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IL-15 is produced by a subset of human melanomas, and is involved in the regulation of markers of melanoma progression through juxtacrine loops

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IL-15 is a novel cytokine active through the IL-2R$\beta\gamma$. Since several human melanoma cell lines display functional IL-2Rs, we studied the IL-15/melanoma cells interactions. Ten out of 17 melanoma cell lines express the IL-15 transcript and four of them express levels of IL-15 mRNA similar to those detected in control activated monocytes. Nine out of ten cell lines also express two transcripts for the IL-15Rα originated by the alternative splicing of exon′3′. Two melanoma cell lines, MELP and MELREO, derived from patients with rapidly progressive primary melanomas, co-express the two IL-15 transcripts, originated by alternative splicing of exon ′A′. Intracellular Il-15 protein was only detected in these two cells lines and it is mainly retained in the Endoplasmic Reticulum (ER). However, a small amount of IL-15 is also found in the Golgi apparatus and in the early endosomes, suggesting production and intercellular trafficking of endogenous IL-15 protein. Nevertheless, no biologically active IL-15 could be detected in the supernatant of all melanoma cells. The anti IL-15 blocking mAb M111 causes the up regulation of HLA Class I in dense MELP and MELREO cultures. These data suggest that IL-15 is probably active through juxtacrine loops negatively controlling HLA Class I molecules expression. These data offer, for the first time, a likely explanation to the controversal issue of IL-15 secretion and constitute a natural model for understanding IL-15 routing. Moreover, we identify a subset of melanoma cells producing IL-15, possibly involved in tumor escape mechanisms.

Keywords: melanomas; IL-15; IL-15R; secretion pattern; tumor progression immunotherapy

Introduction

IL-15 is a novel cytokine which exhibits many biological activities that are similar to those of IL-2. IL-15 was first characterized for its ability to stimulate the proliferation of the murine IL-2-dependent lymphoid cell line CTLL2. It was subsequently shown that IL-15 mimics many other functional properties of IL-2, including stimulation of LAK activity, GM-CSF and IFNγ secretion by NK cells as well as proliferation of T, B, and NK cells (Bamford *et al*., 1994; Grabstein *et al*., 1994; Carson *et al*., 1994, 1996; Armitage *et al*., 1995; Gamero *et al*., 1995). Despite these overlapping activities IL-15 does not share sequence homology with IL-2, even if both cytokines belong to the four α helices cytokine and share the β and γ receptor subunits (Giri *et al*., 1994). The IL2-R is composed of three different chains the 55 kDa α chain, the 70–75 kDa β chain and the 64 kDa γ_c chain. The functional high affinity IL-2R (k_d = 2 to 50 pM) consists of the $\alpha\beta\gamma$ heterotrimer and is present on activated T cells. Scatchard analysis also reveals the existence of biologically active intermediate affinity receptors (k_d = 0.5 to 2 nM) which are composed by the $\beta\gamma$ heterodimer (for review see Waldmann 1993). Functional studies have shown that both the β and γ chains but not the IL2-Rα are necessary for binding and signaling of IL-15. Nevertheless, scatchard analysis reveals in a panel of cell lines, the existence of IL-15 receptors of both high (k_d = 10 to 80 pM) and intermediate (k_d = 0.25 to 2.5 nM) affinity (Giri *et al*., 1994). In this context, the human IL-15Rα chain has been identified and found to display structural similarities to IL-2Rα chain. At lease three molecular forms of the IL-15Rα are generated by alternative splicing mechanisms. In contrast to the IL-2Rα chain, the IL-15Rα chain is constitutively expressed in a broad range of tissues and is able, as a single chain, to bind to IL-15 at high affinity (Anderson *et al*., 1995).

Additional experimental results suggest that IL-15 might also bind to novel receptor molecules that are not utilized by IL-2. For instance, a novel 68 kDa IL-15Rx subunit different from the β and γ chains was found to be expressed and functional in mast cells (Tagaya *et al*., 1996a,b). The common use of the $\beta\gamma$ complex by IL-2 and IL-15 is the first example in which two different T cell growth factors share more than one receptor subunit. Despite their many similarities, IL-15 does not always behave like IL-2. One main difference is that IL-2 is easily secreted by producer cells, whereas IL-15 is not easily released even though the cells express the transcript (Giri *et al*., 1994; Bamford *et al*., 1996; Meazza *et al*., 1996; Mrozek *et al*., 1996; Tagaya *et al*., 1996a,b). Indeed, production and secretion of IL-15 are negatively controlled both at transcriptional- and post- transcriptional levels. Until now, only few cell lines, which contain viral oncogenic informations were shown to secrete detectable amounts of biologically active IL-15 (Grabstein *et al*., 1994; Bamford *et al*., 1996). This may depend, at least in

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part, on a low translation rate of IL-15 mRNA into protein and to a low efficiency of natural occurring signal peptides in driving secretion (Tagaya *et al*., 1996a,b; Meazza *et al*., 1997). Furthermore, while IL-15 transcript is detected in a wide range of tissues, including kidney skeletal muscle, placenta and activated monocytes and macrophages, IL-2 expression is restricted to activated lymphoid cells that do not express IL-15 message (Grabstein *et al*., 1994).

Different groups have shown that certain human solid tumors express functional IL-2R (for review see Azzarone *et al*., 1996) and that in a subset of melanoma and breast cancer cell lines IL-2 may increase their neoplastic potential (Han *et al*., 1996; Katano *et al*., 1994). Moreover few tumor cell lines produce a biologically active IL-2 (Katano *et al*., 1994; Alileche *et al*., 1993).

Since IL-15 gene has been found to be expressed in several normal tissues, in the majority of small lung carcinoma cell lines and in other tumor histiotypes (Meazza *et al*., 1996), we analysed IL-15 melanoma interactions in cell lines expressing a functional Il-2R. Moreover, since Ultraviolet B irradiation up-regulates IL-15 gene transcription in human keratinocytes (Mohamadzadeh *et al*., 1995) it was important to study IL-15 and IL-15R gene expression in these tumors whose evolution is related to sunlight exposure.

Results

Detection of IL-15 and IL-15Rα transcripts by RT-PCR in human melanoma cell lines

RT–PCR analysis performed with specific oligonucleotides primers which allow the amplification of the entire IL-15 coding region (Figure 1a) revealed a 524 bp specific fragment in LPS activated human monocytes used as positive controls in this study.

Four out of the 17 melanoma cell lines (MELP, MELP-CL1, IGR3 AND MEL-REO) displayed an abundant 524 bp amplification product, six (JUSO, GLL19, DOR, ME1477, WM983A and MENTO) expressed low levels of this transcript and the remainders were negative (Figure 1a and Table 1).

Table 1 IL15/IL15 R transcript expression in human melanoma cells

	IL15	*IL2-Rβ*	*IL2-Rγ*	*IL15-Rα*
M14	–	+	+	NT
IGR3	+	+	+	+
ME-1477	±	+	+	+
DOR	±	+	–	+
JUSO	+	+	–	+
GLL-19	±	+	–	+
MELP	+ +	+	–	+
MELP-CL1	+ +	+	+	+
MILG	–	+	+	+
MEL-CAR	±	+	–	+
MEL-REO	+ +	+	+ + +	+
9742	–	+	–	NT
INT35	–	+	–	NT
WM983A	+	+	–	NT
GG5	–	+	–	NT
MENTO	±	+	–	NT
A375P	–	+	–	NT

MELP and MELREO cells also displayed an additional band of 643 bp.

