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IRF4 silencing inhibits Hodgkin lymphoma cell proliferation, survival and CCL5 secretion

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Summary

Interferon regulatory factor 4 (IRF4) expression is detected in many lymphoid and myeloid malignancies, and may be a promising therapeutic target. IRF4 is strongly expressed in classical Hodgkin lymphoma (cHL) and its expression is up-regulated by CD40L and down-regulated by both anti-proliferative and pro-apoptotic stimuli. This study analysed the effects of IRF4 silencing in a panel of HL-derived cell lines. We demonstrated that IRF4 down-modulation determined a remarkable decrease of both cell number and clonogenic growth in L-1236, L-428, KM-H2 and HDLM-2 cells, but not in IRF4-negative L-540 cells. IRF4 silencing induced apoptosis, as evaluated by caspase-3 activation and Annexin-V staining and up-regulation of the pro-apoptotic molecule Bax. CD40 engagement by both soluble and membrane bound-CD40L almost totally reduced IRF4 down-modulation and growth inhibition by IRF4 silencing in both L-1236 and L-428 cells. Finally, IRF4 silencing decreased CCL5 secretion in all HL cell lines tested and CCL17 in KM-H2 cells. Taken together, our results demonstrated that IRF4 down-modulation by IRF4 silencing was reversed by CD40 engagement, inhibited HL cells proliferation, induced apoptosis and decreased CCL5 secretion, thus suggesting that IRF4 may be involved in HL pathobiology.

Keywords: Hodgkin lymphoma, IRF4/MUM1, CD40, microenvironment, chemokines.

Interferon regulatory factor 4 (IRF4), a member of IRF family transcription factors, represents a critical mediator of myeloid-, and dendritic-cell development (Tamura *et al*, 2005) and it is required for lymphocyte activation and generation of immunoglobulin secreting plasma cells during immune responses (Klein *et al*, 2006). IRF4 is induced by mitogenic stimuli, including antigen receptor engagement, lipopolysaccharides, IL-4 and CD40 signalling (Klein *et al*, 2006). These stimuli activate the Nuclear Factor-kappa B (NF-κB) pathway, which leads to IRF4 promoter activation by NF-κB heterodimers (Klein *et al*, 2006; Shaffer *et al*, 2009).

IRF4 is expressed in many myeloid and lymphoid malignancies (Gualco *et al*, 2010), including Hodgkin lymphoma (HL). Recently, Shaffer *et al* (2008, 2009), using a loss-of-function RNA interference-based genetic screen, demonstrated that IRF4 inhibition is toxic to myeloma cell lines, making IRF4 a new target that may be exploited therapeutically (Shaffer *et al*, 2009).

HL cells exhibit constitutively active NF-κB (Kuppers, 2009) and IRF4 is expressed in HL cell lines and in Reed-Sternberg (RS) cells in almost 100% of classical HL (cHL) primary cases, with a moderate-to-strong staining intensity (Falini *et al*, 2000; Carbone *et al*, 2002; Aldinucci *et al*, 2010a; Gualco *et al*, 2010). Recently, we demonstrated that IRF4 expressed by HL cells is consistently up-regulated after CD40 engagement but down-regulated by agonistic anti-CD95 antibodies or after treatment with Adriamycin and Dacarbazine, two chemotherapy agents commonly used for HL treatment (Aldinucci *et al*, 2010a). These latter results demonstrated that IRF4 was modulated in an opposite way by proliferative *versus* apoptotic and anti-proliferative stimuli, suggesting that IRF4 may be potentially involved in RS cells proliferation.

To determine whether the effects detected after CD40 engagement and after treatment with chemotherapy could be a direct consequence of IRF4 modulation, this study analysed, after IRF4 silencing, RS cells proliferation, apoptosis and secretion of molecules involved in proliferation and/or microenvironment formation (Aldinucci *et al*, 2010b). The effects of IRF4 silencing were also evaluated in the presence of

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the HL survival factor CD40L (Carbone *et al*, 1995; Kuppers, 2009).

The results of this study reinforce the notion that IRF4 expression may be involved in the proliferation and survival of RS cells and suggest a role for IRF4 also in the secretion of molecules involved in microenvironment formation.

Materials and methods

Cell lines

The study utilized a set of human HL-derived cell lines: KM-H2, HDLM-2 and L-428 cells were obtained through the German collection of Microorganisms and cell cultures (Braunschweig, Germany) (Drexler, 1993), L-1236 (Wolf *et al*, 1996) and L-540 (Drexler, 1993) cell lines were kindly provided by A. Jox and V. Diehl (University of Koln, Germany), respectively. The mouse fibroblastic Ltk-cell line stably transfected with CD40L (CD40L-L-cells) and the non-transfected Ltk-cell line (L-cells) were kindly provided by Dr P. De Paoli (CRO, Aviano, Italy). All cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) (Biocrom KG, Berlin, Germany) supplemented with 10% heat inactivated fetal calf serum (FCS; Sigma-Aldrich, Milan, Italy), 0·2 mg/ml penicillin/streptomycin and 0·1% (w/v) L-glutamine (Biocrom) at 37°C in a 5% CO_2 fully humidified atmosphere.

