Research

Gene-expression profiling of the response of peripheral blood mononuclear cells and melanoma metastases to systemic IL-2 administration

Monica C Panelli*, Ena Wang*, Giao Phan†, Markus Puhlmann†, Lance Miller†, Galen A Ohnmacht†, Harvey G Klein* and Francesco M Marincola*

Addresses: *Immunogenetics Section, Department of Transfusion Medicine, Clinical Center, †Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA.

Correspondence: Francesco M Marincola. E-mail: marincola@mail.cc.nih.gov

Published: 25 June 2002

Genome ***Biology*** 2002, **3(7)**:research0035.1–0035.17

The electronic version of this article is the complete one and can be found online at http://genomebiology.com/2002/3/7/research/0035

(Print ISSN 1465-6906; Online ISSN 1465-6914)

Received: 4 February 2002
Revised: 9 April 2002
Accepted: 26 April 2002

Abstract

Background: Interleukin-2 (IL-2) has direct pluripotent effects on cells with immune and inflammatory function. Which of these effects has a critical role in mediating tumor regression remains enigmatic. In this study, we compared early changes in transcriptional profiles of circulating mononuclear cells with those occurring within the microenvironment of melanoma metastases following systemic IL-2 administration.

Results: The results suggest that the immediate effects of IL-2 administration on the tumor microenvironment is transcriptional activation of genes predominantly associated with monocyte cell function; minimal effects were noted on migration, activation and proliferation of T cells. However, production of chemokines and markers of adhesion and migration within few hours of IL-2 administration may be responsible for a secondary recruitment of immune cells to the tumor site later.

Conclusion: Our results suggest that IL-2 induces inflammation at tumor sites with three predominant secondary effects: activation of antigen-presenting monocytes; massive production of chemoattractants that may recruit other immune cells to the tumor (including MIG and PARC, which are specific for T cells); and activation of cytolytic mechanisms in monocytes (calgranulin, grancalcin) and NK cells (NKG5, NK4).

Background

The cytokine interleukin-2 (IL-2) has been used extensively as a single agent for immune modulation of the host in cancer therapy [1,2]. Clinical regression of metastatic melanoma and renal cell cancer resulted in long-term cures, and IL-2 has been approved by the Food and Drug Administration for standard therapy. Systemic IL-2 administration also appears to dramatically increase the frequency of tumor regression in specific immunization against cancer [3]. It has been postulated on clinical grounds that the anti-cancer effects of IL-2 are mediated through *in vivo* expansion and activation of cytotoxic lymphocytes [1] and/or promotion of their migration within target tissues [3], but it has become apparent that IL-2 at the doses used therapeutically has broader immune/pro-inflammatory effects [4,5]. In addition, IL-2 may have a role in activation-induced T-cell death,

therefore causing peripheral tolerance [6,7]. IL-2 administration is also associated with potent systemic toxicity that limits its therapeutic usefulness [8], and a relationship between toxicity and clinical effectiveness has been observed, suggesting a common effector pathway [9]. Most peculiarly, the extent of IL-2-induced thrombocytopenia and lymphopenia is associated with increased frequency of clinical responses [10]. Reversal of IL-2-induced toxicity by corticosteroid or anti-tumor necrosis factor-α (TNF-α) antibodies also blunts its therapeutic efficacy, further indicating the mandatory nature of this association [11,12]. The ability to distinguish the effects of IL-2 in target tissues from its systemic effects may yield important insights for the development of improved anti-cancer therapies.

With the advent of functional genomics [13], it has become possible to study complex biological behaviors in concert in order to obtain a panoramic view of downstream events resulting from pathophysiological alterations of entire biosystems. Thus, various aspects of the immune response can be analyzed *in vitro* [14]. To our knowledge, however, little information is available about the effects of IL-2 in controlled *in vitro* conditions [15] and none about the effects of *in vivo* administration. We have therefore characterized the effects of IL-2 administration *in vivo* on the transcriptional program of circulating cells as markers of its systemic effect, and on the tumor microenvironment as a marker of its effect on target tissue.

We compared the time course of gene induction after *in vitro* exposure of peripheral blood mononuclear cells (PBMC) to IL-2 (6,000 IU/ml) with *in vivo* exposure to a dose regimen (720,000 IU/kg every 8 hours), which is associated with fairly high systemic toxicity and clinical effectiveness [16]. The changes in expression profile induced by IL-2 in the tumor microenvironment were monitored by repeated fine-needle aspiration (FNA) biopsies. Amplified antisense RNA (aRNA) obtained from *in vitro* or *ex vivo* samples was fluorescence labeled and hybridized to 6,500-spot cDNA arrays as previously described [17].

