro*.

Additional BL-cell survival mechanisms are likely to be provided by non-malignant elements of the tumour. Here we have considered possible paracrine mechanisms mediated both by direct 'feeder'-cell/tumour-cell interactions and by soluble factors. We considered fibroblasts, follicular dendritic cells (since BL is a malignancy of germinal centre B-cell origin[5]) and macrophages which constitute the major non-malignant cell component of BL.[34] In addition, we assessed the effects of the activated T-cell surface molecule CD40-ligand since activated T cells are also known to infiltrate BL[23] and CD40-ligation is known to

generate anti-apoptosis signals in BL cells.[35–37] CD40-mediated survival, however, was also accompanied by gross phenotypic changes, including CD54 and CD95 up-regulation along with cell-growth arrest, comparable with observations in other studies in which ligation of CD40 on BL cells was achieved either by T-cell-anchored or soluble CD40L or by anti-CD40 antibody.[37,38] It has been noted that a threshold in the level of CD40 ligation determines phenotypic changes in B cells.[39] Our results indicate that ligation of CD40, achieved by multiple mechanisms (cell-bound and soluble natural ligand as well as agonist antibody) consistently alters the group I phenotype of BL and therefore is unlikely to provide a constant pro-survival mechanism in the tumour environment.

Of greater relevance to the biology of BL are our findings that fibroblasts, the follicular dendritic cell-derived line HK and macrophages, together with the cytokines IL-4, IL-10 and TNF-α, each substantially promoted BL-cell survival and tumour-cell yield with retention of the group I phenotype. These results confirm and extend earlier observations that irradiated fibroblasts supply survival factor(s) to BL cells without up-regulating Bcl-2 expression.[40] Furthermore, our observations point to several paracrine pro-survival mechanisms that could contribute to the micro-environment in BL. These mechanisms include (1) undefined direct cell/cell contacts with fibroblasts, HK cells and macrophages (2) undefined soluble factors derived from these cells and (3) defined soluble factors from T cells (IL-4) and macrophages (IL-10 and TNF-α). There may be overlap in the mechanisms underlying (1) and (2) above since the undefined soluble factors observed here appeared to operate over short range. Regardless of this the undefined survival mechanisms could involve cytokines, cytokine mixtures or cell-surface-associated factors in addition to those tested here. Alternatively or additionally, BL-cell survival and tumour-cell yield may be supported by markedly different mechanisms. For example 'feeder' cells may stimulate biosynthesis of anti-oxidants such as glutathione that have proven pro-survival effects on BL cells[41] or they may mediate phagocytic removal of apoptotic tumour cells which could otherwise have detrimental effects on their neighbours. In the present context, survival and net expansion of BL populations may be supported not only by the 'professional' phagocytic activity of macrophages (which have proven ability to phagocytose effectively apoptotic BL cells[25,27]) but also by the potential 'amateur' phagocytic activity of fibroblasts and HK cells. While the potential of HK cells to phagocytose apoptotic cells has not been investigated, fibroblasts have known ability to phagocytose apoptotic cells,[42] including BL cells (CD Gregory, 1999 unpublished observations).

The biological activity of macrophages infiltrating BL has not been studied as far as we are aware. Here we have considered several macrophage functions that might be relevant in BL. We show that the macrophage-derived pro-inflammatory cytokine TNF-α has supportive activity for the tumour cells, particularly in combination with IL-10, an anti-inflammatory cytokine which has been found by some[43] (though not by others[21]) to be produced by macrophages

that have engulfed apoptotic cells. We observed that macrophages engaged in phagocytosis of apoptotic BL cells produced soluble factors that were less supportive for the tumour cells than those produced by untreated macrophages or macrophages co-cultured with viable BL cells, necrotic BL cells or LPS. Furthermore, we found that TGF-β1, which is known to be produced by macrophages that have engulfed apoptotic cells and which can suppress macrophage pro-inflammatory responses,[21] potently promotes apoptosis in BL cells, including those cells supported by IL-4, IL-10 and TNF-α, probably via a mechanism involving cell cycle conflict (G1-arresting signals in the face of de-regulated c-*myc*).[44] These results predict that tumour-infiltrating macrophages with a pro-inflammatory phenotype would be more supportive toward the malignant cells than macrophages with an anti-inflammatory phenotype, although in the present system macrophages of either category supported net tumour-cell growth to a greater or lesser degree. In addition, since certain EBV-positive BL lines fail to growth-arrest following exposure to TGF-β1,[45,46] our results suggest that the 'anti-inflammatory' macrophage phenotype may have differential effects on EBV-positive *versus* EBV-negative BL.

In conclusion, this study has taken the first steps in identifying factors which may function in the micro-environment of BL to promote net growth of a tumour population that retains a high propensity to undergo apoptosis. We identify several mechanisms of potential relevance to BL growth *in vivo*. Significantly, our results suggest that tumour-infiltrating macrophages may provide both positive and negative effects on net tumour growth and highlight the need for further studies focused on macrophage/tumour-cell interactions in BL. It will be important to determine the phenotypes of the macrophages *in situ* and the ways in which they are affected by the tumour cells. For example, are they involved in the impairment of anti-tumour cell-mediated immune responses? A detailed understanding of such macrophage/tumour-cell interactions may yield important information for the development of novel anti-tumour therapeutic strategies.