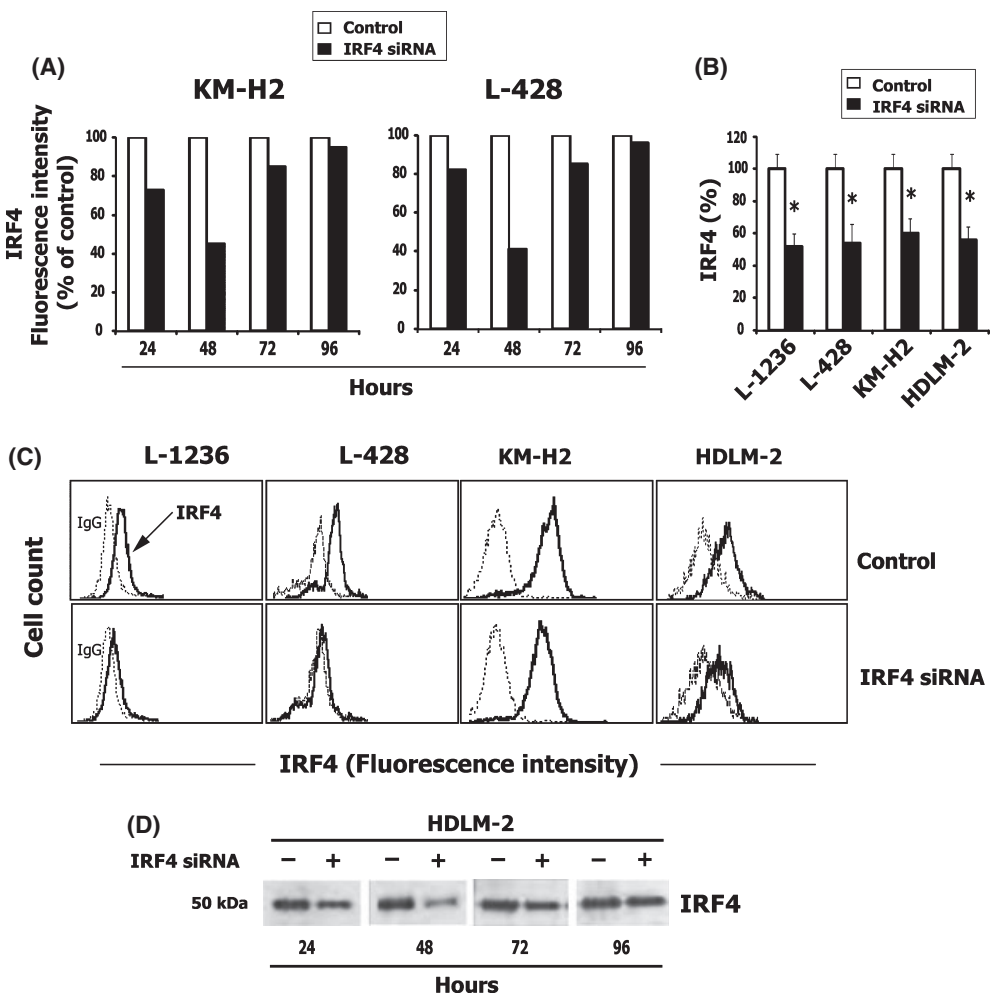

Fig 1. IRF4 down-regulation by IRF4 silencing. HL cells were transfected with IRF4 siRNA or non-specific control siRNA. The efficacy of this method was evaluated by analysing IRF4 expression by Western blotting and flow cytometry. (A) IRF4 expression analysed at different times by flow cytometry with anti-IRF4 antibodies in KM-H2 and L-428 cells. (B) IRF4 expression evaluated 48 h after transfection. Results are expressed as percentage of IRF4 expression after IRF4 silencing with respect with non-specific control siRNA of at least six independent experiments. Asterisks denote a statistically significant decrease of IRF4 expression after IRF4 silencing. (C) Representative flow cytometry overlays showing changes in IRF4 expression after IRF4 silencing. Broken lines indicate background fluorescence, as determined by isotype-matched control immunoglobulins. The *X*- and *Y*-axes indicate the logarithm of the relative intensity of green fluorescence and relative cell number, respectively. (D) IRF4 expression analysed at different times by Western Blotting. Equal amounts of proteins from HDLM-2 cells transfected with IRF4 siRNA or non-specific control siRNA were subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto polyvinylidende difluoride membranes. Blots were then incubated with anti-human IRF4 antibodies and developed by chemiluminescence.