Test samples consisted of gradient-separated PBMC exposed either *in vitro* or *in vivo* to IL-2, labeled with Cy5 (red) and co-hybridized with reference samples labeled with Cy3 (green). Reference samples for PBMC consisted of corresponding material for each experiment obtained before IL-2 exposure. For *in vitro* experiments, for example, reference samples were PBMC cultured in the absence of IL-2. For *ex vivo* experiments, references consisted of PBMC obtained from the respective patient before IL-2 administration. For test FNA samples obtained during the course of IL-2 administration, reference material consisted of FNA from the respective lesion obtained before IL-2 therapy. This unusual strategy was selected to emphasize the differences between pre- and post-IL-2-exposure expression profiles while minimizing differences related to individual lesion and/or patient heterogeneity. The complete data set was filtered, letting through only genes expressed at ratios > 3 or < 0.33 compared to the reference in at least one experiment. A parameter was applied to the filter that selected genes whose fluorescence intensity was above 300 in one channel at least in one experiment. This arbitrary threshold was chosen to exclude genes whose fluorescence intensity was close to background fluorescence. To recover possibly useful information, this filter was removed if the same gene demonstrated high intensity of fluorescence in the other channel (> 3,000) in at least one experiment. A total of 2,393 genes out of the 6,500 clones were selected for subsequent analyses.

Results

Technical challenges

Administration of IL-2 in humans induces dramatic changes in the frequency of different subsets of circulating cells. These changes need to be addressed when considering the more specific and minute changes induced at the transcriptional level by this cytokine. Perhaps the most perplexing effect of IL-2 administration is a rapid (within 1 hour) and paradoxical clearance of monocytes and lymphocytes from the peripheral blood (Figure 1a). IL-2 may directly eliminate mononuclear cells through activation-induced death [6,7]. Contrary to the *in vivo* observation, however, *in vitro* stimulation with IL-2 (6,000 IU/ml) of PBMC obtained from apheresis of melanoma patients did not alter mononuclear cell counts, suggesting that cell death is not a direct effect of IL-2 (Figure 1b). A substantial difference between gradient-separated PBMC and circulating cells *in vivo* is the lack of plasma, platelets and polymorphonuclear cells as a result of the apheresis process. This may directly or indirectly contribute to mononuclear cell death *in vivo* in response to IL-2 administration. However, IL-2 (6,000 IU/ml) stimulation of lepirudin-treated (Aventis Pharmaceuticals, Kansas City, MO) fresh blood did not alter mononuclear cell number (data not shown), suggesting a limited role for lymphocyte apoptosis as the cause of their rapid clearance from the circulation.

Whatever the reason, the dramatic changes in the proportions of different subsets of circulating cells during IL-2 therapy introduce a strong bias into the interpretation of the *in vivo* data. Observed variations in gene-expression profile could be due to differential gene expression in individual cells, shifting proportions of different cell subsets, or a combination of the two. The effect of proportional shifts in cell subsets could not be overcome by *ex vivo* mechanical separation of these subsets because of the limited quantity of material obtained from blood draws (20 ml). However, density gradient separation of PBMC almost totally compensated for these shifts by eliminating polymorphonuclear cells, eosinophils and basophils (Figure 1c), and leaving a mononuclear cell population comparable with pre-IL-2 treatment samples. (PBMC from apheresis used as reference for *in vivo* studies and as source of cells for *in vitro* studies