RT–PCR performed with a specific upper primer, which allows the amplification only of the IL-15 transcript cointaining the alternative exon 'A' (Meazza *et al*., 1996), confirmed that in MELP and MELREO cells the 643 bp transcript (observed in

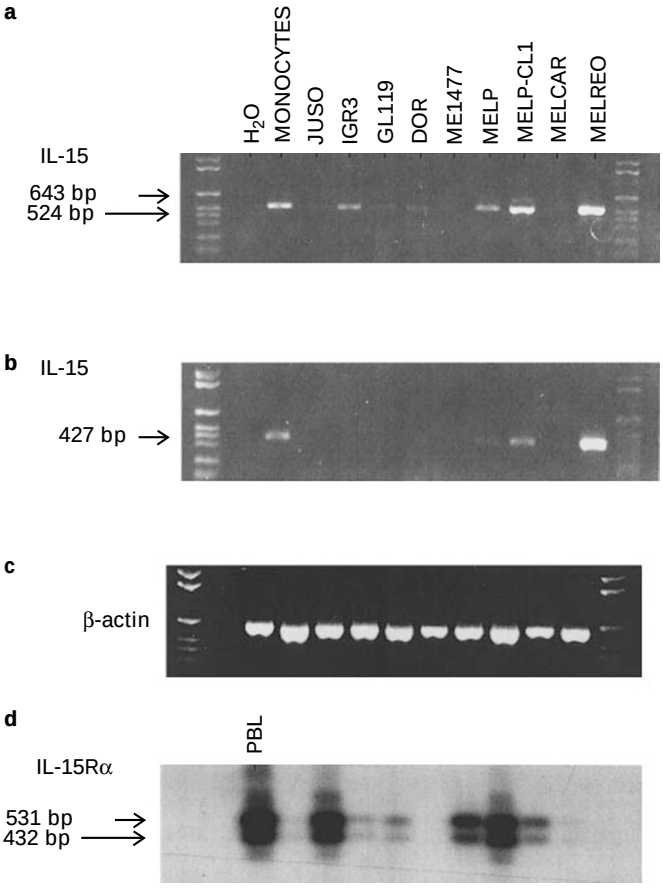

Figure 1 Detection of IL-15 transcripts and IL-15Rα by RT–PCR assay and Southern blot on RT–PCR product respectively in human melanoma cell lines. (**a**) RT–PCR was performed with IL-15 specific oligonucleotides on cDNAs made from human poly-$(A)^+$. RT–PCR was carried out as described in Materials and methods in the presence of $[\alpha^{32}P]$dCTP. An internal PCR control was obtained using primers specific for the house-keeping gene *β*-actin. Human monocytes were used as positive control. A 524 band is expressed in four melanoma cell lines (IGR3, MELP, MELP-CL1, and MEL-REO) with the same intensity scored in the positive control, whereas four other cell lines (JUSO, GLL19, DOR and MELCAR) express low levels of this transcript. MELP, MELP-CL1 and MELREO cells display an additional transcript of 643 bp. (**b**) RT–PCR was also performed with a specific upper primer, which allows the amplification only of the IL-15 transcript containing the alternative exon 'A' (Meazza *et al*., 1996). Activated monocytes, MELP and MELREO cells display the 427 bp transcript corresponding to the IL-15 isoform previously described in SCLC. (**c**) An internal PCR control was performed using primers specific for the house-keeping gene *β*-actin, which was found to be expressed with a similar intensity. (**d**) Detection of IL-15Rα transcripts by Southern blots analysis on RT-PCR amplification products. Ten human melanoma cell lines were tested by RT–PCR assay for the expression of IL-15Rα specific transcripts. RT–PCR amplification products were challenged in Southern blot analysis with a specific cDNA probe corresponding to the sequences between base 160 and base 671 containing the exon 3 (Anderson *et al*., 1995). The analysis reveals the expression of a 432, 531 bp specific doublet (derived from the alternative splicing of exon 3) in human PBL and in nine out of ten melanoma cell lines

Figure 1a) corresponds to an IL-15 isoform previously described in SCLC (Figure 1b). An internal PCR control was performed using primers specific for the house-keeping gene β-actin, which was found to be expressed with a similar intensity (Figure 1c).

Southern blot analysis (Figure 1d) of the electrophoresed PCR-products obtained by the use of IL-15Rd specific primers, with a ^{32}P-labeled IL-15Rα cDNA probe, showed two specific bands of 432 and 531 bp respectively in nine out of ten melanoma cell lines (although Juso and MELREO cells expressed 100-fold less intense transcripts than those scored in control PBL or in MELP cells). The size of the two amplification products is consistent with the presence of two transcripts generated by alternative splicing of the 99 bp exon '3' (Anderson *et al*., 1995).