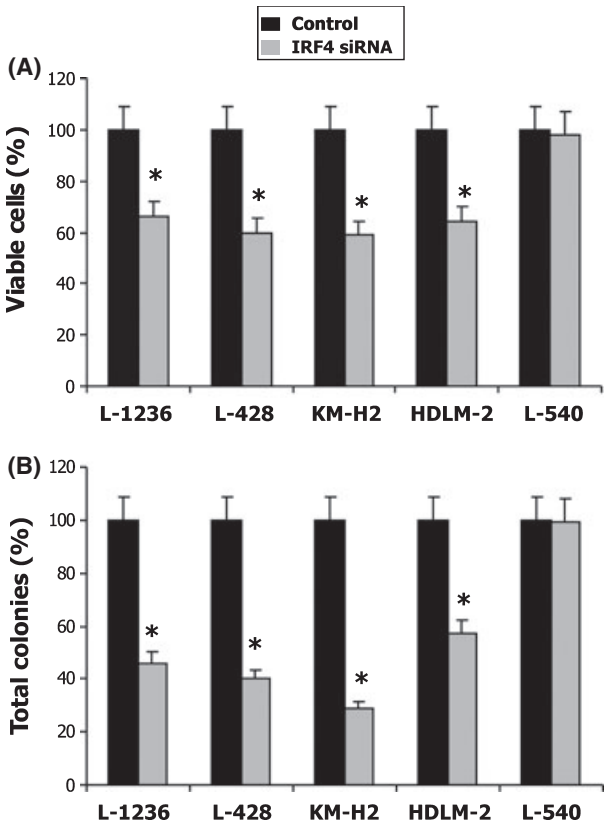

Fig 2. IRF4 silencing inhibits HL cells proliferation. (A) HL cells were counted 48 h after transfection. Viable cells were evaluated by trypan blue staining. Results represent the mean ± SD of at least six independent experiments. (B) Then $2{\cdot}5 \times 10^3$/ml cells were plated in semisolid medium (0·8% methylcellulose). After 14 d of incubation, plates were observed under phase contrast microscopy and aggregates with ≥40 cells were scored as colonies. Results represent the total number of colonies recovered from suspension cultures after transfection, and are presented as percentage relative to cells transfected with nonspecific control siRNA. Asterisks denote a statistically significant decrease in colony growth of HL cells after IRF4 knockdown.

Flow cytometry

IRF4 expression was analysed by indirect immunofluorescence as previously reported (Aldinucci *et al*, 2010a) using an affinity-purified polyclonal goat antibody (M-17) specific for the IRF4 protein (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and revealed by Alexa fluor 488 donkey anti Goat IgG (Invitrogen, Carlsbad, CA, USA). Annexin-V binding was detected by flow cytometry with fluorescein isothiocyanate (FITC)-conjugated Annexin-V protein (Becton-Dickinson [BD]-Pharmingen, Immunocytometry System; San Jose, CA, USA). Caspase activity was ascertained using the fluorochrome inhibitors of caspases (FLICA), CaspaTagTM caspase-3/7 (FAM-DEVD-FMK) (Chemicon International, Milan, Italy) as previously described (Cattaruzza *et al*, 2010). Transfected lymphoma cells were harvested, washed and resuspended in warmed complete medium supplemented with FLICA for 1 h at 37°C under 5% CO_2, and then analysed immediately by flow cytometry. For Bcl-xL and Bax analysis, fixed and permeabilized HL cells were incubated with rabbit anti-human Bcl-xL (Cell Signalling, Danvers, MA, USA) followed by goat anti rabbit IgG-FITC (BD), or with 1 μg/ml of mouse anti-Bax generated from Bax-alpha (BD-Pharmingen), followed by phycoerythrin (PE)-conjugated goat antimouse IgG (BD). Data were collected and analysed on a FACScan flow cytometer (BD), using CELLQUEST software (BD).

Selective inhibition of IRF4 expression by short interfering RNA (siRNA)

siRNA oligonucleotides used to block IRF4 expression (SMART Pool Human IRF4) and nonspecific control (Non-Targeting Pool) siRNA were purchased from Thermo Scientific, Dharmacon (Chicago, IL, USA). HL cell lines were plated at a concentration of $1{\cdot}0 \times 10^6$/ml in 24-wells plates. Double-stranded siRNA were transfected using Interferin (Polyplus Transfection, New York, NY, USA). The final concentration of siRNA was 246 nmol/l. Down-regulation of IRF4 expression by specific siRNA in IRF4-positive cells (L-1236, L-428, KM-H2 and HDLM-2) was evaluated by flow cytometry and Western blotting.