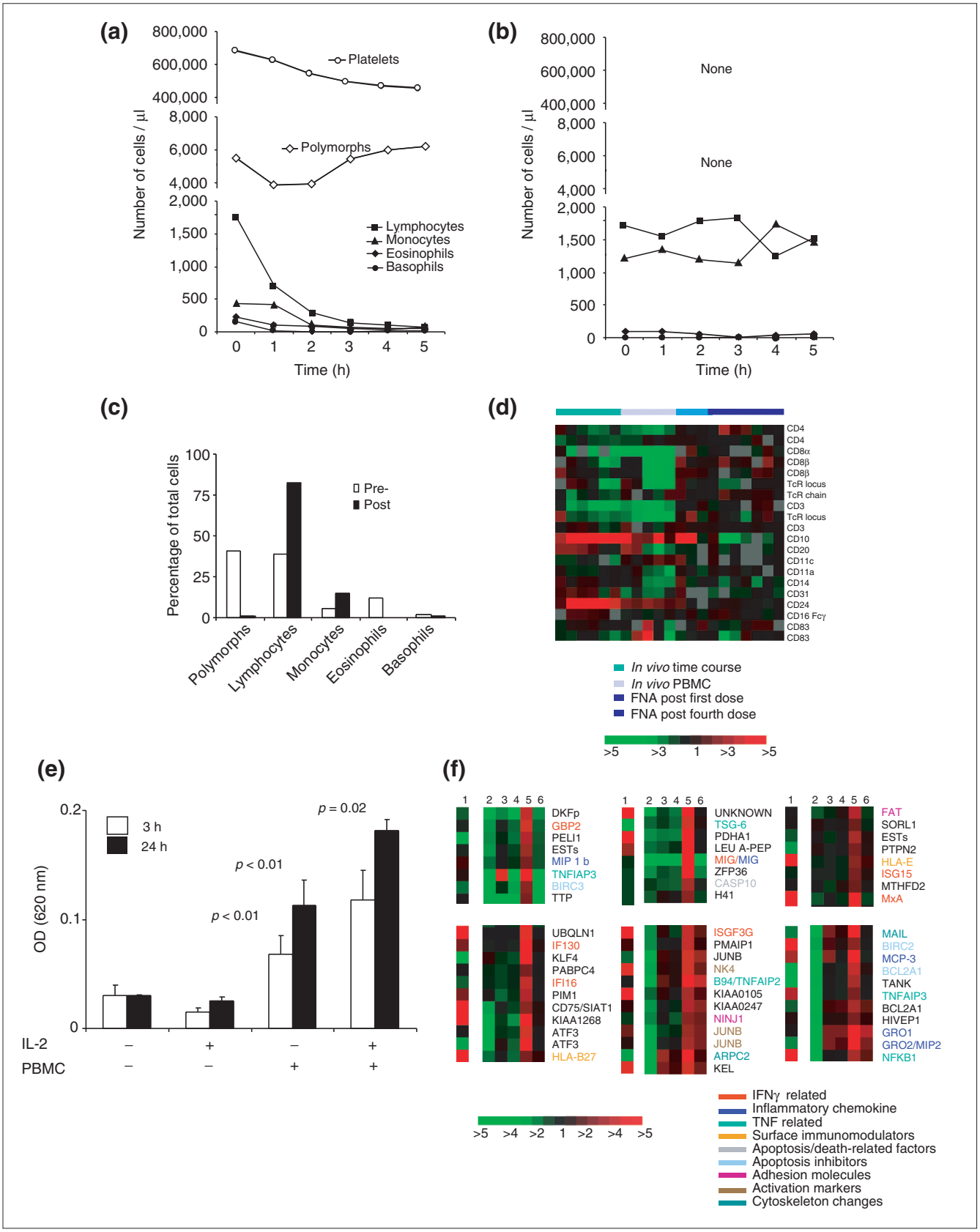

Figure 1 *(see legend on the next page)*

are highly enriched in monocytes and lymphocytes, as seen in Figure 1b.) Therefore, both *in vivo* and *in vitro* studies reflected similar starting populations of mononuclear cells. This, at least in part, mitigated the variation in gene-profile pattern due to shifting cell-subset proportions *in vivo*.

It has been suggested that rapid clearance of mononuclear cells after IL-2 administration is due to increased permeability of vascular endothelium [4], facilitating cell migration into extravascular spaces including tumor sites [3]. However, the level of genes constitutively expressed by various subsets of mononuclear cells did not change significantly within the tumor microenvironment. Differential display of pre- compared with post-IL-2 specimens detected changes only in circulating cells (that is, of genes for CD8, CD3, CD10 and CD24; green and gray bars in Figure 1d). These most probably reflected proportional shifts in cell subsets rather than altered gene expression, because the level of expression of the same genes remained constant in the *in vitro* time course (data not shown). No changes could be detected in FNAs obtained pre-IL-2 administration compared with FNAs obtained 3 hours after one (light-blue bar) or four (dark-blue bar) doses. We could not confirm the aRNA-based results by immunohistochemistry because the material obtainable by FNA was not sufficient for both analyses. However, we have previously shown good correlation between transcript detection of cellular markers in aRNA, total RNA and at the protein level [18].

We further evaluated the effects of IL-2 on endothelial cell permeability as a possible factor mediating migration of circulating immune cells into the tumor site. IL-2 had no direct impact on the permeability of human umbilical vein endothelial cells (HUVEC) (Figure 1e). The addition of PBMC enriched in lymphocytes and monocytes by gradient separation increased the permeability of HUVEC within 3 hours. Treatment of the enriched PBMC with IL-2 (6,000 IU/ml) increased HUVEC permeability (compared to PBMC alone) only after 24 hours (Student *t*-test $p = 0.02$). This finding suggests that IL-2 affects endothelial cell permeability through activation of PBMC. The delayed effect of IL-2 on endothelial cell permeability observed with this functional assay was supported by gene profiling. IL-2 alone had minimal effects on HUVEC. Supernatant from PBMC cultures in serum-free medium had extensive effects on HUVEC transcriptional profile (data not shown). However, supernatants from PBMC conditioned for 24 hours with IL-2 (6,000 IU/ml) induced differential expression in six additional groups of genes (Figure 1f) that could not be induced with supernatants conditioned for 3 hours. These results suggest that the effect of IL-2 on endothelial cells is predominantly mediated indirectly, through activation of immune cells, and occurs at a later stage of IL-2 treatment. This conclusion is in line with the clinical observation that fluid shifts and vascular leak occur in the later phases of high-dose IL-2 therapy.