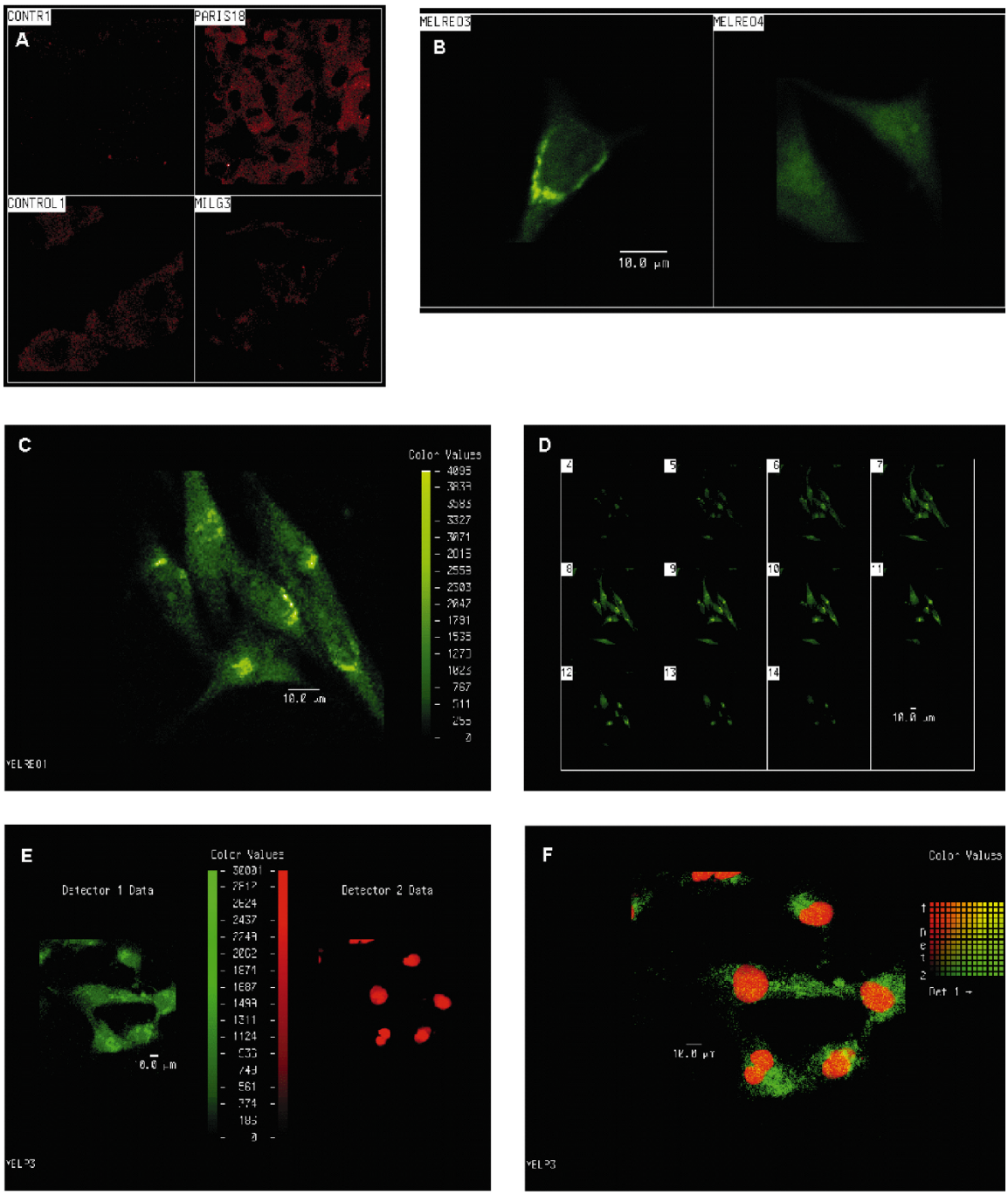

Figure 2 Confocal microscopy in MELP and MELREO cells and analysis of IL-15 intracellular localization using the anti IL-15 mAB M111. (**a**) A positive intracellular staining is observed in MELP (red staining, mAb M111) but not in MILG cells (right panels). No staining is observed in the control cultures (left panels) treated with a non relevant mAb. (**b**) Left panel: intracellular IL-15 in a single MELREO cell (green staining, mAb M111) and right panel: control cultures treated with a non relevant mAb. (**c**) Intracellular IL-15 in a field of MELREO cells (green staining, mAb M111). (**d**) Confocal microscopy analysis of serial cuts of 1 μm thickness from the outside of the cell toward the inner compartments (performed on the MELREO cells analysed in (**c**). Staining is only observed in the internal sections confirming the specificity of the localization. (**e**) Left panel, intracellular IL-15 staining in MELP cells (green staining); Right panel, MELP nuclei stained with propidium iodide (red staining). (**f**) Overlay of the two previous pictures: the absence of yellow staining shows that no IL-15 is localized inside the nuclei

Lack of detection of IL-15 protein in the supernatant of melanoma cell lines

ELISA analysis performed with two different kits (Predicta kit Genzyme and Bio-Source) did not allow the detection of IL-15 in the supernatant of melanoma cells (limit of detection: 10 pg/ ml). Moreover, no biological activity was found both in the supernatant (even concentrated tenfold) and in co-culture experiments performed with the IL-15 dependent CTLL2 murine lymphoid cell line (limit of detection: 0.1 ng/ ml).

Detection of intracellular IL-15 protein by flow cytometry and confocal microscopy

Since IL-15 transcripts were detected in some melanomas, without detectable IL-15 in the supernatant of these cells and of many other cell types (Giri *et al*., 1994; Bamford *et al*., 1996; Meazza *et al*., 1996; Mrozek *et al*., 1996; Tagaya *et al*., 1996a,b), we investigated the presence and localization of the IL-15 protein inside the cells.

Immunofluorescence performed with the anti-IL15 mAb M111 and confocal microscopy showed a specific cytoplasmic staining in all permeabilized MELP (Figure 2a upper panels) and MELREO cells (Figure 2b,c) displaying the strongest positivity for IL-15 mRNAs. Confocal microscopy analysis of serial cuts of 1 μm thickness from the outside of the cell toward the inner compartments showed staining only in the internal sections confirming the specificity of the localisation (Figure 2d). No staining was observed in MILG cultures (Figure 2a lower panels) which do not express IL-15 transcripts (Azzarone *et al*., 1996), suggesting the specificity of intracellular staining with M111 mAb.

In Figure 2e, nuclei of MELP cells are specifically stained in red by Propidium Iodide right panel) and the green staining identifies the intracellular IL-15 (mAb M111, left panel). Colocalization experiments, show the absence of intranuclear IL-15 (Figure 2f) since no yellow staining was detected.

In Figure 3a (MELP) and Figure 3d (MELREO), endoplasmic reticulum is defined by the green staining obtained with the anti β1 integrin C9 mAb, whereas intracellular IL-15 is stained in red by a rabbit polyclonal anti IL-15 in MELP (Figure 3b) and MELREO cells (Figure 3e).

In Figure 3c (MELP) and Figure 3f (MELREO), the presence of an intense and diffuse cytoplasmic yellow staining shows the massive localization of IL-15 inside the Endoplasmic Reticulum (ER).

In Figure 4b (MELP) and Figure 4e (MELREO), the Golgi apparatus is defined by the red staining using the specific Rab-6 rabbit polyclonal serum, whereas the green staining identifies the intracellular IL-15 in MELP (Figure 4a) and MELREO cells (Figure 4d).