Western blotting

Lysates obtained from cell culture samples were loaded onto a SDS-polyacrylamide gel (4–20% polyacrylamide gradient), subjected to electrophoresis under reducing conditions and transferred to a nitrocellulose membrane. The membranes were saturated with Tris-buffered saline (20 mmol/l Tris and 0·15 mol/l NaCl) containing 0·1% Tween-20 and 5% non-fat dried milk for 1 h at room temperature and then incubated with IRF4-specific goat antibody (M-17) (Santa Cruz Biotechnology Inc.) at 4°C overnight. The membranes were incubated with horseradish peroxidase (HRP)-conjugated appropriate secondary antibodies (Amersham, GE Healthcare BioSciences AB, Buckinghamshire, UK) and then revealed with the ECL Plus™ chemiluminescence kit (Amersham, GE Healthcare). Total protein content was analysed with the protein assay dye reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Proliferation assay

HL cell viability was assessed, 48 h after transfection, by trypan blue exclusion. Clonogenic growth was assessed as previously described (Cattaruzza *et al*, 2009). Briefly, $2{\cdot}5 \times 10^3$/ml cells were suspended in 1 ml of IMDM medium containing 0·8% methyl-cellulose and 15% FCS. The cell suspension was dispensed in 0·1 ml aliquots (eight replicates) in 96-well flat-bottomed micro-plates. After 14 d of incubation, plates were observed under phase-contrast microscopy and aggregates with ≥40 cells, were scored as colonies. The number of clonogenic cells was expressed as the total number of colonies adjusted for the number of cells initially seeded, as reported

previously (Pinto *et al*, 1992). In other experiments, after transfection, HL cells were cultured in IMDM medium supplemented with 3% FCS in the presence of sCD40L (1 μg/ml) and the enhancer (1 μg/ml), an additional product that increase the biological activity of sCD40L (Alexis, San Diego, CA, USA) or in the presence of mitomycin C-treated (50 μg/ml for 30 min at 25°C) parental L-cells or CD40L-L cells.

