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FULL PAPER

IL-10 stimulatory effects on human NK cells explored by gene profile analysis

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The molecular mechanisms underlying the increase of natural killer (NK) cell anticancer activity mediated by interleukin (IL)-10 have not been elucidated. The aim of this study was to identify potential molecular mediators of IL-10 stimulatory effects by exploring the NK cell gene display induced by this cytokine. Gene profile was determined by high-throughput cDNA microarray and quantitative real-time PCR. In vitro, NK cells resting or conditioned with IL-10 were tested for cytotoxicity, migration and proliferation. IL-10 enhanced mRNA levels of cell activation/cytotoxicity-related genes (eg secretogranin, TIA-1, HMG-1, interferon-inducible genes) not upregulated by IL-2. In line with these findings, IL-10 increased NK cell in vitro cytotoxicity against Daudi cells. Unlike IL-2, IL-10 did not show any significant effect on NK cell in vitro proliferation and migration. However, gene profile analysis showed that IL-10 increased the expression of cell migration-related genes (eg L-selectin, vascular endothelium growth factor receptor-1, plasminogen activator, tissue; formyl peptide receptor, lipoxin A4 receptor), which might support a stimulatory effect not evident with the in vitro functional assay. Overall, gene profiling allowed us to formulate new hypotheses regarding the molecular pathways underlying the stimulatory effects of IL-10 on NK cells, supporting further investigation aimed at defining its role in cancer immune rejection.
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Keywords: *human NK cell; IL-10; gene profile*

Introduction

Interleukin (IL)-10 is a pleiotropic cytokine produced by a variety of immune cells as well as tumor cells.[1] *In vitro*, the best-known effects of IL-10 on the immune system are the inhibition of HLA-dependent cell-mediated immune response. In fact, IL-10 inhibits CD28 expression by cytotoxic T lymphocytes (CTL), decreases the secretion of IL-2, interferon-gamma (IFNγ) and granulocyte macrophage colony-stimulating factor (GM-CSF) as well as the proliferative response of Th1 cells, and counteracts the activity of dendritic cells.[2] Despite these considerations, the role of this cytokine in tumor immunology is controversial.[3] Some authors raised the teleological hypothesis that IL-10 acts as an immunosuppressive molecule secreted by tumors to escape from immune surveillance.[4–6] However, a large body of evidence in different animal tumor models opposes this theory, showing that IL-10 can favor immune-mediated cancer rejection.[7–14] In humans, the knowledge on IL-10's role in tumor immunology is scarce. In the search for a cytokine pattern that could predict tumor response to IL-2/peptide-based melanoma patient vaccination,[15] we studied cytokine mRNA abundance in fine-needle aspirate material from metastatic lesions by using quantitative real-time PCR.[16,17] We found that pretreatment IL-10 mRNA levels were significantly higher in responding than in nonresponding tumor lesions, suggesting that high IL-10 levels in the tumor microenvironment might be conducive to an effective immune response.

Others have observed that the IL-10 promoter polymorphism leading to a reduced IL-10 expression phenotype was associated with an increased incidence of melanoma and prostate cancer, thus suggesting a natural role of this cytokine against cancer.[18,19]

The mechanism underlying this hypothetical immune stimulating effect of IL-10 remains obscure. Some authors have suggested that the IL-10 antitumor activity observed in animal models might be mediated by NK cells.[12,20] NK cell *in vitro* cytotoxicity has been reported to be enhanced by IL-10.[3,7,21–23] However, IL-10 also inhibits the production of IFNγ and tumor necrosis factor alpha (TNFα) by NK cells[24,25] and downregulates NK cell target molecules on tumor cells,[26] thus potentially reducing NK cell antitumor effects.[27]

As NK cell genes targeted by IL-10 are largely unknown and given the central role NK cells are likely to play in innate and adaptive immune response against tumors,[28] we investigated the effects of IL-10 on NK cells at genetic and functional level to identify molecular mechanisms potentially responsible for the functional effects of the cytokine on these cells. We used IL-2 as a

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reference cytokine in order to compare the gene pathways induced by IL-10 with those proper of a classic NK cell activator.

Results

NK cells isolation and RNA amplification

Microbeads purification yielded 2×10^4 cells per 1×10^6 peripheral blood mononuclear cells (PBMC). The percentage of $CD3^-/CD56^+$ cells was on average 92% (range 89–95%), as assessed by flow cytometry analysis (Figure 1). On average, starting from 1.1 μg (range 0.8–1.5 μg) of total RNA extracted from purified NK cells, we yielded 10 μg of amplified RNA (range 8.2–12.1 μg) after the first round of amplification and 4300 μg (range 3500–6600 μg) of aRNA at the end of the second round.

cDNA microarray analysis

After slide scanning, microarray spots with size lower than 50 pixels and fluorescence intensity lower than 300 were discarded. These high-stringency settings were arbitrarily chosen to minimize background or labeling/hybridization bias.[29,30] This filter was removed if the same gene demonstrated high-intensity fluorescence in the other channel (>3000) in at least one experiment in order to recover possibly useful information. The complete data set was adjusted by log-transforming the absolute value of fluorescence ratios, which were then median-centered and normalized as suggested by Eisen *et al*.[31] Adjusted data were filtered to remove genes with missing values in more than one experiment and only allowing test genes expressed at a ratio >3 or <0.33 compared to the reference in at least one experiment. Using these settings, a total of 2631 genes out of the 6400 clones were sorted out for subsequent analysis (Figure 2).

Adjusted ratios were shown according to the central method for display using a normalization factor that emphasizes differences in expression between experimental groups analyzed by resetting the equality parameter (ratio = 1) to the mean of all the experiments.[32]

Genes differentially expressed as a result of IL-10 or IL-2 exposure were identified by comparing the gene profile of unstimulated with that of cytokine-conditioned NK cells. To this aim, we queried the data set by selecting genes differentially expressed according to a paired sample *t*-test. The cutoff p_2-value of ≤ 0.02 was arbitrarily chosen and was not corrected by the number of hypotheses tested (ie, genes).[30] In this fashion, 136 and 353 genes displayed differential expression following