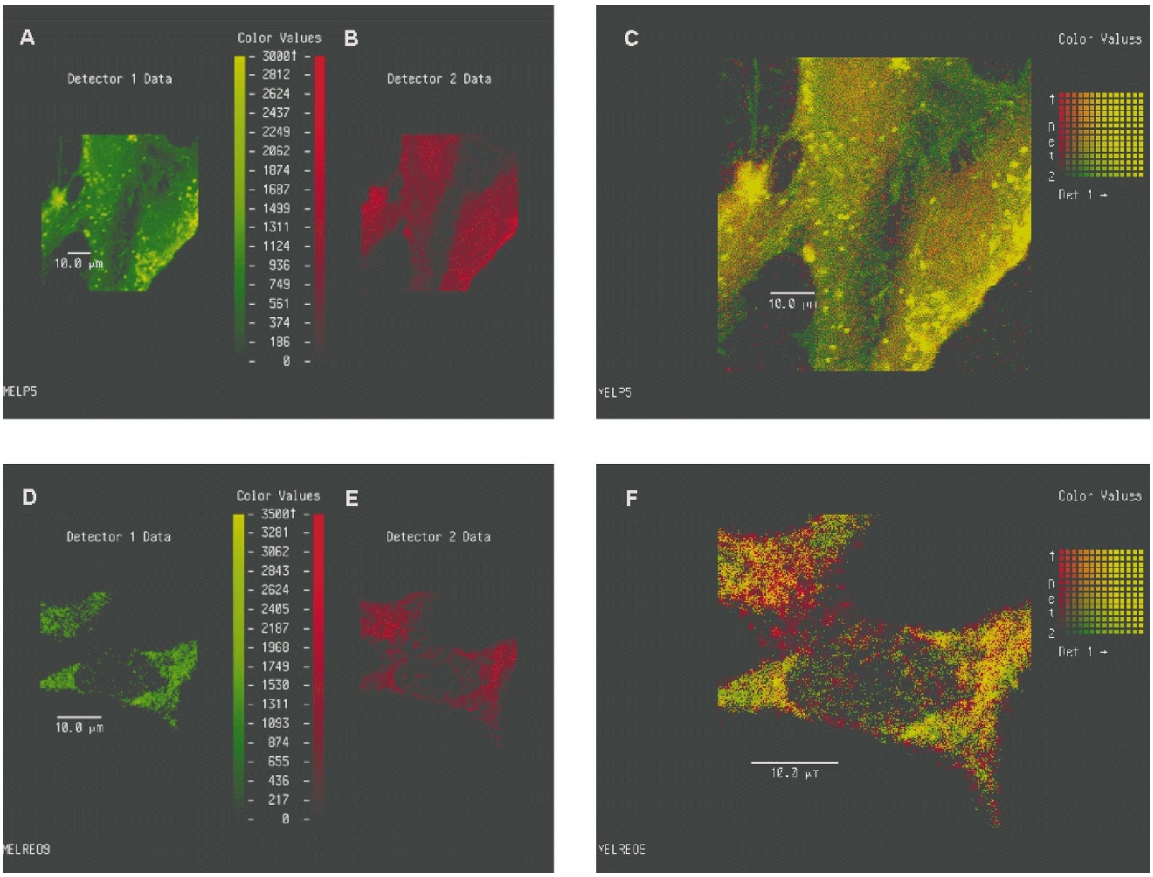

Figure 3 Confocal microscopy in MELP and MELREO cells and analysis of IL-15 intracellular localization using the rabbit polyclonal anti IL-15 serum and the anti integrin β_1 mAb C9 as marker of the endoplasmic reticulum. (**a**) MELP cells Endoplasmic Reticulum (green staining). (**b**) MELP cells intracellular IL-15 (red staining). (**c**) Overlay of the two previous pictures: the presence of a diffuse yellow staining, shows that the majority of intracellular IL-15 is inside ER. (**d**) MELREO cells Endoplasmic Reticulum (green staining). (**e**) MELREO cells intracellular IL-15 (red staining). (**f**) Overlay of the two previous pictures: the presence of a diffuse yellow staining, shows that the majority of intracellular IL-15 is inside ER

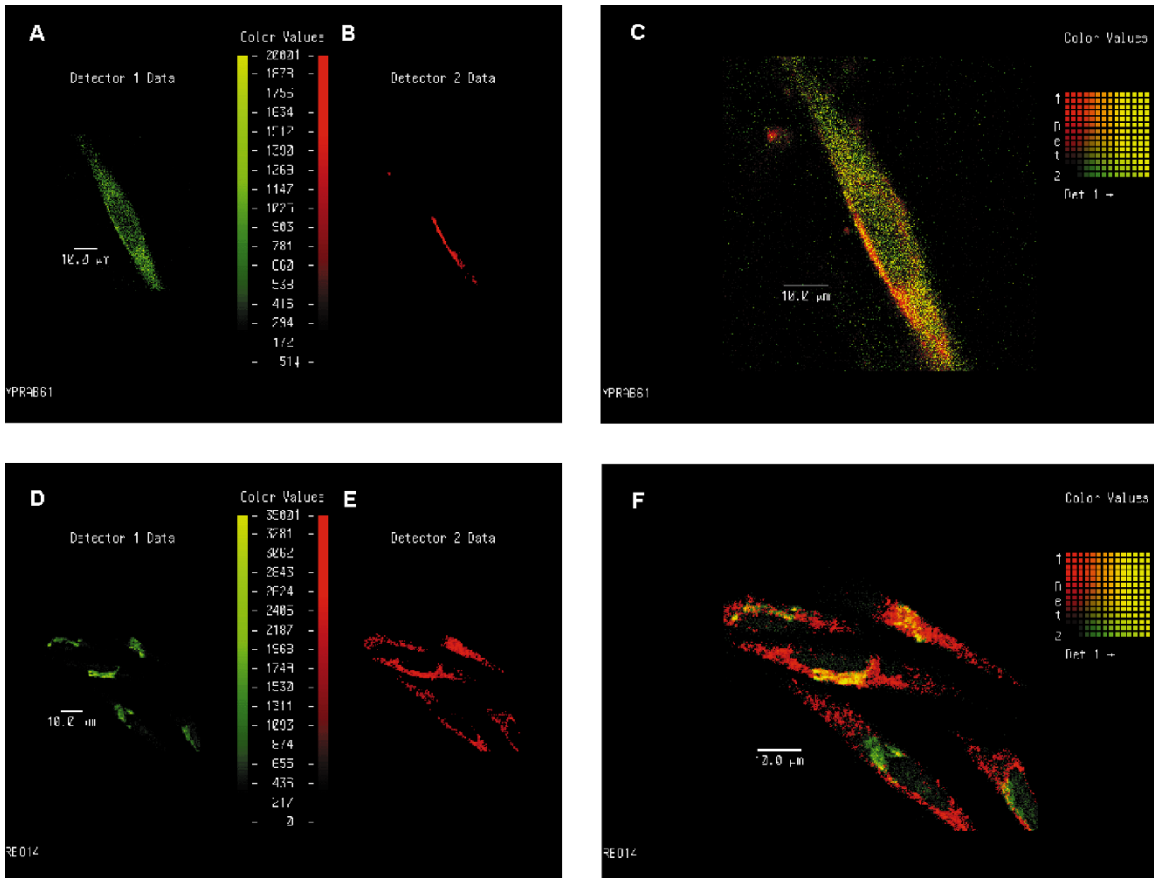

Figure 4 Confocal microscopy in MELP and MELREO cells and analysis of IL-15 intracellular localization using the anti IL-15 mAb M111 and the rabbit polyclonal Rab6 serum as marker of the Golgi apparatus. (**a**) MELP cells, intracellular IL-15 in a single cell (green staining). (**b**) MELP cells, Golgi apparatus in a single cell (red staining). (**c**) MELP cells, overlay of the two previous pictures. The presence of yellow spots inside the red staining shows that some IL-15 is localized inside the Golgi apparatus. (**d**) MELREO cells intracellular IL-15 in a field on cells (green staining). (**e**) MELREO cells Golgi apparatus in a single cell (red staining). (**f**) Overlay of the two previous pictures. The presence of yellow spots inside the red staining shows that some IL-15 is localized inside the Golgi apparatus of 3 out of 4 cells analysed

In Figure 4c (MELP) and Figure 4f (MELREO), the presence of yellow spots shows that some IL-15 is also localized inside the Golgi apparatus. Interestingly, in MELREO cells (Figure 4f) one of four cells does not show IL-15 localization in the golgi apparatus (this feature has been detected in several cellular fields).

In Figure 5 intracellular IL-15 (red staining) is identified by the mAB M111 (Figure 5b, MELP and Figure 5e, MELREO) and the early endosomes (green staining) are defined by the FITC-transferrin (MELP Figure 5a and MELREO Figure 5d). Overlay of the previous pictures shows the presence of a yellow staining at submembrane level, shows the localization of endogenous IL-15 inside these organelles in MELP (Figure 5c) and MELREO cells (Figure 5f, about 40% of positive cells).