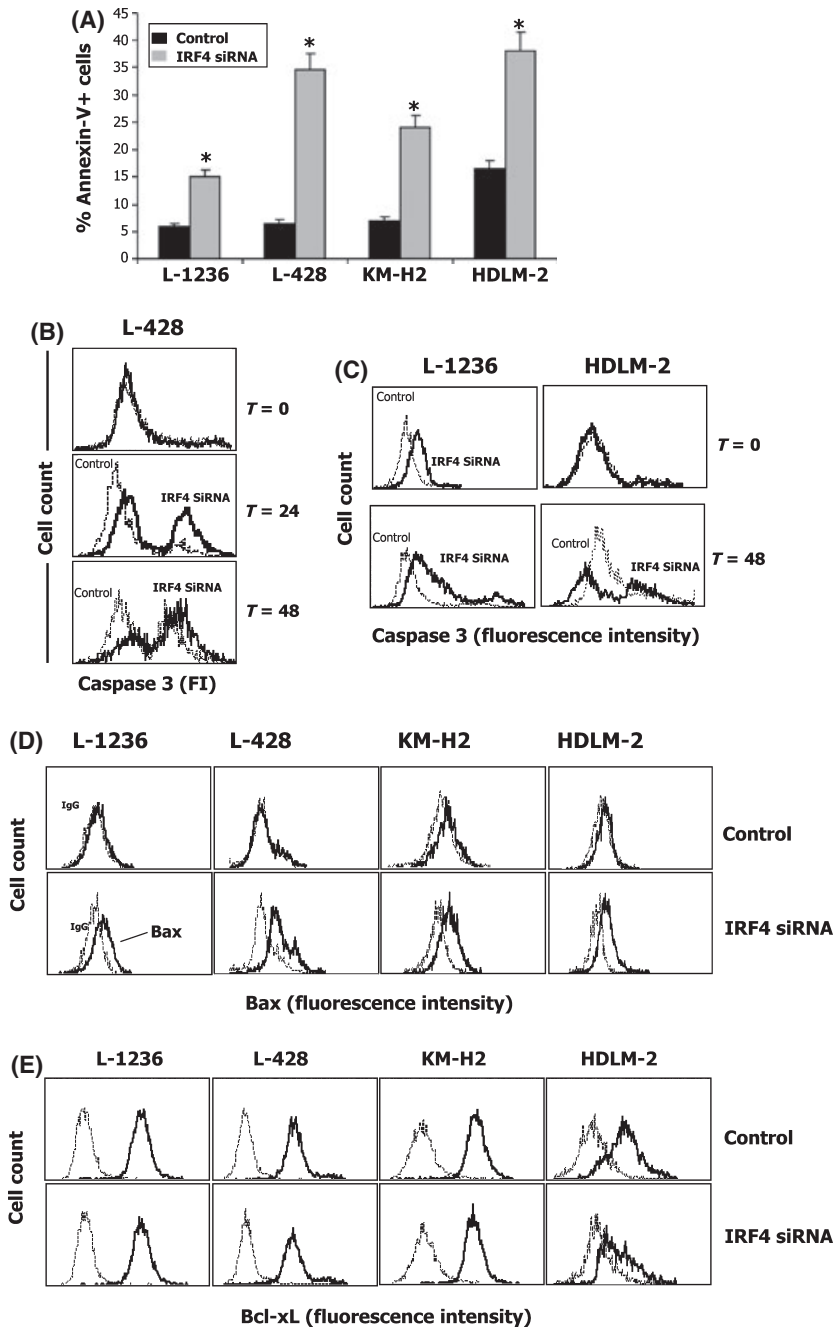

Fig 3. Induction of apoptosis by IRF4 silencing. FACS analysis of apoptosis. Forty-eight hours after transfection with IRF4 siRNA or non-specific control, (A) HL cells were stained with AnnexinV-FITC. Bars indicate the percentage of AnnexinV-FITC-positive cells. Results represent the mean ± SD of three independent experiments. Asterisks denote a statistically significant increase of AnnexinV staining after IRF4 knockdown. (B) At 24 and 48 h after transfection, Caspase-3 activity was evaluated by incubating L-428 cells with the Fluorocrome Inhibitors of Caspase (FLICA) for 1 h at 37°C under 5% CO_2 and then analysed immediately by flow cytometry. (C) Forty-eight hours after transfection Caspase-3 activity was evaluated with FLICA. The overlays are representative of three separate experiments, (D) 48 h after transfection with IRF4 siRNA or non-specific control, Bax expression was evaluated with mouse anti-Bax, followed by PE-conjugated goat anti-mouse IgG (E) and Bcl-xL with rabbit anti-Bcl-xL followed by FITC-conjugated goat anti-rabbit IgG. Broken lines indicate background fluorescence, as determined by isotype-matched control immunoglobulins. The *X*- and *Y*-axes indicate the logarithm of the relative intensity of fluorescence and relative cell number, respectively.

ELISA assay

At 48 h after transfection, concentration of the following cytokines and chemokines was measured using commercially available kits following procedures recommended by the manufacturer. Interleukin (IL)-6 and CCL5 (Pierce Biotecnology, Rockford, IL, USA), IL-10 (Boster Biological Tecnology, Wuhan, China), IL-13 and CCL17 (R&D Systems, Minneapolis, USA), IL-21 (Cusabio Biotech, Co. Ltd., Newark, USA), CCL17 (R&D System).

Statistical analysis

Mean data values are given with their standard deviation (mean ± SD). Statistical comparisons were drawn using Student's *t*-test. Differences were considered significant where $P < 0{\cdot}05$. The Kolmogorov–Smirnov statistical test (K–S) was used for immunofluorescence histogram comparison (Lampariello, 2000).

Results

IRF4 down-regulation inhibited proliferation of HL cells

IRF4 expression and its modulation in HL cell lines have been previously demonstrated (Falini *et al*, 2000; Carbone *et al*, 2002; Aldinucci *et al*, 2010a). However, whether IRF4 plays a role in HL cells proliferation and survival is currently unclear. To explore the function of IRF4 in HL cells, we knocked down *IRF4* mRNA using siRNA in a panel of HL cell lines. First, the efficacy of IRF4 silencing was verified by evaluating IRF4 expression by flow cytometry at 24, 48, 72 and 96 h after transfection in both L-428 and KM-H2 cells and we found the greatest down-regulation of IRF4 48 h after IRF4 silencing (Fig 1A). IRF4 silencing was then evaluated after 48 h in all HL cell lines (Fig 1B) and representative flow cytometry profiles are shown in Fig 1C. IRF4 silencing was also evaluated at 24, 48, 72 and 96 h after transfection by Western blotting in HDLM-2 cells (Fig 1D). We evaluated the consequences of IRF4 silencing on HL cell proliferation, as determined by viable cell count and clonogenic growth. IRF4 silencing significantly ($P < 0{\cdot}05$) decreased proliferation of all IRF4-positive HL cell lines tested, but not of the IRF4-negative L-540 cells (Fig 2A). No significant cell cycle modifications were observed under these experimental conditions (data not shown). Moreover, growth inhibition was most evident when evaluated by clonogenic growth respect to viable cell count (Fig 2B).

IRF4 down-regulation induced apoptosis in HL cells

We then evaluated if apoptosis was responsible for the reduced growth observed after IRF4 silencing. IRF4 silencing determined a significant increase in Annexin-V staining (Fig 3A) in all HL cell lines tested and a significant time-dependent caspase-3 activation in L-428 (Fig 3B) and L-1236 and HDLM-2 cells (Fig 3C), as demonstrated using a potent fluorescent-labelled caspase inhibitor that covalently binds to active caspase within the cells. Moreover, because Bcl-xL and Bax play a crucial role in regulating the intrinsic apoptotic pathway in HL, we analysed their expression after IRF4 silencing. IRF4 down-regulation significantly increased the expression of the pro-apoptotic molecule Bax in all HL cell lines tested (Fig 3C), whereas the anti-apoptotic molecule Bcl-xL was down-regulated only in HDLM-2 cells (Fig 3D) (Table I).

CD40 engagement rescued the effects of IRF4 silencing in HL cells

We previously showed that CD40 engagement by both soluble- and membrane-bound-CD40L determined IRF4 up-regulation in starved HL cells (Aldinucci *et al*, 2010a). We evaluated here the effects of CD40 engagement after IRF4 silencing in two

Table I. IRF4 silencing modulates Bax and Bcl-xL expression in HL cell lines.