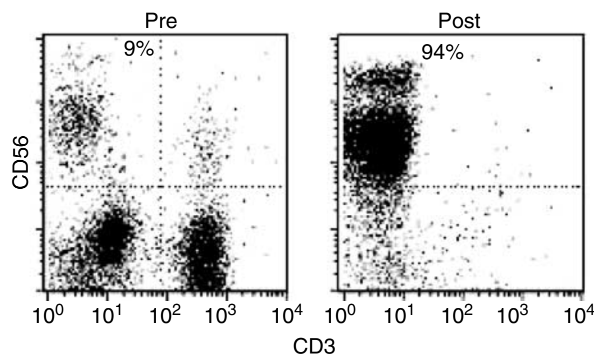
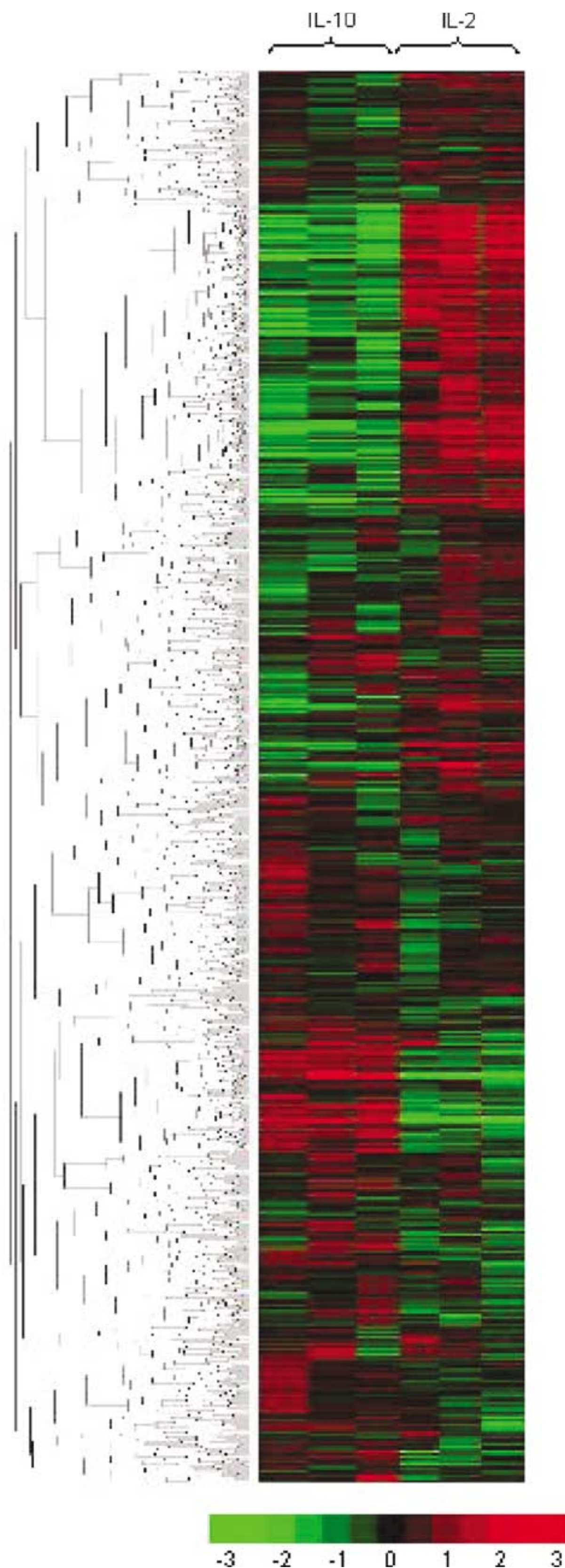


Figure 1 NK cell isolation from PBMC. FACS data before (PRE, left panel) and after (POST, right panel) negative selection. NK cells are identified as $CD3^-/CD56^+$ cells.



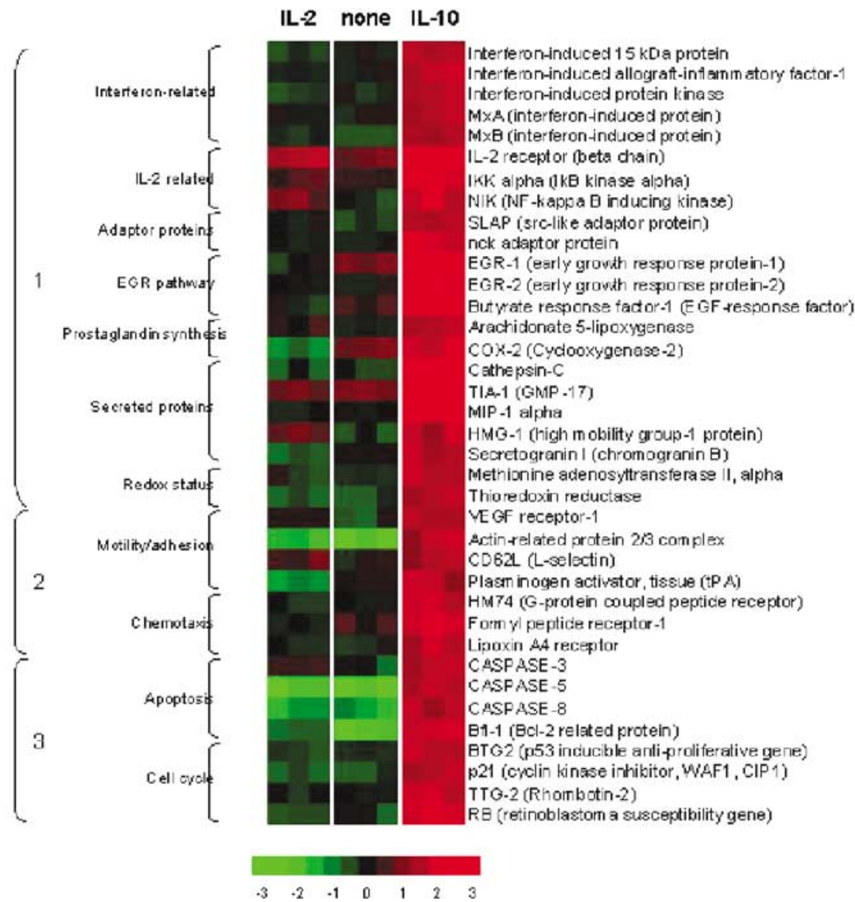


Figure 3 NK cell gene profile following IL-10 conditioning. Gene expression of IL-10-conditioned NK cells (right column) was compared with that of unconditioned NK cells (middle column). The data set including genes expressed by unconditioned and conditioned (IL-10) NK cells was queried for selecting genes differentially expressed according to a paired sample t-test (cutoff p_2-value $\leqslant 0.02$). Among the 136 genes overexpressed by IL-10-conditioned NK cells, we selected 37 genes potentially involved in cell activation/cytotoxicity (1), adhesion/migration (2) or proliferation/apoptosis (3). For comparison purposes, the behavior of the same set of genes following IL-2 conditioning is shown (left column).

IL-10 or IL-2 exposure, respectively. For IL-10, we selected 37 genes according to their known or potential involvement in NK cell activation/cytotoxicity (eg MxA, secretogranin and several IFN-inducible genes), adhesion/migration (eg CD62L, actin-related protein, formyl peptide receptor) and proliferation/apoptosis (eg BCL2-related protein, CASPASE-3, CASPASE-5, CASPASE-8) (Figure 3). Similarly, for IL-2 we chose 40 genes related to NK cell activation/cytotoxicity (eg granzyme-A, several cytokine receptors), adhesion/migration (eg tubulin, MMP-12, integrins) and proliferation/apoptosis (eg cyclin-E, CDK4, mitotic kinesin like protein-1) (Figure 4).

Figure 2 Gene expression (2631 genes sorted out starting from 6400 genes by means of filtering criteria described in the text) of NK cells stimulated with either IL-10 (left column) or IL-2 (right column) as displayed by hierarchical clustering analysis. One experiment (done in triplicate) out of three is shown. Similar results were obtained in the other two experiments. Rows represent individual genes; columns represent individual samples. Each cell in the matrix represents the expression level of a single transcript in a single sample, with red indicating transcript levels above and green indicating transcript levels below the median for that gene across all samples, respectively. Color saturation is proportional to magnitude of the difference from the median. Hierarchical clustering is an agglomerative approach in which single expression profiles are joined to form groups, which are further joined until the process has been carried to completion, forming a single hierarchical tree. The process of hierarchical clustering proceeds as follows. First, the pairwise distance matrix is calculated for all of the genes to be clustered. Second, the distance matrix is searched for the two most similar genes or clusters. Third, the two selected clusters are merged to produce a new cluster that now contains at least two objects. Fourth, the distances are calculated between this new cluster and all other clusters. Last, steps 2–4 are repeated until all objects are in one cluster.