Endogenous IL15 modulates surface antigen expression in melanoma cells

The finding that IL-15 co-localizes in the early endosomes compartment in MELP and MELREO cells, suggested that small amounts of IL-15 secreted by these melanoma cells could be released, trapped by surface receptors and possibly modulate biological effects by autocrine or juxtacrine loops. This possibility was also suggested by the expression of the IL-15Rα and IL-2Rβ chains in MELP cells and by the expression of IL-2 R β and γ chains in MELREO cells (Table 1).

To test this hypothesis, we analysed the effect of endogenous IL-15 inhibition, obtained with blocking mAbs directed against IL-15 (M111 at 5 μg/ml), IL-2Rβ chain (MIKβ1 at 5 μg/ml), IL-2Rγ chain (3G11 at 5 μg/ml) and IL-15Rα chain (Rabbit polyclonal P1) on the modulation of HLA class I antigens which are down-regulated by IL-2 in some melanoma cell lines (Plaisance *et al*., 1993).

Flow cytometric analysis (Figure 6) showed that the basal expression of HLA-Class I antigens in MELP cells (Figure 6a, peak 2) was strongly increased (seven fold) by anti IL15 mAb in 72 dense h cultures (Figure 6a, peak 3) whereas no modifications were observed in 24 h sparse cultures (not shown). An increased expression of HLA Class I antigens (2–3-fold) was also observed in MELP cells treated with MIKβ1 mAb (Figure 6a, peak 5) or P1 polyclonal anti IL-15Rα serum (Figure 6a, peak 6), but not in cultures treated with the isotype control CF1 (Figure 6a, peak 4).

A similar behaviour was observed in MELREO cells (Figure 6b) where the basal surface expression of HLA class I antigenes (Figure 6b peak 2) was increased (2.5-fold) in 72 h cultures, by anti IL-15 mab M111 (Figure 6b, peak 3) and by MIK β1 mAB or anti IL-15Rα

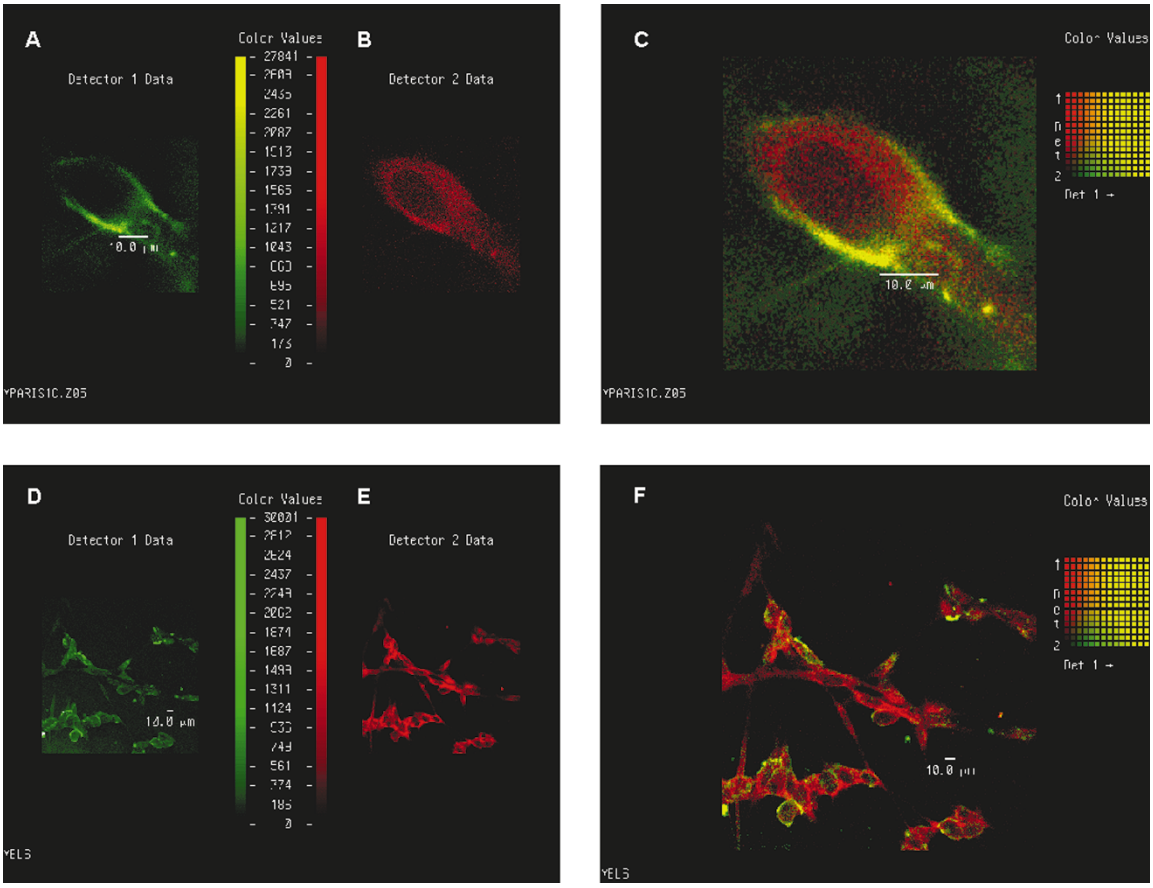

Figure 5 Confocal microscopy in MELP and MELREO cells and analysis of IL-15 intracellular localization using the anti IL-15 mAb M111 and the FITC transferrin as marker of the early endosomes. The pictures represent optical sections inside the entire cells. (**a**) MELP cells, early endosomes (green staining; analysis of a single cell). (**b**) MELP cells, intracellular IL-15 (red staining; analysis of a single cell). (**c**) Overlay of the two previous pictures. The presence of a yellow staining at submembrane level shows the localization of some IL-15 inside the early endosomes. Most of the intracellular IL-15 is localized outside the early endosomes. (**d**) MELREO cells, early endosomes (green staining; analysis of a cell field). (**e**) MELREO cells, intracellular IL-15 (red staining; analysis of a cell field). (**f**) MELREO cells, overlay of the two previous pictures. The presence of a yellow staining at submembrane level shows the localization of some IL-15 inside the early endosomes of about 40% of the cells in the field

serum (Figure 6b peak 4), but not by 3G11 mAb nor by the isotype control CF1.

In no instances could anti-IL-15 mAb alter the *in vitro* proliferation rates of the melanoma cell lines.

Discussion

Human melanoma cells may express functional IL-2R complex composed of α, β and γ chains, but produce little (Alileche *et al*., 1993) or no IL-2. In these cells exogenous IL-2 modifies their cell surface phenotype (Plaisance *et al*., 1993; Rimoldi *et al*., 1993) and could select both *in vivo* and *in vitro* more aggressive clones (Han *et al*., 1996; Doucet *et al*., 1997). Since IL-15 shares with IL-2 the $\beta\gamma$ complex (Giri *et al*., 1994) it was of interest to investigate the possible expression and role of IL-15 in human melanomas.