Taken together, these results suggest that immediate migration of mononuclear cells to the tumor microenvironment is not the primary effector mechanism of systemic IL-2 administration. This observation, of course, does not exclude the possibility that, at a later time, mononuclear cells might migrate or proliferate at the tumor site. The reason for the clearance of circulating cells immediately following IL-2 administration remains unsolved, and may be related to their sequestration in the reticular endothelial system, a hypothesis that could not be evaluated by this study.

Time-course analysis of IL-2-induced gene expression in PBMC *in vitro*

To assess the direct effect of IL-2 on mononuclear cells without the experimental noise caused by proportional shifts

Figure 1 *(see figure on the previous page)*
Effects of IL-2 on circulating immune cells. **(a)** Changes in peripheral blood cell count following administration of one dose of IL-2. **(b)** Cell counts in PBMC obtained by apheresis. PBMC were further purified by density gradient and stimulated *in vitro* with IL-2 (6,000 IU/ml). At each time point, adherent and non-adherent cells were harvested and counted with an automated cell counter. Symbols as in (a). **(c)** Effect of density-gradient separation on peripheral blood-cell populations. Monocytes and lymphocytes are enriched after gradient separation (Post-) compared with before (Pre-), whereas polymorphonuclear cells, eosinophils and basophils are excluded. **(d)** Expression of marker genes for leukocytes derived from a 6,500-spot cDNA array analysis. Expression profiles are displayed in an *in vivo* time course. Samples were obtained from blood 1, 2, 3, 4, 6 and 8 h after the first dose of IL-2 (green bar); from a group of PBMC obtained from different patients 3 h after IL-2 administration (gray bar); and from FNAs of melanoma metastases obtained 3 h after one (light blue) or four (dark blue) doses of IL-2. In the clusterograms, colors are presented according to the central method for display using a normalization factor as recommended by Ross *et al.* [22]. The results represent the ratio of hybridization of fluorescent test samples to reference samples. Ratios are depicted according to the color scale bar underneath indicating the degree of gene upregulation (red) or downregulation (green). **(e)** Effect of IL-2 and/or lymphocyte/monocyte-enriched PBMC on HUVEC permeability. Confluent monolayers of HUVEC were incubated for 3 h (white bars) or 24 h (black bars) with IL-2 (6,000 IU/ml) and/or PBMC. Permeability was measured as increase of optical density in abluminal chamber medium. PBMC increased HUVEC permeability in all conditions, compared with those in which no PBMC were present (Student *t*-test, $p < 0.01$). IL-2 addition to PBMC increased permeability significantly compared to PBMC alone only after 24 h incubation (Student *t*-test, $p = 0.02$). **(f)** Effect of IL-2 and/or lymphocyte/monocyte-enriched PBMC on HUVEC gene expression. HUVEC were exposed for 3 h to IL-2 (column 1), culture medium alone (column 2) or supernatant obtained 3 h (column 3) or 24 h (column 4) after exposure of PBMC to IL-2. Controls: supernatant obtained from PBMC after 3 and 24 h of culture (columns 4 and 6) was used for HUVEC conditioning. Total RNA was extracted from HUVEC immediately after 3 h exposure to supernatants. Six signatures encompassing all genes specifically upregulated by IL-2-stimulated PBMC are shown (Student *t*-test, $p < 0.05$, supernatant from IL-2-conditioned PBMC for 24 h compared to PBMC alone).

in cell subsets, we first determined the transcriptional changes occurring on exposure of PBMC to IL-2 *in vitro*. IL-2 has direct effects on immune cells carrying the IL-2 receptor, which in turn release soluble factors and/or activate other cells by cell-cell interactions. Thus, to match as closely as possible the *in vitro* conditions with those occurring *in vivo*, we stimulated lymphocyte/monocyte-enriched PBMC preparations with 6,000 IU/ml IL-2 in serum-free medium. This concentration approximates the pharmacokinetic distribution of IL-2 following intravenous bolus administration with mean peak levels around 250 ng/ml, corresponding to approximately 5,000 IU/ml [19]. This time-course analysis identified 155 genes expressed more than threefold over pre-IL-2 samples (Figure 2). According to their kinetics of expression, the large majority of genes were identifiable approximately 3 hours after IL-2 exposure, with the exception of a subgroup including transcription and regulatory factors (c-Fos, MKP1) which underwent early and short-lived upregulation. An overview of the genes most consistently affected by IL-2 therapy revealed a large array of transcripts with functions ranging from regulation of cell division and apoptosis to the secretion of immune-stimulatory and/or pro-inflammatory factors. In addition, several surface activation markers in lymphocytes and monocytes that may influence migration and interaction with other cells were induced. Thus, the effects of IL-2 on PBMC span, as expected, a broad range related to the function of several different types of mononuclear cells.