Quantitative real-time PCR

Several genes known to be involved in NK cell activities were not included in the cDNA array gene list. We therefore utilized quantitative real-time PCR to measure the expression of some of these genes (Table 1). The results are shown in Figure 5. Compared to PBMC (the reference sample chosen for cDNA array analysis), unstimulated NK cells showed higher mRNA levels of killing inhibitor receptor (KIR), CD16, CD2, perforin and TIA-1. TIA-1, which codes for a cytotoxic granule-associated protein, was specifically increased at the mRNA level by IL-10 stimulation of NK cells. IL-2 showed broader effects that resulted in the upregulation of mRNA levels of TNFα, IFNγ, granzyme-B, perforin, FAS ligand (FAS-L) and inducible nitric oxide synthase

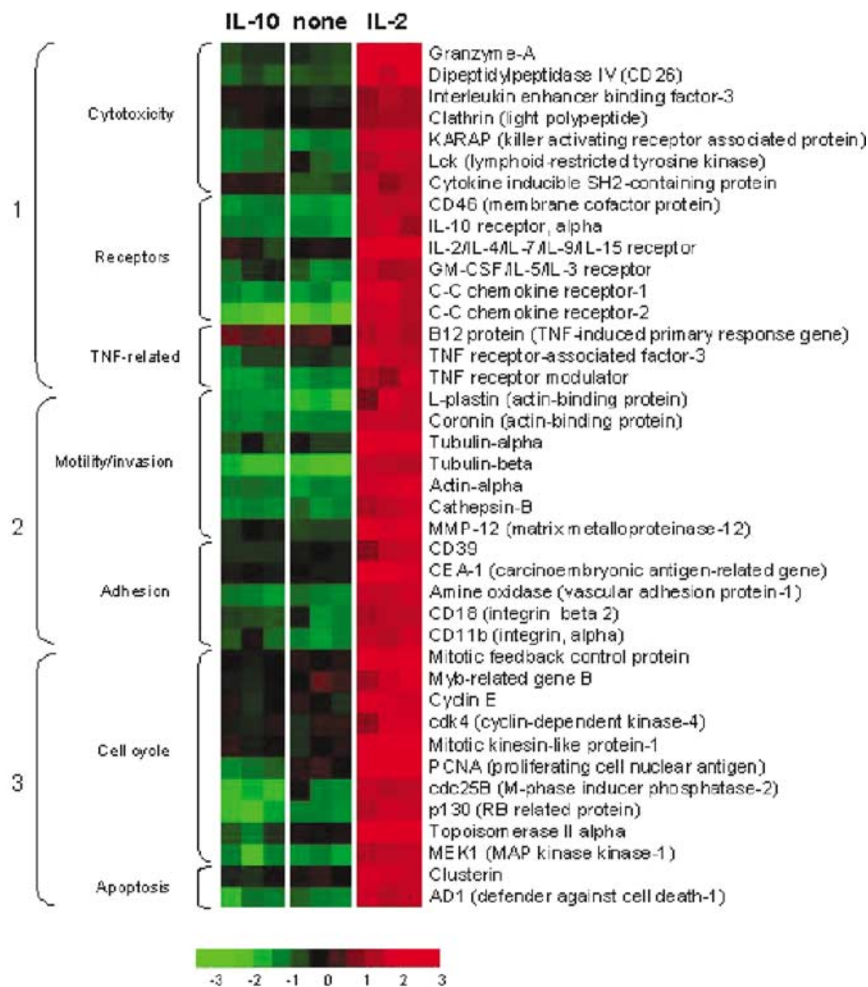


Figure 4 NK cell gene profile following IL-2 conditioning. Gene expression of IL-2-conditioned NK cells (right column) was compared with that of unconditioned NK cells (middle column). The data set including genes expressed by unconditioned and conditioned (IL-2) NK cells was queried for selecting genes differentially expressed according to a paired sample *t*-test (cutoff p_2-value $\leqslant 0.02$). Among the 353 genes overexpressed by IL-2-conditioned NK cells, we selected 40 genes potentially involved in cell activation/cytotoxicity (1), adhesion/migration (2) or proliferation/apoptosis (3). For comparison purposes, the behavior of the same set of genes following IL-10 conditioning is shown (right column).

(iNOS). Quantitative PCR confirmed the overexpression of some genes found at microarray analysis, although this validation was possible only for genes for which we could design appropriate probe/primers triplets (ie GRO2, EBI3, IL1, IL8, IL2-R, TGFbeta-R, IL10-R, VEGF-R, CD59, CD29, CASPASE-3, CASPASE-5, CASPASE-8, NKG5, NFkappaB, p21, RB, COX-2).

TIA-1 protein expression evaluation

To confirm the information obtained at mRNA level, TIA-1 protein expression was tested in resting and IL-10- or IL-2-conditioned NK cells by intracellular flow cytometry using the corresponding goat anti-human polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were permeabilized by incubation in PBS-saponin (0.2%, Sigma, St Louis, MO, USA) in 5% milk for 6 h.[33] A donkey anti-goat secondary antibody (Molecular Probes, Eugene, OR, USA) was used for the fluorescent staining.

IL-10, but not IL-2, stimulated TIA-1 protein expression in NK cells (Figure 6), confirming the genetic findings.

Proliferation and migration assay

As shown in Figure 7, we found that IL-2 stimulated NK cell proliferation, while IL-10 did not show any significant effect on this NK cell activity. This finding was very much in line with the genetic profile shown in Figures 3 and 4: while IL-2 induced the expression of several genes associated with active cell cycle, IL-10 rather enhanced the mRNA abundance of apoptosis-related genes (eg caspases). As for cell migration, the functional assay showed that IL-2 but not IL-10 has a stimulatory effect on NK cells. In this case, the functional assay, which confirmed the genetic data observed for IL-2, was at least partially in contrast with the IL-10-induced gene profile that showed an upregulation of migration-related genes (eg L-selectin, VEGF receptor-1, t-PA, HM74, formyl peptide receptor-1 and lipoxin A4 receptor) (Figures 3 and 4).

Cytotoxicity assay

As expected, unconditioned NK cells did not show any significant cytolytic activity against Daudi cells. At 20 : 1