Ten out of 17 melanoma cell lines express the IL-15 transcript when tested by RT-PCR. Six of them (30%) express, however, low levels of this transcript, whereas four of them (25%) express the IL-15 transcript with the same intensity observed in the LPS activated monocytes. Moreover, in two cell lines of the latter group, MELP and MELREO, an isoform containing an alternative exon 'A' which encodes for a preditected pre-protein with a shorter signal peptide of 21 amino acids (Meazza *et al*., 1996) was also expressed. Nevertheless, no IL-15 was detected in the supernatant of all these melanoma cell lines using two different ELISA assays and the biological assay with the IL-15 dependent cell line CTLL2. A similar behaviour has been reported by other groups which did not detect IL-15 in the supernatant of several cell types that express high levels of the transcript. This phenomenon may relate to a low efficency of IL-15 mRNA translation into protein due to the presence of several AUG codons in the 5′ untranslated region of the mRNA, which have been shown to impair translation (Bamford *et al*., 1996). Moreover, the presence of an unusually long signal peptide (48 a.a) seems to interfere with IL-15 secretion. Indeed, only the substitution of this sequence with the signal peptide of IL-2 (Tagaya *et al*., 1996a,b) or IgK light chain (Meazza *et al*., 1997) allows the secretion of detectable amounts of IL-15 in transfected cells.

In addition, our present and previous results show that also the cell lines expressing the SCLC IL-15 mRNA isoform, bearing the shorter signal peptide, do not release measurable levels of IL-15 (Meazza *et al*., 1996; Azzarone *et al*., 1996; Ferrini *et al*., 1996). These

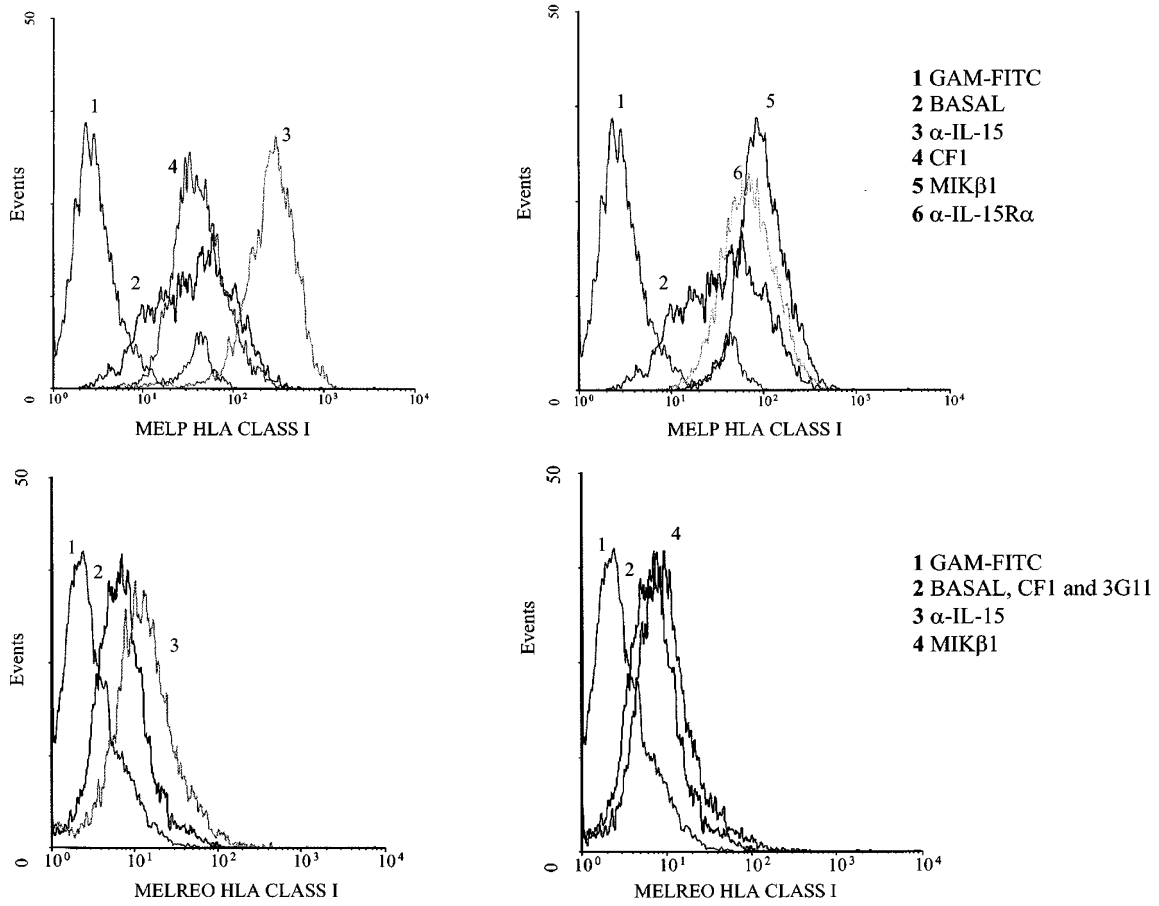

Figure 6 Flow cytometric analysis of HLA-Class-1 expression and effect of anti IL-15 mAb M111 in human melanoma MELP and MELREO cell lines. Data show the mean fluorescence intensity expressed in arbitrary units: negative control (FITC-GAM alone) value. The figure is representative of at least three different experiments. The same number of cells (1×10^5) was suspended in the same amount of medium (2 ml) and seeded onto six microwell plates. The cells were analysed after 24 h (sparse cultures) or after 72 h (dense cultures). Anti IL-15 mAb M111 causes the up-regulation of HLA Class 1 (mAb W632) in 72 h dense cultures

data and previous ones (Giri *et al*., 1994; Bamford *et al*., 1996; Meazza *et al*., 1996; Mrozek *et al*., 1996; Tagaya *et al*., 1996a,b) suggest that IL-15 should be considered as a juxtacrine factor secreted in low amounts. The latter hypothesis is supported by recent papers showing that NK activation by LPS-stimulated monocytes or $CD34^+$ hematopoietic precursors differentiation directed by bone marrow stromal cells involved IL-15. However, only minimal amounts of IL-15 could be detected in the supernatant of these cells and the IL-15 dependent activation of NK or $CD34^+$ cells was observed in co-culture experiments requiring intimate cell/cell contacts (Carson *et al*., 1994; Mrozek *et al*., 1996).

In order to gain a better insight on this phenomenon, we analysed the intracellular localization and trafficking of IL-15 in melanoma cells. By confocal microscopy, using the M111 mAb, we could show the presence of the intracellular IL-15 only in MELP and MELREO cells. Endogenous IL15 was mainly localized inside the Endoplasmic Reticulum of all cells, whereas no nuclear staining was detected.

However we could also localize some IL-15 inside the Golgi apparatus of 80% of the cells and the early endosomes of 40% of the cells. IL-15 detection inside the Endoplasmic Reticulum suggests that most of the intracellular IL-15 is retained inside this compartment. On the other hand, the detection of some IL-15 inside the Golgi apparatus and the early endosomes strongly suggests that a small amount of this cytokine follows the intracellular routing common to other secreted cytokines. The fact that IL-15 localization inside Golgi apparatus and early endosomes is detected in 80 and in 40% of the cells respectively, strongly suggests that the phenomenon is dynamic, regulated on time and probably dependent on the metabolic state of the cells.