Time-course analysis of IL-2-induced gene expression in PBMC *in vivo*

A similar time-course analysis was performed in the patient who donated the PBMC for the *in vitro* time course, by obtaining blood at intervals following intravenous administration of the first dose of IL-2 (720,000 IU/ml). The blood was separated by density gradient, which left a population of cells enriched in lymphocytes and monocytes. In the *in vivo* time course, 167 genes displayed differential expression (> 3-fold change in at least 70% of the samples) with IL-2 treatment. We then compared the expression profiles in the *in vivo* time course for the 155 genes differentially expressed as a result of IL-2 exposure during the *in vitro* time-course analysis. Differences between the two data sets could be due to shifts in cell-subset frequencies *in vivo* and/or true alterations in the gene-expression profile of individual cells. We hypothesized that expression profiles concordant *in vitro* (where no change in cell-subset frequency is observed) and *in vivo* may represent with most confidence the pattern of gene expression induced by IL-2. A set of 93 out of 155 genes concordantly expressed in the *in vitro* and *in vivo* experiments were identified (Figure 3a-e) by cluster analysis of the combined *in vitro* and *in vivo* time-course samples. Genes for chemokines and cytokines related to inflammatory processes (IP-10, MIP-1β, MCP1, MCP-3, GM-CSF/IL-15/IL-3Rβ, IL-1 receptor) showed a concordant early upregulation *in vitro* and *in vivo*. Downregulation of growth factors/cell cycle/transcription factor-related genes (for ERF-2, CDC16, L-Myc) occurred in a similar fashion *in vitro* and *in vivo*. As the majority of genes appeared to be concordantly expressed *in vitro* and *in vivo,* we postulate that, for these at least, the effect of IL-2 on gene expression overrides its effect on population shift. Although variability in the kinetics of expression of individual gene was noted *in vivo*, as for the *in vitro* time course, the 3-hour time point appeared to yield the most comprehensive view of alterations in gene-expression profile following IL-2 administration. Thus, this data point was used in a larger cohort of patients to identify genes consistently modulated by IL-2 administration.

Genes whose expression is consistently altered in peripheral blood following IL-2 administration

The expression pattern of the 155 genes identified by the *in vitro* time course was evaluated in PBMC samples obtained 3 hours after IL-2 administration in a larger cohort of patients (Figure 4). A first node including 52 genes consistently upregulated (at least 3-fold change in ≥ 70% of samples) was identified. Of these genes, all but 6 were included among the 93 genes coordinately expressed in the *in vitro* and *in vivo* time courses (Figure 3). A second node included 28 genes consistently downregulated, most of them represented among those coordinately downregulated in the time-course studies. These data suggest that the genes identified were highly representative of the effects of IL-2 administration on circulating cells.

For some genes it was possible to extrapolate a potential cell subset responsible for their expression by testing different mononuclear cell subsets separated by negative selection after *in vitro* exposure to IL-2 (monocytes, $CD4^+$ T cells, $CD8^+$ T cells, total lymphocytes and neutrophils, respectively, under the orange bar, Figure 4). Several were selectively expressed by either monocytes or CD4 and/or CD8 T cells. Interestingly, IL-2 also appeared to stimulate the expression of some of these genes in neutrophils, which, however, could not be accounted for in the analysis of PBMC samples enriched in monocytes and lymphocytes and almost completely depleted of neutrophils.

Genes specifically expressed in the tumor microenvironment in response to IL-2 administration

The genes whose expression was consistently altered in PBMC following IL-2 administration were analyzed in a set of FNAs obtained from the same patients plus an additional group of patients (Figure 4). Because there are practical limits on the number of FNAs that can be carried out in the same lesion without causing excessive disturbance of its structure, we sampled lesions 3 hours after one dose (first four samples in the array) and four doses (remaining six samples) of IL-2. The fourth dose (24 hours after the administration of the first) was selected as a later point likely to be reached by all patients before treatment was discontinued because of limiting toxicity [20]. The post-IL-2 FNA material was co-hybridized with reference RNA obtained pre-IL-2