	L-1236		L-428		KM-H2		HDLM-2	
Molecule	Control	siRNA	Control	siRNA	Control	siRNA	Control	siRNA
Bax								
MFI	10	**36**	6	**94**	59	**103**	12	**58**
K–S	4	**11**	2	**25**	18	**31**	6	**17**
Bcl-xL								
MFI	179	164	188	170	166	163	137	**65**
K–S	46	45	42	40	45	44	27	**16**

After 48 h from transfection with IRF4 siRNA or a non-specific control siRNA, Bax and Bcl-xL expression were evaluated with mouse anti-Bax, followed by PE-conjugated goat anti-mouse IgG with rabbit anti-Bcl-xL followed by FITC-conjugated goat anti-rabbit IgG, respectively. Data are presented as mean fluorescence intensity (MFI) and as D/s(n), which is an index of similarity for the two curves, according to Kolmogorov–Smirnov statistical test (K–S) calculated with CellQuest software. In this equation, D is the greatest difference between the two curves and s(n) equals the square root of $(n_1 + n_2)/(n_1 \times n_2)$, where n_1 is the number of events in the first histogram and n_2 is the number of events in the second histogram. Experimental conditions in which IRF4 silencing elicited a significant modulation of Bax or Bcl-xL are reported as bold figures.

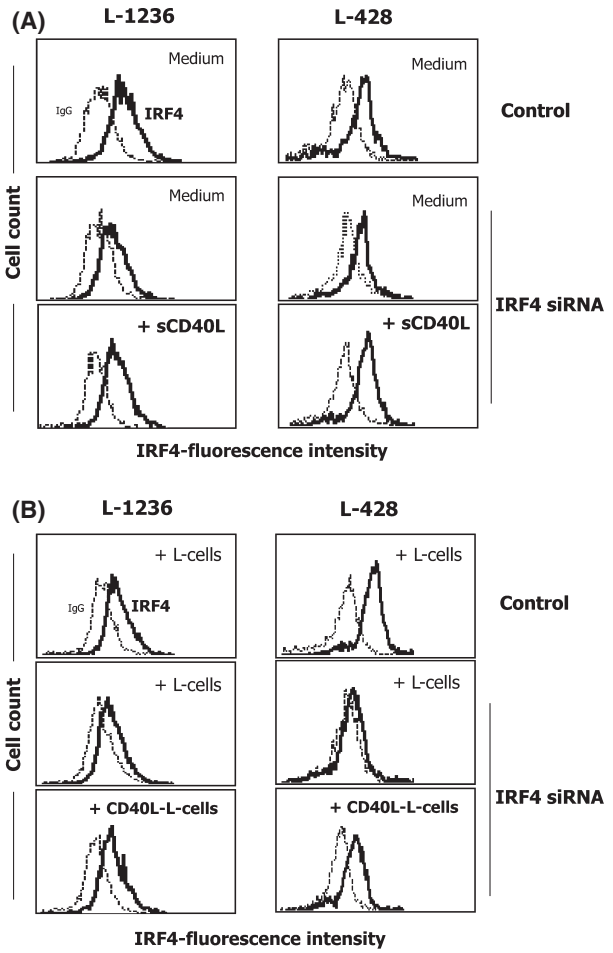

Fig 4. sCD40L rescues IRF4 down-regulation obtained by IRF4 silencing. After transfection with IRF4 siRNA or non-specific control, L-1236 and L-428 cells were cultured in IMDM with 3% FCS (A) in the presence of sCD40L (1 μg/ml) and the enhancer (1 μg/ml). (B) Alternatively, HL cells were cultured in the presence of non-transfected (L-cells) or human CD40-ligand transfected murine Ltk-cells (CD40L-L-cells). After 48 h, IRF4 expression was assessed by flow cytometry. The *X*- and *Y*-axes indicate the logarithm of the relative intensity of green fluorescence and relative cell number, respectively.