Table 1 Quantitative real-time PCR probes and primers

Gene	*Probes and primers*
β-actin	(f) 5′-GGCACCCAGCACAATGAAG-3′ (r) 5′-GCCGATCCACACGGAGTACT-3′ (p) 6FAM-TCAAGATCATTGCTCCTCCTGAGAGCGC-TAMRA
IFNγ	(f) 5′-AGCTCTGCATCGTTTTGGGTT-3′ (r) 5′-GTTCCATTATCCGCTACATCTGAA-3′ (p) 6FAM-TCTTGGCTGTTACTGCCAGGACCCA-TAMRA
TNFα	(f) 5′-CCCCAGGGACCTCTCTCTAATC-3′ (r) 5′-TACAACATGGGCTACAGGCTTG-3′ (p) 6FAM-CAGTCAGATCATCTTCTCGAACCCCGAG-TAMRA
Perforin	(f) 5′-TGGAGTGCCGCTTCTACAGTT-3′ (r) 5′-GCCCTCTTGAAGTCAGGGTG-3′ (p) 6FAM-CCATGTGGTACACACTCCCCCGC-TAMRA
Granzyme-B	(f) TCCTAAGAACTTCTCCAACGACACT (r) GCACAGCTCTGGTCCGCT (p) TGCTACTGCAGCTGGAGAGAAAGGCC
FAS-L	(f) 5′-GCTGAGGAAAGTGGCCCA-3′ (r) 5′-CATAGGTGTCTTCCCATTCCAGA-3′ (p) 6FAM-TAACAGGCAAGTCCAACTCAAGGTCCATG-TAMRA
iNOS	(f) 5′-GGGAGGATCCAGTGGTCCA-3′ (r) 5′-AAACATTTCCCGGGCAGTG-3′ (p) 6FAM-TGCAGGTCTTCGATGCCCGCA-TAMRA
TIA-1	(f) 5′-CTCACTCGGGCCTCTGGCC-3′ (r) 5′-GGTCTGCGTCACGTGGATG-3′ (p) 6FAM-CAGGGCATGGGGACATCATATCAGG-TAMRA
CD16	(f) 5′-TGCAGCTAGAAGTCCATATCGG-3′ (r) 5′-CCTCCTTGAACACCCACCG-3′ (p) FAM-TGGCTGTTGCTCCAGGCCCC-TAMRA
KIR[a]	(f) 5′-AGTGGGTGGTCGGCACC-3′ (r) 5′-GGTTTTCTGTGGACTCGACCTG-3′ (p) FAM-AGCAACCCCCTGGAGATCACGGTC-TAMRA
CD2	(f) 5′-CCAGCCTGAGTGCAAAATTCA-3′ (r) 5′-CAGGCTCGACACTGGATTCC-3′ (p) FAM-TGCACAGCAGGGAACAAAGTCAGCA-TAMRA

(f) forward primer; (r) reverse primer; (p) probe.
[a]KIR2DS4.

E : T ratio, NK activity was increased by IL-10, although to a lower extent with respect to IL-2. The percentage of cell lysis by unconditioned, IL-10- and IL-2-conditioned NK cells was 5, 13 and 19%, respectively (Figure 8). Cytotoxicity-related genes upregulated by IL-10 and IL-2 appeared to be different, suggesting that a separate and specific pathway mediates the stimulatory properties of the two cytokines (Figures 3 and 4). The inhibition of TIA-1 activity by specific blocking antibodies significantly reduced NK cell cytotoxicity, which confirmed the relationship between IL-10-mediated increase in NK cytotoxicity and the induction of TIA-1 expression by the cytokine.

Discussion

The molecular mechanisms underlying the postulated immunostimulatory activity of IL-10 has not been elucidated. We addressed this issue by studying IL-10 effects on the functional and gene profile of NK cells *in vitro*. To this aim, beside traditional functional assays, we adopted high-throughput cDNA microarray and quantitative real-time PCR to portrait the gene patterns of IL-10-conditioned NK cells. Brown *et al*[34] suggested a tight correlation between the function of a protein and the expression patterns of its gene, which should provide a compelling reason for a gene profile-based formulation of scientific hypotheses.[35] However, it must be taken into account that other investigators have shown that changes in mRNA expression cannot always predict biological outcome, which is basically dictated by protein activity. Therefore, our findings only allowed us to formulate some hypothesis on the role of IL-10-induced activation of NK cells.

Furthermore, some considerations must be made regarding the study design we adopted for the gene profile analysis. Firstly, the use of pooled PBMC instead

of unstimulated NK cells allowed us to analyze the gene expression of unstimulated NK cells; in addition, this type of reference sample makes the present experiments directly comparable with others already performed with this strategy[29,30] or to be performed in the next future in our laboratory. Secondly, we studied gene expression at a single time point (4 h), which implies that genes with later or earlier up/downregulation were overlooked. However, our choice was based on the results of a previous time course microarray study,[30] in which we observed that PBMC upregulated most genes 3 h after the exposure to IL-2. Finally, the choice of IL-10 dose was derived from dose–effect experiments, which showed a maximum number of genes changing their transcriptional levels IL-10 concentrations equal/greater than 30 ng/ml.

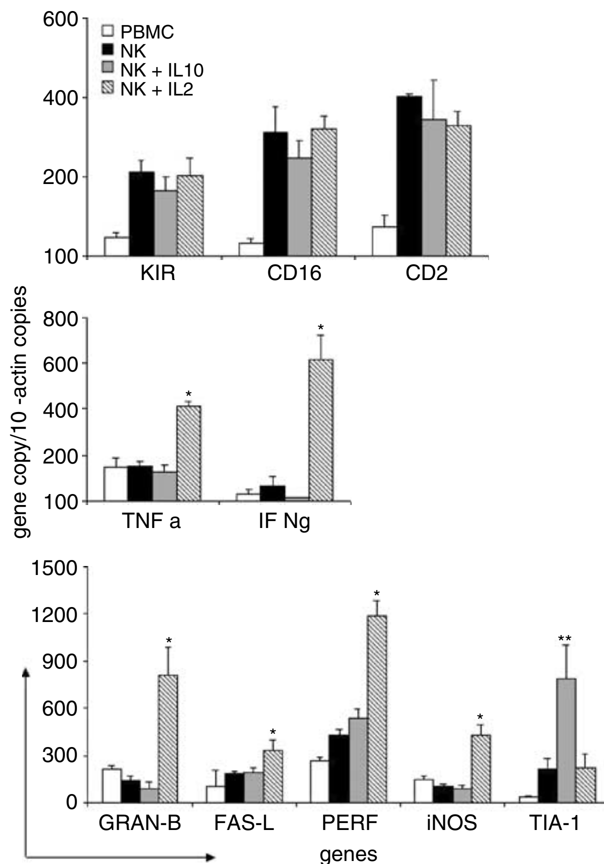


Figure 5 Pooled PBMC (white bars), unconditioned NK cells (black bars) and NK cells conditioned with IL-10 (gray bars) or IL-2 (crossed bars) were tested for the expression of nine genes not included in the microarray gene list by means of quantitative real-time PCR. β-actin was used as housekeeping gene. Significant differences in gene expression between unconditioned NK cells and NK cells conditioned with IL-2 or IL-10 are marked with a single or double asterisk, respectively. GRAN-B: granzyme-B; PERF: perforin.

Regarding the reference cytokine, our results confirmed the notion that IL-2 induces a general activation of NK cells, by augmenting their proliferation rate, migration capability and cytotoxic activity. Accordingly, several proliferation, migration and cytotoxicity-related genes were found to be upregulated by IL-2 (Figure 4). Noticeably, IL-2 influenced the expression of genes (IL-10 receptor, IL-2/IL-4/IL-7/IL-9/IL-15 receptor, GM-CSF/IL-5/IL-3 receptor, TNF-induced primary response gene, TNF receptor-associated factor 3, TNF receptor modulator) involved in the signal transduction pathways of several other cytokines, which corroborates its pivotal role in modulating NK cell activities.

IL-10 and NK cell migration/proliferation activity

Compared to IL-2, IL-10 showed a smaller but consistent range of effects on the gene display and functional activities of NK cells. Although IL-10 can act as a growth factor for B-cell tumors and melanomas,[36,37] the functional assay showed that IL-10 had no effect on NK cells proliferation. In addition, gene pattern was characterized by the upregulation of some proapoptotic (caspase-3, caspase-5, caspase-8) and cell cycle suppression (p21, RB, BTG-2) genes (Figure 3).