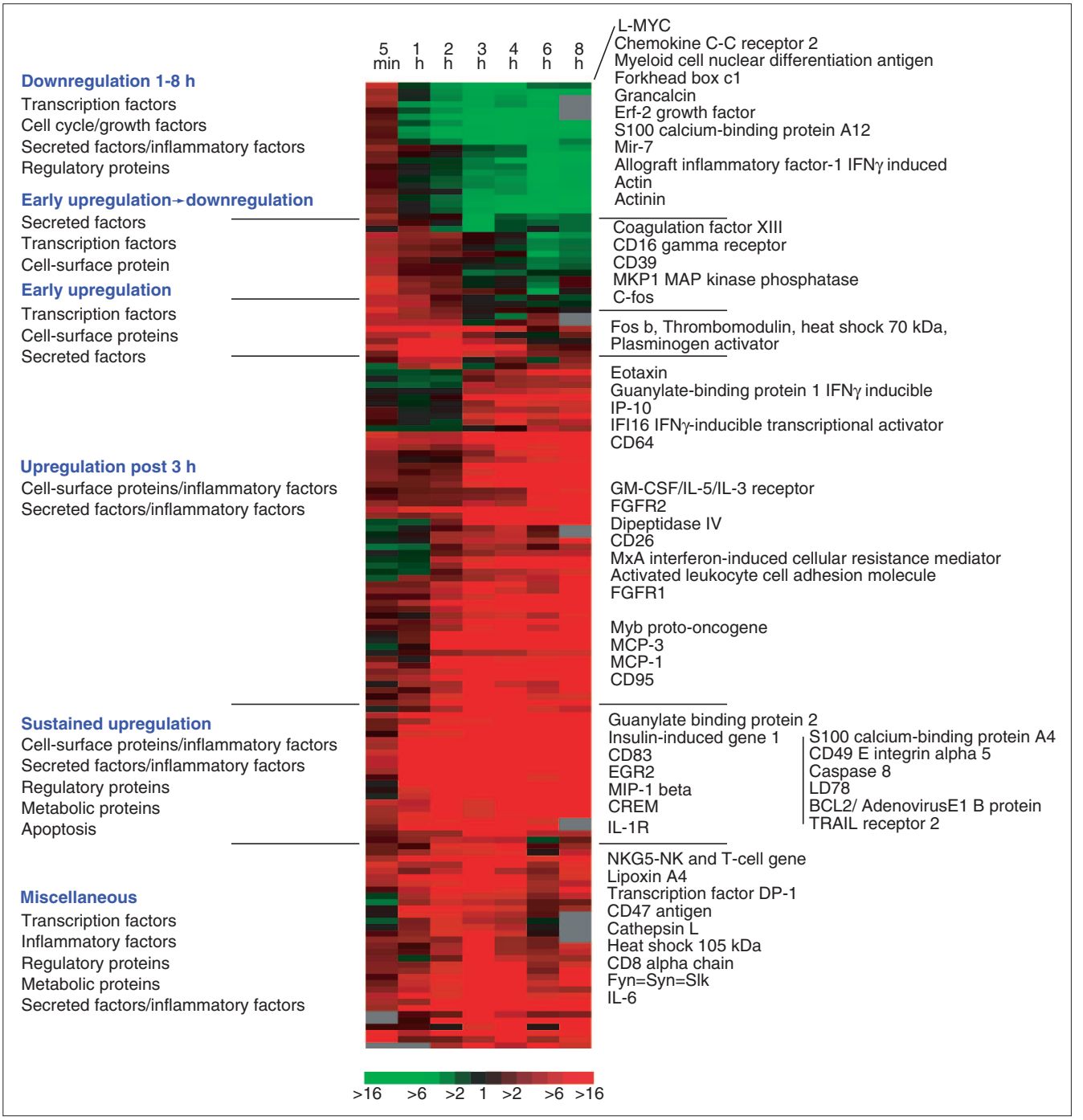

Figure 2
Time-course analysis of changes in gene expression in PBMC in response to IL-2 stimulation *in vitro*. The clustergram represents a group of genes filtered from 6,500 cDNA clones expressed ≥ 3-fold between reference and test samples in at least 70% of the experiments. Red indicates upregulation in test samples compared with the pre-IL-2 reference sample and green indicates downregulation. Suggested functional signatures based on kinetics of expression and gene annotations are given on the left. On the right, selected genes from individual clusters relevant to immune regulation are shown.

from each lesion as previously discussed. Only a small proportion of these genes were consistently upregulated in FNA samples and belonged to a closely related subcluster consisting of genes for inflammatory chemokines (MCP-1, MCP-3, IP-10, LD-78) and interferon-γ (IFN-γ)-related genes (guanylate-binding protein IFN-inducible, MxA, IP-10). Analysis of mechanically separated subsets related their expression to activation of either monocytes or T cells.