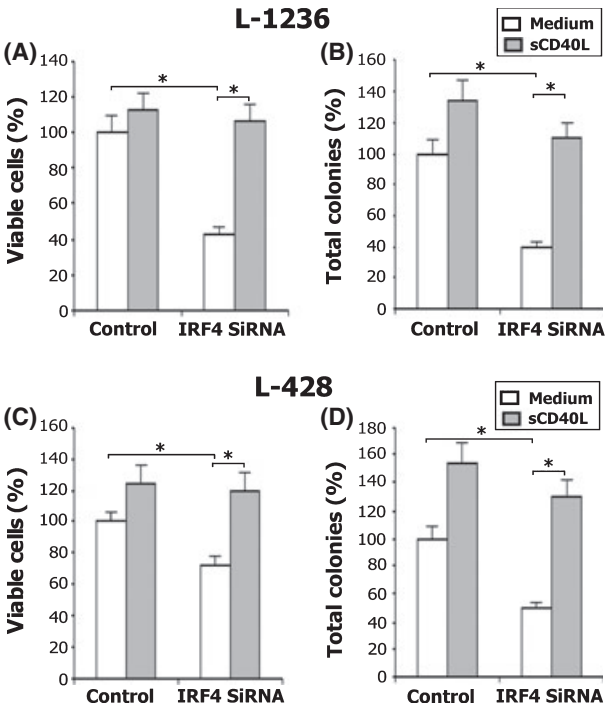

Fig 5. sCD40L rescues the effects of IRF4 silencing on growth inhibition. After transfection with IRF4 siRNA or non-specific control, L-1236 and L-428 cells were cultured in IMDM with 3% FCS in the presence of sCD40L (1 μg/ml) and the enhancer (1 μg/ml). After 48 h, viable cells, evaluated by trypan blue staining, were counted (A, C). Then, $2{\cdot}5 \times 10^3$/ml cells were plated in semisolid medium (0·8% methylcellulose) (B, D). After 14 d of incubation, plates were observed under phase contrast microscopy and aggregates with ≥40 cells were scored as colonies. Results represent the total number of colonies recovered from suspension cultures after transfection, and are presented as a percentage relative to cells transfected with nonspecific control siRNA. Results represent the mean ± standard deviation of at least three independent experiments. Asterisks denote a statistically significant modulation of viable cell count or colony growth.

CD40-positive HL cell lines, L-1236 and L-428 cells. Both soluble (Fig 4A) and membrane-bound CD40L (Fig 4B) were able to reverse IRF4 down-modulation following IRF4 silencing, as evaluated by flow cytometry, in both L-1236 (Fig 4A, B, left panel) and L-428 (Fig 4A, B, right panel) cells. Consistent with the increased IRF4 expression, sCD40L was able to counteract growth inhibition due to IRF4 silencing. In fact, the effect of IRF4 down-modulation on viable cell numbers and clonogenic growth was almost totally reversed by sCD40L in both L-1236 (Fig 5A, B) and L-428 cells (Fig 5C, D).

Effect of IRF4 down regulation on cytokines/chemokines secretion by HL cells

The abnormal cytokine/chemokine pattern seemed to contribute not only to RS cells proliferation but also to the maintenance of an environment in which an effective host immune response to RS cells cannot be achieved (Aldinucci *et al*, 2010b). To verify whether IRF4 down-modulation affected the secretion of molecules involved in RS cells proliferation and survival (IL-13, IL-21 and CCL5), microenvironment formation (CCL5, CCL17, IL-21), immunesuppression (IL-10) and bad prognosis (IL-6) (Herbst *et al*, 1996; van den Berg *et al*, 1999; Skinnider *et al*, 2002; Aldinucci *et al*, 2008; Biggar *et al*, 2008; Lamprecht *et al*, 2008), we measured the levels of several cytokines in cell supernatants 48 h after IRF4 silencing. Under our experimental conditions, IRF4 down-regulation significantly affected CCL5 and CCL17 secretion (Fig 6). CCL5 was decreased in all cell lines tested, whereas CCL17 secretion decreased significantly only in KM-H2 cells. IRF4 silencing did not affect HL cell secretion of IL-6, IL-10, IL-13 and IL-21 (Fig 6).