Materials and Methods

Cells

All cell lines were routinely cultured in suspension in RPMI 1640 medium (Gibco) containing 10% Serum Supreme (BioWhittaker). L3055 cells are EBV-negative and carry the t(8;14) translocation.[47] Although the group I BL surface phenotype (CD10$^+$ CD23$^-$ CD39$^-$ CD77$^+$) – that of the freshly isolated biopsy cells – was originally described for EBV-positive BL, it can also be applied to EBV-negative cells.[7,47] L3055 cells have been previously classified as group I and are also Bcl-2-negative.[13,47] This phenotype was confirmed here using established immuno-phenotyping methods.[13,47] Cells were used in early passage (<50) only. Stable Bcl-2 transfectants were obtained as described.[48] Human foetal skin fibroblasts were subcultured using trypsin/EDTA (Gibco) and used between passage 8 and 25. The follicular dendritic cell-like line HK[22] was obtained from Dr. Y Choi (Alton Ochsner Medical Foundation, New Orleans) and subcultured using trypsin/EDTA. L cells expressing CD40L,[24] together with their parental counterparts, were obtained from Dr. J Banchereau,

Schering Plough, Lyon and subcultured using 0.02% EDTA. L cells were irradiated (7000 rads) before use in experiments. Macrophages were obtained by culture of human monocytes isolated by Percoll fractionation of de-fibrinated venous blood as described.[25,27] Adherent cells (>99% monocytes at the start of the culture period[25]) were maintained for 7 days in IDMEM (Gibco) containing 10% heat-inactivated autologous serum.

Autocrine culture

L3055 cells were pre-incubated for 24 h in RPMI containing 1% Serum Supreme, subsequently seeded at the indicated cell densities in 1% Serum Supreme in 25 cm^2 tissue culture flasks (Nunc, Life Technologies) and incubated for 72 h at 37°C. Of each pair of duplicate flasks, one was incubated horizontally with cells on the 25 cm^2 surface, the other vertically with the cells on the 7.6 cm^2 surface. Alternatively the L3055 cells were seeded into duplicate 96-well plates (Nunc, Life technologies) with either flat or round-bottomed wells and were incubated in 10% Serum Supreme for 96 h at 37°C. In some experiments L3055 cells were cultured in transwells above viable, apoptotic, or necrotic L3055 cells (10^5/well). Apoptosis was induced by cold-shock[20] and necrosis by heat shock.[27]

Paracrine culture

L3055 cells were co-cultured with fibroblasts, HK cells, L cells, or macrophages either in 25 cm^2 tissue culture flasks or in 24-well plates (Nunc). In flasks, fibroblasts and HK cells were used at approximately 75% confluence; L cells were seeded at 4×10^6 per flask followed by removal of non-adherent cells. L3055 cells were added in RPMI containing 10% Serum Supreme (10 ml, 2×10^5 per ml) and incubated for 72 h at 37°C. In wells, L3055 cells were cultured for 48–96 h either in direct contact (10^5 cells per well in 1.25 ml) with fibroblasts, HK cells or macrophages, or were separated from them using Millicell-CM (Millipore) inserts containing Biopore membranes with pore size of 0.4 μm (10^5 cells per insert in 0.5 ml with 0.75 ml of medium below the insert). Cytokines were added at concentrations indicated in the Figure legends. Where neutralising antibodies were used, they were incorporated at doses known to neutralise >90% of the effects of the requisite cytokines. In some experiments, macrophages were incubated with 10^5 viable, apoptotic or necrotic BL cells or with LPS (5 ng/ml). Apoptosis and necrosis were induced as described above.

Assessment of apoptosis and cell production

Harvested cells were fixed in 2% Formaldehyde (Analar, BDH). Apoptosis was assessed either by fluorescence microscopy of acridine orange-stained samples[11] or by flow cytometric light scatter analysis of unstained samples[49] using either the Becton Dickinson FacScan or the Coulter XL flow cytometer. Total cell counts were obtained from the number of cellular events gathered using the 40 μl volume stop on the Coulter XL. Viable cell counts were obtained from the number of events in the viable light scatter zone as described.[49] Net production of viable cells was subsequently calculated by subtracting the viable cell count of untreated L3055 cells from the viable cell count of treated L3055 cells.

Soluble factors and neutralising antibodies

PDGF BB, IGF-1, bFGF, IL-1β, IL-4, IL-6, IL10 and TNF-α were obtained from R&D Systems (Abingdon, UK). GM-CSF and TGF-β1 were obtained from Peprotech (London, UK). EGF was from Upstate

Biotechnology (TCS Biologicals, Buckingham, UK). Neutralising polyclonal goat antibodies against IL-10 and TNF-α were purchased from R&D Systems. Neutralising polyclonal rabbit anti-IL-4 was from Peprotech.

Expression of surface antigens and Bcl-2

Indirect immunofluorescence staining of cell surface antigens was carried out on unfixed cells as previously described[25] using saturating concentrations of primary antibodies and relevant FITC-conjugated secondary antibodies for visualisation. Bcl-2 staining was performed on ethanol-fixed cells.[33] The following mouse IgG mAbs were used: 55 (CD10), BU17 (CD11a), MHM6 (CD23), AC2 (CD39), S2C6 (CD40), BU49 (CD49d) and anti-Bcl-2 (Dako), all visualised using goat anti-mouse IgG-FITC (Sigma). N3D4 (CD54) and CH11 (CD95, Upstate Biotechnology) are mouse IgM mAbs which were visualised using goat anti-mouse IgM-FITC (Southern Biotechnology Associates). 38.13 is rat IgM mAb which was visualised using goat anti-rat IgM-FITC (Southern Biotechnology Associates). Immunofluorescence profiles were analyzed using the Coulter XL flow cytometer. Light scatter properties were used to gate viable and apoptotic cells as described.[49]

Cell cycle analysis

Cells were fixed in PBS containing 1% paraformaldehyde and 0.1% Triton X-100 and stained with propidium iodide (20 μg/ml). Histograms were obtained using the Coulter XL.

Acknowledgements

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