According to the functional assay, IL-10 appeared to have no influence on *in vitro* NK cell migration. By contrast, microarray results showed that IL-10 increased mRNA levels of adhesion/migration-related genes such as L-selectin, VEGF receptor-1, t-PA, HM74, formyl peptide receptor-1 and lipoxin A4 receptor (Figure 3). The last three genes are involved in the synthesis of peptide receptors[38–40] that mediates leukocyte chemotaxis by binding peptides derived from protein degrada-

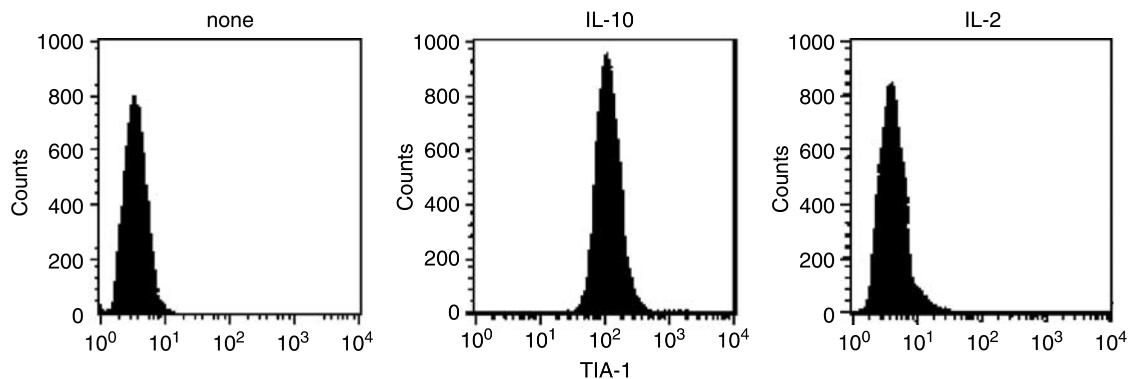


Figure 6 TIA-1 protein expression in unstimulated NK cells (left panel) and NK cells conditioned with IL-10 (middle panel) or IL-2 (right panel). NK cells were isolated with magnetic beads and then permeabilized to primary (TIA-1 specific) and secondary (fluorescence conjugated) antibodies for intracellular flow cytometry analysis. A significant increase in TIA-1 protein expression was observed following IL-10 (but not IL-2) administration.

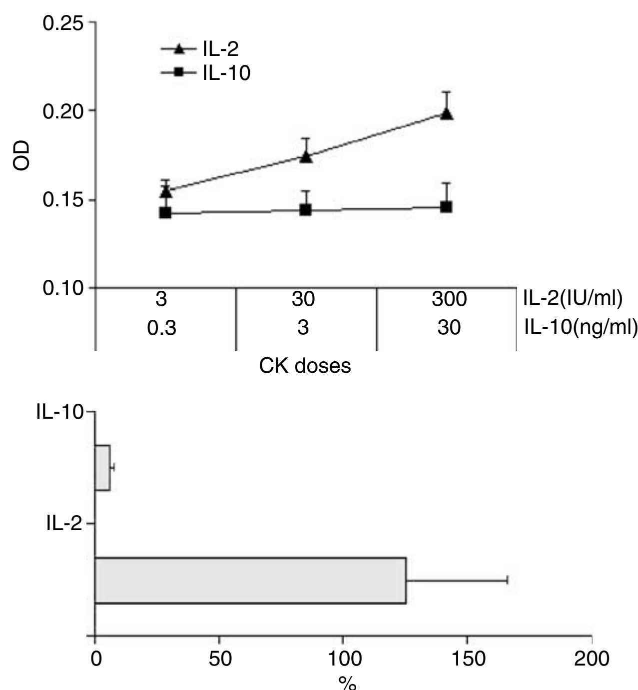


Figure 7 Upper panel. NK cell proliferation assay. Using a nonradioactive colorimetric assay (XTT assay) and growing doses of cytokines, IL-10 effect on NK cells was assessed. Unlike IL-2, IL-10 did not influence NK cell proliferation. Lower panel. NK cell migration assay. Cell migration was evaluated under the effect of interleukin-10 (IL-10) or interleukin-2 (IL-2), using the Boyden chamber method. Results are reported as the ratio (expressed as a percentage) between the test (conditioned NK cells) and the control (unconditioned NK cells) number of migrated cells. The average control migration was 150×10^3 migrated cells/4 h incubation. IL-2 increased NK cell migration by 125% (s.e.m.: 30%), while IL-10 showed no significant effect on this cell activity.

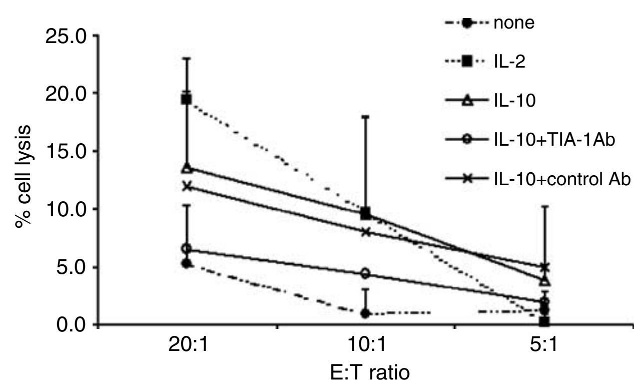


Figure 8 Cytotoxicity assay. NK cells were incubated with target cells (Daudi cells) alone (none) or in the presence of interleukin-10 (IL-10) or interleukin-2 (IL-2) at different E : T ratios. A nonradioactive fluorescence-based assay was used to assess the influence of IL-10 on NK cell cytotoxicity. As a comparison, IL-2-stimulated NK cells were also analyzed. At 20 : 1 E : T ratio, IL-10-conditioned NK cells showed a significantly higher cytotoxic activity as compared to unconditioned NK cells.

tion occurring in damaged/diseased areas.[41] The overexpression of these receptors might play an important role in NK cell migration in response to the liberation of danger signals (eg peptides) freed in the tumor microenvironment. The discrepancy between genetic and functional findings might just depend on the inability of the *in vitro* assay to reveal the influence of the genes upregulated by IL-10 on NK cell migration.

IL-10 and NK cell activation/cytotoxicity

Concordantly with the cytotoxicity assay results, gene profile showed that IL-10 increased the expression of genes potentially involved in cytotoxicity/cell activation, although for most of them the role in NK cell activities has not yet been proved. These genes code for proteins belonging to the following groups:

(a) interferon-inducible proteins (eg allograft inflammatory factor-1, interferon-induced 15 kDa protein[42]);
(b) signal transduction pathways (eg nuclear factor kappa B (NF-kB) modulator, NF-kB-inducing kinase, src-like adaptor protein (SLAP),[43] nck,[44] early growth response (EGR) family proteins[45]);
(c) prostaglandin synthesis (COX-2,[46] 5-lypooxygenase);
(d) redox status regulation (adenosyl-methionine synthetase, thioredoxin reductase[47]);
(e) secreted proteins (eg MIP-1α, HMG-1,[48] cathepsin-C, secretogranin and TIA-1).