Thus, IL-15 may move from the ER and through the Golgi and early endosomes in response to yet unknown cellular signals and may be then exposed at the outer cell surface and/or released in low amount and finally trapped by the secreting cells or by the surrounding ones. We then tried to understand if the endogenous IL-15 was biologically active on melanoma cells. The finding that *in vitro* proliferation of melanoma cells was not affected by anti-IL-15 mAb suggests that the endogenous IL-15 does not act as an autocrine growth factor, displaying a biological role which is different from that observed for endogenous IL-15 in HTLV-1 ATL cells (Bamford *et al*., 1994).

Indeed, our data show that the anti IL-15 or anti IL-2Rβ blocking mAbs caused an increase in the surface expression of HLA Class 1 in 72 h (dense cultures) but not in 24 h cultures (sparse cultures) of two (MELP

and MELREO) out five melanoma cells. This shows that the endogenous IL-15 is biologically active in melanoma cells but that intimate cell/cell contacts are necessary in order to detect this activity. Thus, endogenous IL-15 contributing to maintain a low of expression of HLA Class-1 molecules may umbalance the efficency of cytolytic cells.

These effects could therefore favour tumor escape from the control of HLA Class I restricted cytolytic cells.

All together these data support the hypothesis that IL-15 is a slowly released cytokine which could be retained in the intercellular microenvironment where it could act through cell/cell contacts. In this case the lack of CTLL-2 stimulation in coculture experiments with MELP cells or MELREO may be explained by the fact that the minimal concentration of IL-15 necessary to stimulate CTLL2 cells (0,1ng/ml) is not achieved *in situ*. On the other hand, MELP cells secrete important amounts of soluble immunosoppressive factors (Doucet *et al*., 1997) such as s-ICAM-1 (Altomonte *et al*., 1992) and s-CD25 (Kawami *et al*., 1993) which could interfere on CTLL2 cell activation. In addition, IL-15Rα expressed by MELP cells and other melanoma cells may also act as a decoy receptor, trapping and inactivating the secreted IL-15 (Anderson *et al*., 1995).

It has been recently shown that in lymphoid cells IL-15 acts trough the IL-2R$\beta\gamma$ complex and that in some cell models also the expression of the IL-15Rα chain is required (Giri *et al*., 1995). Our data suggest that the juxtacrine action of IL-15 in human melanomas may occur in the absence of the γ chain (MELP cells IL-15R$\alpha^+\beta^+\gamma^-$) or with a borderline detection of the transcripts IL-15Rα by Southern blot on the PCR products (MELREO cells IL-15R $\alpha^-\beta^+\gamma^+$). In both cells the β chain would play an important role, since anti-β mAb have the same effects observed with the anti IL-15 mab M111. However, the expression of IL-15 Rα in MELP cells as well as the expression of the IL-2Rγ chain in MELREO cells could improve the affinity of the β chain for Il-15 (DeJong *et al*., 1996).

Our data suggest that in melanoma cells there is a gradient in the capacity to produce a potentially immunostimulatory agent such as IL-15. The cell lines expressing the intracellular protein could represent a more aggressive subset competent for a rapid mobilization of the IL-15 peptide. Indeed the two cells lines exhibiting this behaviour have been derived from patients bearing rapidly developing primary melanomas. Moreover, endogenous IL-15 may act on melanoma cells themselves by downregulating the expression of surface molecules involved in the recognition by the immune system (HLA class I).

The present demonstration of an intracellular and intercellular trafficking of IL-15 in some melanomas, suggests the existence of a juxtacrine mechanism of action. These data shed a new light on the controversial issue of IL-15 secretion (Grabstein *et al*., 1994; Bamford *et al*., 1996; Meazza *et al*., 1996; 1997; Azzarone *et al*., 1996) and offer an explanation on the mode of action of this cytokine. This implies that a better knowledge of the balance between endogenous cytokines, their receptors and the recombinant factors is necessary in order to improve their use in melanoma biotherapy.

Materials and methods

Cell lines

The growth characteristics, IL-2R surface phenotype as well as IL-2 production of the human melanoma cell lines M14, IGR3, ME1477, DOR, JUSO, GLL19, MELP, MELP-CL1 and MILG has been reported elsewhere (Han *et al*., 1996; Plaisance *et al*., 1993; Rimoldi *et al*., 1993) and are summarized in Table 1.

The origin and the characteristics of INT35, WM983A, 9742, GG5, MENTO and A3758 cells have been detailed elsewhere (Caré *et al*., 1996). MELP, MELCAR and MEL-REO cell lines have been recently adapted to *in vitro* culture from biopsies of rapidly progressing primary melanomas. Briefly, biopsies had been obtained from lesions of patients who had not yet received any treatment. After mincing freshly resected tumor into small pieces, melanoma cell suspension was filtered throughout a sterile gauze and then run on a phicoll gradient. Melanoma cells were then resuspended in RPMI 1640 supplemented with 20% Foetal Calf Serum (FCS), 2% Glutamine and 2% Insulin-Transferrin-Sodium Selenite (ITS) and seeded into 25 cm^2 flasks. Expression of IL-2R subunits is summarized in Table 1, interestingly MEL-REO cells express, by flow cytometry, descrete surface levels of IL-2R β and γ chains. Human recombinant IL-15 was a generous gift of Immunex (Seattle, USA).

Flow cytometric analysis

The analysis of melanoma cells for the expression of the surface phenotype was performed by flow cytometry. Briefly, the plastic adherent cells were detached with a non-enzymatic cell dissociation solution (Sigma, France), washed in DMEM and incubated for 30 min at 4°C with 10 μg/ml of MoAbs against human ICAM-1 (84H10, Immunotech, Luminy, France), CD44 (T61/7) which recognizes the hyaluronan binding domain (Morimoto *et al*., 1994), HLA class I molecules (W632, a generous gift of Dr S Ferrone, NYMC, Valhalla, NY).

Afterwards, the cells were washed with DMEM and then incubated for an additional 30 min at 4°C with FITC-conjugated goat anti-mouse (FITC-GAM, Immunotech, Luminy, France). As controls, the cells were incubated under similar conditions with FITC-GAM or with antibodies of the same isotype, followed by FITC-GAM. Then, the cells were washed and analysed in a Coulter Profile II (Coulter, Hialeah, FL) flow cytofluorograph. At least 10^4 viable cells were analysed in each instance.

Proliferation and surface phenotype modulation experiments were performed in the presence of 1 to 10 μg/ml of anti IL-15 mAbs M111 (Genzyme, Cambridge USA). M110 (kindly provided by Imunex Seattle, USA) of anti IL-2Rβ chain MIKβ1 mAb (10 μg/ml) which inhibits IL-15 stimulation of lymphpoid T cells (DeJong *et al*., 1996), of the anti-IL-2Rγ chain mAb 3G11 (10 μg/ml) which inhibits IL-2 binding and function (Nakarai *et al*., 1994) as well as IL-15 stimulation of human PBL and NK cells (data not shown) and of a rabbit polyclonal anti IL-15Rα blocking serum (kindly provided by Imunex Seattle, USA).

MELP or MELREO cells were suspended in 3 ml of medium, seeded either onto six microwell plates (1×10^5 cells per plate) and incubated 72 h with the different blocking mAbs, as isotype control mab we used a non blocking anti IL-2Rβ chain (CFI 10 μg/ml, a generous gift of Jannick Jacques U211 INSERM, Nantes, France). After 24 h (sparse cultures) or 72 h (dense cultures) cells were detached and tested by flow cytometry.