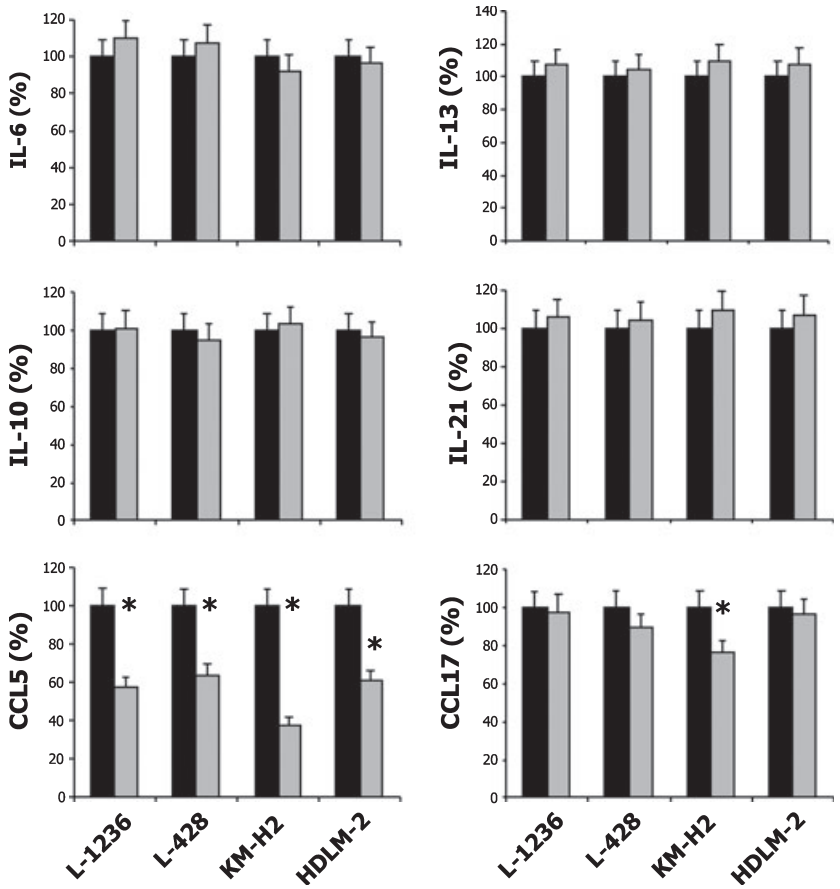

Fig 6. Constitutive production of cytokines/chemokines upon IRF4 silencing in HL cells. After 48 h from transfection, cytokine and chemokine concentrations in HL cells supernatants was measured using commercially available kits following procedures recommended by the manufacturer. Results are expressed as a percentage of control (non-specific control siRNA) and represent the mean ± SD of three different experiments. Asterisks denote a statistically significant decrease in chemokine secretion by IRF4 silencing.

Discussion

Recent reports have demonstrated that IRF4 inhibition is toxic to Multiple Myeloma cells (Shaffer *et al*, 2008, 2009) and Epstein–Barr virus-transformed cells (Xu *et al*, 2008). We recently provided evidences that IRF4 is up-modulated by CD40 engagement and down-modulated by apoptotic and chemotherapy agents (Aldinucci *et al*, 2010a), raising the possibility that knocking down IRF4 could affect cell viability also in HL.

This study provided, for the first time, evidence that IRF4 may be a key mediator of HL proliferation, survival and secretion of molecules involved in both HL proliferation and microenvironment formation. Using a panel of HL cell lines, we demonstrated that IRF4 down-regulation by IRF4 silencing decreased HL cell lines proliferation, as evaluated by both viable cell count and confirmed by clonogenic growth in methylcellulose medium. IRF4-silencing-mediated cell growth inhibition may reflect a decrease in the rate of cell proliferation and/or an increase in the level of apoptosis. No significant cell cycle modifications were observed (data not shown), but IRF4-silencing induced apoptosis because the early apoptosis marker Annexin-V was increased and Caspase 3 was activated in HL cells. Consistently with apoptosis induction we found increased levels of the pro-apoptotic molecule Bax in all cell lines tested and a decrease of the anti-apoptotic molecule Bcl-xL in HDLM-2 cells.

It is well known that CD40, a molecule expressed by RS cells (Carbone *et al*, 1995), is involved in HL cells proliferation and survival, and protects HDLM-2 cells from FAS-induced apoptosis (Re *et al*, 2000; Skinnider & Mak, 2002). CD40 engagement on normal B cells activates NF-κB, which in turn induces IRF4 expression (Saito *et al*, 2007). Given that CD40 engagement increased IRF4 basal levels in starved HL cells (Aldinucci *et al*, 2010a), we inferred that CD40L could reverse IRF4 down-modulation induced by IRF4 silencing. Our study demonstrated that both soluble and membrane-bound-CD40L almost totally reversed the effect of IRF4 silencing not only on IRF4 expression but also on growth inhibition in L-428 and L-1236 cells, suggesting that the high levels of IRF4 expressed by RS cells '*in vivo*' (van den Berg *et al*, 1999; Carbone *et al*, 2002) may be a

consequence of CD40 engagement. The positive correlation between IRF4 expression and cell growth reinforced the notion that CD40/CD40L signalling is involved in RS cells proliferation and survival (Carbone *et al*, 1995).

RS cells growth is regulated by interactions among tumour cells and reactive cells accumulating in HL-involved tissues. These non-malignant cells, recruited and/or induced to proliferate by tumour cells, produce soluble or membrane bound molecules involved in tumour cell growth and survival (Skinnider & Mak, 2002; Aldinucci *et al*, 2010b; Steidl *et al*, 2010). Under our experimental conditions, IRF4 silencing did not affect HL cell secretion of IL-6, IL-10, IL-13 and IL-21. On the contrary, IRF4 down-modulation resulted in a significant decreased secretion of CCL5, a chemokine produced by RS cells that has a direct effect on RS cells survival, proliferation and microenvironment formation (Fischer *et al*, 2003; Aldinucci *et al*, 2008). Therefore, as CD40 engagement on RS cells increased both IRF4 expression (Aldinucci *et al*, 2010a) and CCL5 secretion (Aldinucci *et al*, 2008), we can speculate that CCL5 secretion in HL is due, at least in part, to IRF4 induction by the rosetting CD40L T-cells of the HL microenvironment. IRF4 down-modulation determined a significantly reduced secretion of CCL17 only in KM-H2 cells. Thus, given that B cells produce CCL17 if stimulated with anti-CD40 and IL-4 (Lin *et al*, 2003), two factors involved in IRF4 induction in B cells (Klein *et al*, 2006), we can speculate a concerted action involving CD40 engagement, IRF4 expression and CCL17 secretion by RS cells.

These results, demonstrating for the first time that IRF4 is involved in HL survival and proliferation and in the secretion of molecules involved in HL microenvironment formation, suggest that IRF4 might represent a new therapeutic target for HL.

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