Among the above mentioned genes, we focused on TIA-1, a gene encoding a 15 kDa cytotoxic granule-associated protein whose expression is restricted to CTL and NK cells.[49] This RNA-binding protein, lately renamed GMP-17 (granule membrane protein-17),[50] is considered a candidate effector of apoptotic cell death because of its ability to trigger DNA fragmentation of target cells. *In vivo*, we recently found that TIA-1 mRNA was overexpressed in melanoma metastases responding to peptide-based vaccination in humans,[29] suggesting that this molecule might be part of the tumor microenvironment gene profile that makes cancer cells responsive to immunotherapeutic manipulations. In the present work, we showed that IL-10 induced TIA-1 mRNA overexpression by NK cells. We validated the microarray finding by quantitative real-time PCR (Figure 5), and verified a similar increase at protein level (Figure 6). This, along with the increased cytotoxicity of NK cells in the presence of IL-10, led us to hypothesize that NK cell cytotoxic activity might be enhanced by IL-10 through a cascade of molecular events including TIA-1 overexpression, as supported by the *in vitro* findings with the use of anti-TIA-1 blocking antibodies. Furthermore, we found that this IL-10 effect on TIA-1 expression was selective for NK cells, as no changes in TIA-1 levels were observed in CTL (data not shown). Yet, IL-2 did not induced TIA-1 expression either in CTL or NK cells, while IL-10 had no effect on NK cell expression of other classical markers of cytotoxicity (ie perforin, granzyme-B, iNOS, FAS-L; Figure 5) and did not affect their upregulation by IL-2 when the two cytokines were used together (data not shown). Taken together, these findings suggest that IL-10 might affect NK cell cytotoxicity through a specific pathway distinct from that used by IL-2 to activate NK cells. However, just to underscore the complexity of the cytokine network signal transduction system, we also found that IL-2 increased the mRNA levels of IL-10 receptor, and that IL-10 increased both IL-2 receptor and IL-2 transduction pathway-related (ie IkB kinase α and NF-kB-inducing kinase) genes. This might appear to be conflicting with the common notion by

which IL-10 inhibits IL-2-mediated proinflammatory functions in T lymphocytes.[51] Nevertheless, some investigators have already shown quite opposite results in monocytes,[52,53] which highlights the wide spectrum of effects the same cytokine can exert on different cell populations.

Finally, it is noteworthy that IL-10 increased the expression of some IFN-related genes (Figure 3). In fact, although IL-10 is related to type-I interferons (IFN-α and -β) on the basis of its structure, to our knowledge this is the first time that the gene expression profile induced by IL-10 is reported to significantly overlap that induced by type-I interferons.

Materials and methods

Study design

Previously isolated NK cells (1×10^5/ml) were incubated for 4 h with IL-10 (30 ng/ml, Genzyme, Cambridge, MA, USA). As a comparison, cells were also exposed to a well-known NK cell activator, that is, IL-2 (300 UI/ml, Chiron, Emeryville, CA, USA). Gene profile of conditioned and unconditioned NK cells was assessed by high-throughput cDNA microarray. The expression of nine additional NK cell-related genes not included in the microarray gene list was measured by quantitative real-time PCR.

NK cells unconditioned and conditioned with IL-10 (30 ng/ml) or IL-2 (300 UI/ml) were used for the motility and cytotoxicity assay. Growing doses of cytokines were used for the proliferation assay.

All experiments, performed in triplicate, were repeated three times. Results are reported as the mean values $\pm$ s.d.

IL-10 activity

Human IL-10 activity was measured by measuring human IL-1β (IL-1) in the supernatant fluids of cultured human PBMC. IL-1 was measured using a commercially available human IL-1 ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions. Recombinant human IL-10 was evaluated for its ability to inhibit human IL-1 production by LPS (*Escherichia coli* 0127 : B8, Sigma)-stimulated human PBMC. Human IL-10 was serially diluted 1/2 starting at a concentration of 120 to 1 ng/ml. The control groups were incubated without IL-10 in the positive control and without IL-10 and LPS in the negative control. Inhibition (100%) was achieved at IL-10 doses greater than 15 ng/ml.

NK cells isolation

NK cells were purified from buffy coats obtained from healthy donors. PBMC were first isolated by Ficoll gradient separation. Then, NK cells were negatively isolated by using the NK cell Isolation Kit (Miltenyi Biotec, Auburn, CA, USA), following the manufacturer's guidelines. Briefly, this is an indirect magnetic labeling system for the isolation of untouched NK cells from human PBMC. Non-NK cells, that is, T-cells, B-cells, dendritic cells, monocytes, granulocytes and erythroid cells, are indirectly magnetically labeled by using a cocktail of biotin-conjugated antibodies against CD3, CD4, CD14, CD15, CD19, CD36, CD123 and Glycophorin A, and anti-biotin microbeads. Consequently, isolation of highly pure NK cells is achieved by depletion of magnetically labeled cells. Purity of isolated NK cells was assessed by flow cytometry (FACScan, Becton Dickinson; San Jose, CA, USA) using purified mouse anti-human CD3 and CD56 monoclonal antibody (BD Pharmingen, San Jose, CA, USA).

RNA extraction and amplification

Total RNA from isolated NK cells was isolated and linearly amplified as we described elsewhere.[54]

High-throughput cDNA microarray

A 6400-spot human cDNA microarray slide (including 5492 unique clones from sequence-verified genes and 537 expression sequence tags) printed at Advanced Technology Center, NCI was utilized. For complete gene list, please visit web site at http://nciarray.nci.nih.gov/gal_files/index.shtml (Mm-FCRF-GEM2 file). Pooled PBMC from six healthy donors were used to prepare reference aRNA to be cohybridized in all experiments with test aRNA.[29] For probe preparation, aRNA obtained after two rounds of amplification was labeled in a reverse transcription reaction in the presence of Cy3 (reference sample: pooled PBMC)- or Cy5 (test samples: NK cells unconditioned or exposed to IL-10 or IL-2)-labeled dUTP (Amersham, Piscataway, NJ, USA), as we have already described.[30] Slides were scanned at 10-mm resolution on a GenePix 4000 Scanner (Axon Instruments, Foster City, CA, USA) at variable PMT voltage to obtain maximal signal intensities with $<1\%$ probe saturation. Resulting tiff images were analyzed via ArraySuite software (National Human Genome Research Institute, Bethesda, MD, USA). Data were analyzed using Cluster and TreeView software by Michael Eisen.[31]

cDNA synthesis and quantitative real-time PCR

Appropriately diluted amplified RNA (1 µl) was reverse transcribed into cDNA using 1 µl of random hexamers, 5 µl first-strand buffer, 2 µl 0.1 M DTT, 2 µl magnesium sulfate, 1 µl 10 mM dNTP and 1 µl SS-II. The reaction mixture was heated to 65°C for 10 min before adding SS-II, then synthesis was continued with 1 h of 42°C incubation. cDNA was stored at −20°C until use.

The expression of 11 genes (including β-actin, which was used as the housekeeping gene) by unstimulated and IL-10- or IL-2-conditioned NK cells was assessed by means of quantitative real-time PCR, as we reported elsewhere.[18,19] Quantitative PCR was also employed to validate the cDNA microarray expression data relative to genes for which we could design specific probes and primers (GRO2, EBI3, IL1, IL8, IL2-R, TGFβ-R, IL10-R, VEGF-R, CD59, CD29, CASPASE-3, CASPASE-5, CASPASE-8, NKG5, NFkappaB, p21, RB, COX-2).

For each sample, target transcript values were corrected by the housekeeping gene (β-actin) copies to obtain normalized values independent from variation in starting material. Gene levels were reported as number of gene copies per 1×10^5 copies of β-actin.