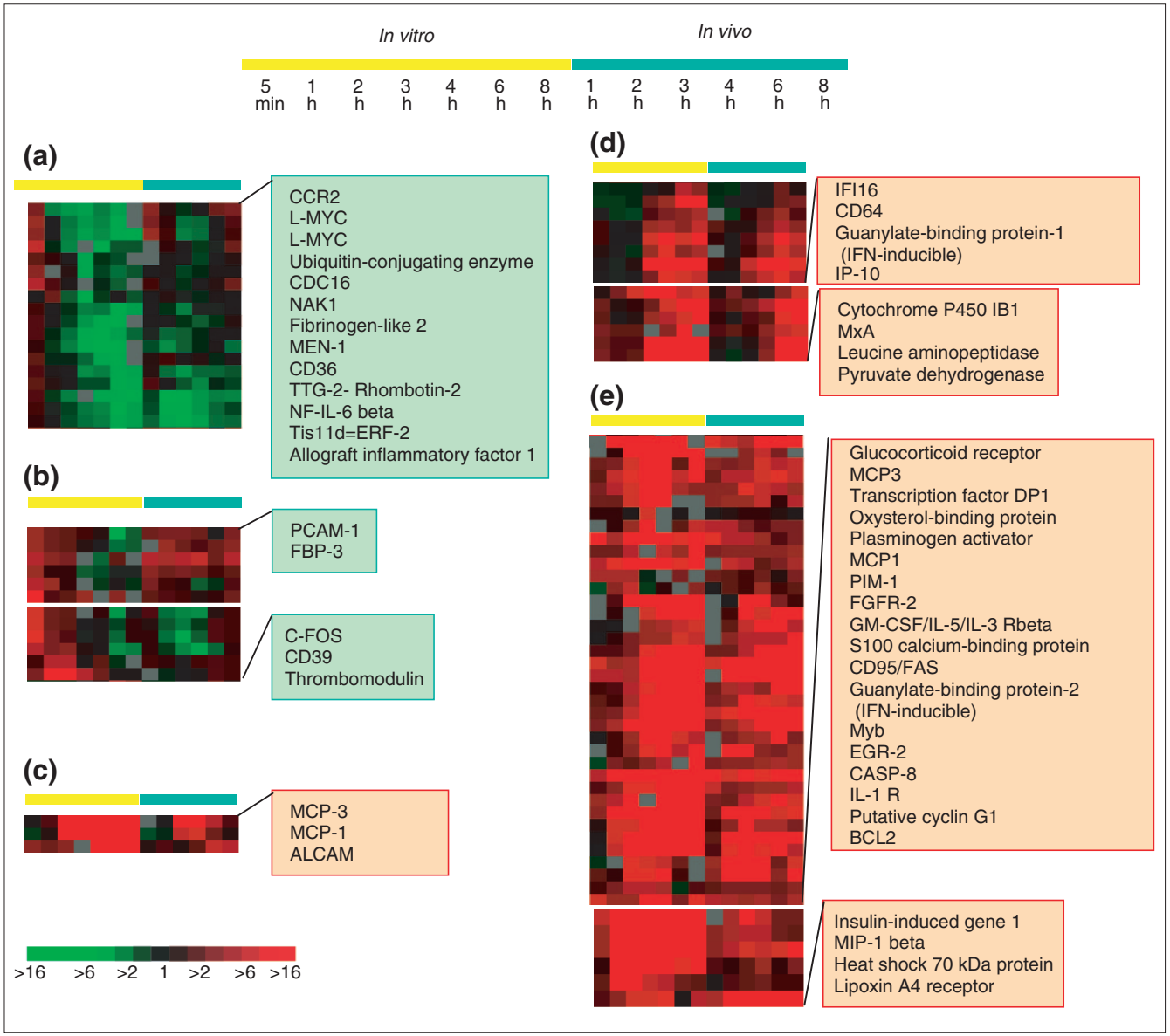

Figure 3
Genes concordantly expressed *in vitro* and *in vivo* following IL-2 exposure. The pattern of expression of genes identified as outliers (≥ 3-fold differential expression between test and reference samples) after IL-2 stimulation of cells *in vitro* (yellow bar), as in Figure 2, was compared to the expression pattern in lymphocyte/monocyte-enriched PBMC from gradient-separated blood of the same patient following intravenous IL-2 administration (720,000 IU/kg) (green bar). **(a-e)** Nodes from a cluster of 155 genes identified as differentially expressed in the *in vitro* time course. Only 51 of these are shown here. A smaller subset of 62 discordant genes is not shown.