Modulation of proliferation was studied by tritiated thymidine incorporation, MTT assay and cell count with an electronic Coulter counter (Alileche *et al*., 1993).

Confocal microscopy

Melanoma cells were cultured for 48 h on glass coverslips (Inter Med). After washing twice with Phosphate Buffered Saline (PBS), cells were fixed in 0.25% paraformaldehyde in PBS for 1 h at 4°C, the cell membranes were permeabilized with 0.2% Tween 20 in PBS for 15 min at 37°C. Cells were incubated with blocking solution 0.5% Human-IgG PBS 10 min. Monoclonal antibody (mAb M111) against IL-15 (Peprothech) was subsequently diluted to final concentration of 10 μg/ml in a solution of 0.5% Azide, 2% FCS. Cells were incubated for 45 min with mAb M111 at 4°C or with a non relevant mAb of the same isotype (anti CD34 Immunotech, Luminy, France). Then washed twice before incubation with fluorescein-conjugated-goat anti-mouse (F(ab′)2 (FITC-GAM, 1/50, Immunotech, Luminy, France).

For double staining of IL-15 and the endosomal compartments, Fluoresceine-transferrin (FITC-Tf) was used. Cells were incubated in DMEM deprived of serum for 1 h, following by incubation with FITC-Tf at 37°C for 30 min. After this internalization period, cells were washed twice, fixed, permeabilized and stained with mAb M111. Subsequently, cells were washed twice, incubated for 30 min at 4°C with a biotinylated-goat anti-mouse and finally exposed for 20 min at 4°C to streptavidine-RED_{670} (strep-RED_{670}, Gibco). For staining the medial- and cis-Golgi compartments we have used rabbit polyclonal antibodies anti-Rab6 (rabbit anti-Rab6) a gift of Dr B Goud.

After cells fixation and permeabilization double indirect immunofluorescence was performed. The first staining was accomplished using the anti IL-15 mAb M111, followed by FITC-GAM incubation. The second staining was performed using the rabbit polyclonal-Rab6 antibody followed by biotinylated-sheep anti rabbit incubation and finally revealed by strep-RED_{670} incubation.

For staining the Endoplasmic Reticulum (ER) we have used the IgG1 mAb C9 (a generous gift of Dr Luciano ZARDI, IST, Genoa) which recognizes the integrin β1 chain. Indeed, in the cytoplasm this chain associates with calnexin, a membrane-bound chaperone and resident protein of the endoplasmic reticulum which is involved in the retention of a pool of immature integrin β1 chains in the ER (Lenter and Vestweber, 1994). IL-15 was identified using a rabbit polyclonal anti IL-15 antibody (Peprothech).

After cells fixation and permeabilization double indirect immunofluorescence was performed. The first staining was accomplished using the anti IL-15 rabbit polyclonal antibody followed by biotinylated-sheep anti rabbit incubation and finally revealed by strep-RED_{670} incubation. The second staining was performed with anti-β1 integrin C9 mAb followed by FITC-GAM incubation. Nuclei were identified by Propidium Iodine staining followed by RNAse incubation.

Laser scanning confocal microscopy (LSCM) and three-dimensional image reconstruction

Labeled specimens were scanned with an ACAS 570 Interactive Laser Cytometer (Meridian Instruments, Inc., Okemos, MI, USA), equipped with confocal optics. The system consisted of a 5W argon ion laser tuned to 488 nm, an Olympus IMT-2 inverted microscope with a 100× oil immersion objective (N.A.1.3) Z-axis control, an XY scanning stage, and a variable pinhole aperture, all under 80486 computer coordination. The ACAS 570 was set up in fluorescence mode. FITC and strep-RED_{670} were excited at 488 nm. The computer converts fluorescence intensity into color as output image. The excitation was done at 20 NW power and the filter constitution for emission detection was the conventionally used for FITC 520 nm BP and strep-RED 670 nm BP. To have a good resolution, pinhole and photomultiplier voltage was set at 40 and 25% respectively.

Supernatant generation and IL-15 assay

Melanoma cells were cultured in 25 cm^2 flasks for 24, 48 and 72 h at 37°C in 5% CO_2 atmosphere. Supernatant were collected, filtered through a 0.22 μm (Millipore, France) and frozen at −80°C until use. Aliquots of supernatants were also concentrated tenfold using a Centricon Concentrator (Amicon, Beverly, USA). All samples were assayed for human IL-15 using two commercially available ELISA kit (Genzyme, Cambridge, MA. and BioSource International, Camarillo, CA.) with a sensitivity of 10 and 11 pg/ml respectively. The samples were also used for stimulating the IL-15- dependent murine lymphoid cell line CTLL2 (sensitivity 0.75 ng/ml). CTLL2 cells were also cultured on a feeder layer of MELP cells.

Reverse transcription-polymerase chain reaction (RT–PCR)

Total RNA was isolated from the relevant cell lines for RT–PCR, according to a previously described method (Plaisance *et al*., 1993). The RNA samples were transcribed into cDNAs in a reverse transcription mixture containing poly-d(T) primers (Pharmacia, Orsay, France) and Moloney Mouse Leukemia Virus (M-MLV) reverse transcriptase. PCR was performed in a solution consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.01% gelatin, 2 mM of each primers, 200 mM each deoxynucleotide and 2.5 U Taq polymerase (Boehringer, France). The PCR cycles were 1 min at 94°C; 2 min annealing (60°C for IL-2Rβ and IL-2Rγ, 57°C for IL-15 and 59°C for IL-15Rα and 3 min (IL-2Rβ, γ), 30 s (IL-15) and 45 s (IL-15Rα) extension at 72°C for 30 cycles followed by final extension of 7 min at 72°C. The oligonucleotide primers were prepared in an Applied Biosystem Synthesizer). For human IL-2Rβ (upstream: 5′-TCA TCC TCC TCC TGC CCC TGG C-3′ and downstream: 5′-ACC CTC CAT CGC ACC CCC TCA C-3′). For IL-2Rγ (upstream: 5′-CCA GGA CCC ACG GGA ACC CA-3′ and downstream: 5′GGT GGG AAT TCG GGG CAT CG-3′). For human IL-15 (upstream 5′-GGA TTT ACC GTG GCT TTG AGT AAT GAG-3′, or 5′-GCC TTC ATG GTA TTG GGA A-3′, corresponding to a sequence inside exon A (Meazza *et al*., 1996) and downstream 5′-GAA TCA ATT GCA ATC AAG AAG TG-3′).

For human IL-15Rα (upstream 5′-GGC GAC GCG GGG CAT CAC-3′ and downstream 5′-TCG CGTTGG CCC TGT GGA TA-3′). PCR products were electrophoresed in a 1% agarose gel, transferred to a nylon membrane (Biodyne) and hybridized with a [^{32}P]cDNA probe spanning from base 160 to base 671, obtained by RT–PCR on human PHA-blast. Sequence analysis revealed identity with previously published IL-15Ra sequences containing the exon 3 (Anderson *et al*., 1995).

For β-actin (upstream 5′-GTG GGG CCC CAG GCA CCA-3′ and downstream 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′).

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