Probes and primers (Table 1) were designed to span intron–exon junctions to avoid amplification of genomic DNA and to obtain amplicons less then 150 bp in length in order to enhance PCR efficiency. Genomic DNA contamination was further reduced by the use of RNA

amplification that selectively amplifies mRNA and requires multiple purification steps.

Proliferation assay
The nonradioactive colorimetric assay system using XTT (Boehringer, Mannheim, Germany) was used, following the manufacturer's instructions. Briefly, 10^4 cells/well were seeded in 100 μl culture medium containing growing concentrations of IL-10 or IL-2 (0.3, 3, 30 ng/ml, and 3, 30, 300 IU/ml, respectively). NK cells were then incubated 48 h at 37°C and 6.5% CO_2. At the end of incubation, 50 μl XTT labeling mixture was added per well and cells were incubated for 4 h at 37°C and 6.5% CO_2. Finally, spectrophotometrical absorbance was measured by means of an ELISA reader.

Migration assay
The Boyden chamber migration assay described by Axelsson *et al*[55] was used. Polycarbonate filters with pores of 3 μm diameter (Nuclepore Corporation, Pleansanton, NY, USA) were used between the lower and upper compartments of the Boyden chambers. The lower compartments were filled with 400 μl of RPMI 1640 supplemented with 0.5% human serum albumin. In total, 200 μl of conditioned and unconditioned NK cells were added to the upper compartments. After incubation for 6 h at 37°C, the amount of migratory cells in the lower chambers was counted and the mean values in triplicate chambers were calculated. The results of three experiments (mean ± s.e.m.) were expressed as percent increase compared to untreated control cells.

Cytotoxicity assay
The cytotoxic activity of NK cells unstimulated or conditioned with IL-10 or IL-2 was tested against Daudi cells at different E:T ratios (5:1, 10:1 and 20:1). This assay was performed using the nonradioactive fluorescence-based assay we described elsewhere.[56] Briefly, target cells were resuspended in complete medium at a final concentration of 10^6/ml and incubated with 15 μM calcein-AM (Molecular Probes, Eugene, OR, USA) for 60 min at 37°C with occasional shaking. After two washes in CM, cells were adjusted to 10^5/ml. NK cells unconditioned or after overnight exposure to cytokines were then added. The test was performed in V-bottom 96-well microtiter plates with six replicate wells for spontaneous (only target cells in complete medium) and maximum release (only target cells in medium plus 2% Triton X-100). After incubation at 37°C in 5% CO_2 for 6 h, 5 μl FluoroQuench (One Lambda, Canoga Park, CA, USA) was added to each well and the trays were centrifuged for 1 min at 60 *g*. Then, fluorescence (excitation at 485 nm, emission at 530 nm) was measured using the automated FluorImager 595 scanning system (Molecular Dynamics, Sunnydale, CA, USA). A decrease in the fluorescence emission is proportional to the degree of lysis of target cells, once the released dye is quenched by the hemoglobin contained in the FluoroQuench reagent. The percentage of lysis was calculated as follows: % lysis = (1−(mean experimental emission−mean min/max−mean min)) × 100%.

In order to study the effect of TIA-1 in NK cytotoxicity, this test was also performed in the presence of both anti-TIA-1 antibodies (goat anti-human polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and control antibodies (irrelevant goat IgG) at 50 μg/ml concentration.

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References

1 Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001; **19**: 683–765.
2 Mocellin S, Marincola F, Rossi CR, Nitti D, Lise M. The multifaceted relationship between IL-10 and adaptive immunity: putting together the pieces of a puzzle. *Cytokine Growth Factor Rev* 2004; **15**: 61–76.
3 Mocellin S, Panelli MC, Wang E, Nagorsen D, Marincola FM. The dual role of IL-10. *Trends Immunol* 2003; **24**: 36–43.
4 Hsieh CL, Chen DS, Hwang LH. Tumor-induced immunosuppression: a barrier to immunotherapy of large tumors by cytokine-secreting tumor vaccine. *Hum Gene Ther* 2000; **11**: 681–692.
5 Mocellin S, Wang E, Marincola FM. Cytokines and immune response in the tumor microenvironment. *J Immunother* 2001; **24**: 392–407.
6 Marincola FM, Jaffee EM, Hicklin DJ, Ferrone S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* 2000; **74**: 181–273.
7 Salazar-Onfray F, Petersson M, Franksson L *et al.* IL-10 converts mouse lymphoma cells to a CTL-resistant, NK-sensitive phenotype with low but peptide-inducible MHC class I expression. *J Immunol* 1995; **154**: 6291–6298.
8 Giovarelli M, Musiani P, Modesti A *et al.* Local release of IL-10 by transfected mouse mammary adenocarcinoma cells does not suppress but enhances antitumor reaction and elicits a strong cytotoxic lymphocyte and antibody-dependent immune memory. *J Immunol* 1995; **155**: 3112–3123.
9 Gerard CM, Bruyns C, Delvaux A *et al.* Loss of tumorigenicity and increased immunogenicity induced by interleukin-10 gene transfer in B16 melanoma cells. *Hum Gene Ther* 1996; **7**: 23–31.
10 Adris S, Klein S, Jasnis M *et al.* IL-10 expression by CT26 colon carcinoma cells inhibits their malignant phenotype and induces a T cell-mediated tumor rejection in the context of a systemic Th2 response. *Gene Ther* 1999; **6**: 1705–1712.
11 Barth Jr RJ, Coppola MA, Green WR. *In vivo* effects of locally secreted IL-10 on the murine antitumor immune response. *Ann Surg Oncol* 1996; **3**: 381–386.
12 Kundu N, Beaty TL, Jackson MJ, Fulton AM. Antimetastatic and antitumor activities of interleukin 10 in a murine model of breast cancer. *J Natl Cancer Inst* 1996; **88**: 536–541.
13 Suzuki T, Tahara H, Narula S, Moore KW, Robbins PD, Lotze MT. Viral interleukin 10 (IL-10), the human herpes virus 4 cellular IL-10 homologue, induces local anergy to allogeneic and syngeneic tumors. *J Exp Med* 1995; **182**: 477–486.
14 Ding Y, Qin L, Kotenko SV, Pestka S, Bromberg JS. A single amino acid determines the immunostimulatory activity of interleukin 10. *J Exp Med* 2000; **191**: 213–224.
15 Rosenberg SA, Yang JC, Schwartzentruber DJ *et al.* Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat Med* 1998; **4**: 321–327.
16 Mocellin S, Ohnmacht GA, Wang E, Marincola FM. Kinetics of cytokine expression in melanoma metastases classifies immune responsiveness. *Int J Cancer* 2001; **93**: 236–242.

17 Mocellin S, Rossi C, Pilati P, Nitti D, Marincola F. Quantitative real time PCR: a powerful ally in cancer research. *Trends Mol Med* 2003; **9**: 189–195.
18 McCarron SL, Edwards S, Evans PR *et al*. Influence of cytokine gene polymorphisms on the development of prostate cancer. *Cancer Res* 2002; **62**: 3369–3372.
19 Howell WM, Turner SJ, Bateman AC, Theaker JM. IL-10 promoter polymorphisms influence tumour development in cutaneous malignant melanoma. *Genes Immun* 2001; **2**: 25–31.
20 Zheng LM, Ojcius DM, Garaud F *et al*. Interleukin-10 inhibits tumor metastasis through an NK cell-dependent mechanism. *J Exp Med* 1996; **184**: 579–584.
21 Petersson M, Charo J, Salazar-Onfray F *et al*. Constitutive IL-10 production accounts for the high NK sensitivity, low MHC class I expression, and poor transporter associated with antigen processing (TAP)-1/2 function in the prototype NK target YAC-1. *J Immunol* 1998; **161**: 2099–2105.
22 Cai G, Kastelein RA, Hunter CA. IL-10 enhances NK cell proliferation, cytotoxicity and production of IFN-gamma when combined with IL-18. *Eur J Immunol* 1999; **29**: 2658–2665.
23 Parato KG, Kumar A, Badley AD *et al*. Normalization of natural killer cell function and phenotype with effective anti-HIV therapy and the role of IL-10. *AIDS* 2002; **16**: 1251–1256.
24 Conti P, Kempuraj D, Kandere K *et al*. IL-10, an inflammatory/inhibitory cytokine, but not always. *Immunol Lett* 2003; **86**: 123–129.
25 Fiorentino DF, Zlotnik A, Vieira P *et al*. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991; **146**: 3444–3451.
26 Tsuruma T, Yagihashi A, Hirata K *et al*. Interleukin-10 reduces natural killer (NK) sensitivity of tumor cells by downregulating NK target structure expression. *Cell Immunol* 1999; **198**: 103–110.
27 Seo N, Tokura Y, Takigawa M, Egawa K. Depletion of IL-10- and TGF-beta-producing regulatory gamma delta T cells by administering a daunomycin-conjugated specific monoclonal antibody in early tumor lesions augments the activity of CTLs and NK cells. *J Immunol* 1999; **163**: 242–249.
28 Kelly JM, Darcy PK, Markby JL *et al*. Induction of tumor-specific T cell memory by NK cell-mediated tumor rejection. *Nat Immunol* 2002; **3**: 83–90.
29 Wang E, Miller LD, Ohnmacht GA *et al*. Prospective molecular profiling of melanoma metastases suggests classifiers of immune responsiveness. *Cancer Res* 2002; **62**: 3581–3586.
30 Panelli MC, Wang E, Phan G *et al*. Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration. *Genome Biol* 2002; **3**: RESEARCH0035.
31 Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 1998; **95**: 14863–14868.
32 Ross DT, Scherf U, Eisen MB *et al*. Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* 2000; **24**: 227–235.
33 Nielsen MB, Monsurro V, Migueles SA *et al*. Status of activation of circulating vaccine-elicited CD8+ T cells. *J Immunol* 2000; **165**: 2287–2296.
34 Brown PO, Botstein D. Exploring the new world of the genome with DNA microarrays. *Nat Genet* 1999; **21**: 33–37.
35 Wang E, Marincola FM. A natural history of melanoma: serial gene expression analysis. *Immunol Today* 2000; **21**: 619–623.
36 Gu ZJ, Costes V, Lu ZY *et al*. Interleukin-10 is a growth factor for human myeloma cells by induction of an oncostatin M autocrine loop. *Blood* 1996; **88**: 3972–3986.
37 Yue FY, Dummer R, Geertsen R *et al*. Interleukin-10 is a growth factor for human melanoma cells and down-regulates HLA class-I, HLA class-II and ICAM-1 molecules. *Int J Cancer* 1997; **71**: 630–637.
38 Nomura H, Nielsen BW, Matsushima K. Molecular cloning of cDNAs encoding a LD78 receptor and putative leukocyte chemotactic peptide receptors. *Int Immunol* 1993; **5**: 1239–1249.
39 Campbell JJ, Qin S, Bacon KB, Mackay CR, Butcher EC. Biology of chemokine and classical chemoattractant receptors: differential requirements for adhesion-triggering *versus* chemotactic responses in lymphoid cells. *J Cell Biol* 1996; **134**: 255–266.
40 Resnati M, Pallavicini I, Wang JM *et al*. The fibrinolytic receptor for urokinase activates the G protein-coupled chemotactic receptor FPRL1/LXA4R. *Proc Natl Acad Sci USA* 2002; **99**: 1359–1364.
41 Le Y, Murphy PM, Wang JM. Formyl-peptide receptors revisited. *Trends Immunol* 2002; **23**: 541–548.
42 D'Cunha J, Knight Jr E, Haas AL, Truitt RL, Borden EC. Immunoregulatory properties of ISG15, an interferon-induced cytokine. *Proc Natl Acad Sci USA* 1996; **93**: 211–215.
43 Griffiths EK, Krawczyk C, Kong YY *et al*. Positive regulation of T cell activation and integrin adhesion by the adapter Fyb/Slap. *Science* 2001; **293**: 2260–2263.
44 Gil D, Schamel WW, Montoya M, Sanchez-Madrid F, Alarcon B. Recruitment of Nck by CD3 epsilon reveals a ligand-induced conformational change essential for T cell receptor signaling and synapse formation. *Cell* 2002; **109**: 901–912.
45 Decker EL, Nehmann N, Kampen E, Eibel H, Zipfel PF, Skerka C. Early growth response proteins (EGR) and nuclear factors of activated T cells (NFAT) form heterodimers and regulate proinflammatory cytokine gene expression. *Nucleic Acids Res* 2003; **31**: 911–921.
46 Iniguez MA, Punzon C, Fresno M. Induction of cyclooxygenase-2 on activated T lymphocytes: regulation of T cell activation by cyclooxygenase-2 inhibitors. *J Immunol* 1999; **163**: 111–119.
47 Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res* 2001; **61**: 4756–4760.
48 Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ. HMGB1 as a DNA-binding cytokine. *J Leukoc Biol* 2002; **72**: 1084–1091.
49 Tian Q, Streuli M, Saito H, Schlossman SF, Anderson P. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell* 1991; **67**: 629–639.
50 Medley QG, Kedersha N, O'Brien S *et al*. Characterization of GMP-17, a granule membrane protein that moves to the plasma membrane of natural killer cells following target cell recognition. *Proc Natl Acad Sci USA* 1996; **93**: 685–689.
51 Romano MF, Lamberti A, Petrella A *et al*. IL-10 inhibits nuclear factor-kappa B/Rel nuclear activity in CD3-stimulated human peripheral T lymphocytes. *J Immunol* 1996; **156**: 2119–2123.
52 Dokter WH, Koopmans SB, Vellenga E. Effects of IL-10 and IL-4 on LPS-induced transcription factors (AP-1, NF-IL6 and NF-kappa B) which are involved in IL-6 regulation. *Leukemia* 1996; **10**: 1308–1316.
53 Takeshita S, Gage JR, Kishimoto T, Vredevoe DL, Martinez-Maza O. Differential regulation of IL-6 gene transcription and expression by IL- 4 and IL-10 in human monocytic cell lines. *J Immunol* 1996; **156**: 2591–2598.
54 Wang E, Miller LD, Ohnmacht GA, Liu ET, Marincola FM. High-fidelity mRNA amplification for gene profiling. *Nat Biotechnol* 2000; **18**: 457–459.
55 Axelsson LG, Nilsson G, Bjorksten B. Statistical aspects of cell motility determinations with a modified chemotaxis assembly for multiwell filter assays. *J Immunol Methods* 1981; **46**: 251–258.
56 Roden M, Lee K, Panelli M, Marincola F. A novel cytolysis assay using fluorescent labeling and quantitative fluorescent scanning technology. *J Immunol Methods* 1999; **226**: 29–41.