Whereas the previous analysis was based on a pool of genes identified by studying the effects of IL-2 on circulating T cells, an independent analysis was carried out by selecting, from the complete data set, genes whose expression was preferentially altered in FNA samples following IL-2 administration. Genes were ranked according to their median ratio of expression among all FNA samples (FNAs obtained after one or four doses were pooled together to identify genes associated with IL-2 administration independent of dose number). The 75 genes with the highest median ratio and the 75 with the lowest were selected (Figure 5). The expression of most of these genes seemed to be specifically modulated by IL-2 and not by the trauma of needle aspiration, as only a few differentially expressed genes (in parentheses in Figure 5) were detected in control FNA samples obtained at 24-hour intervals in the absence of IL-2 administration.

Eisen's hierarchical clustering analysis [21] was applied to this data set to compute relatedness among transcriptional profiles of different FNA samples. This was done to evaluate

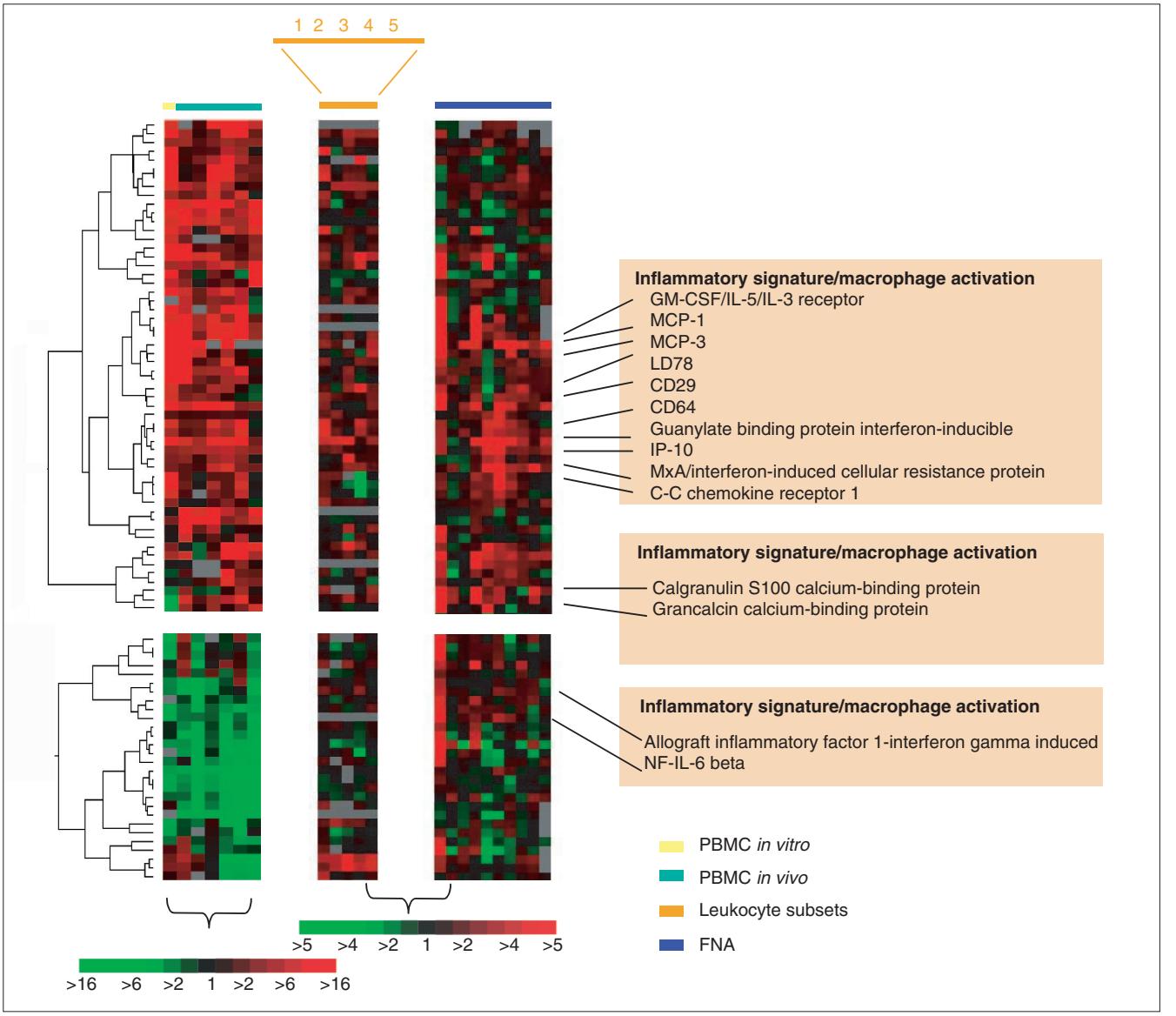

Figure 4
IL-2 activates genes involved in inflammation in peripheral blood cells and in the tumor microenvironment. The profile of genes similarly expressed after 3 h stimulation with IL-2 *in vitro* and *in vivo* are shown. The 155 genes that were ≥ 3-fold up- or downregulated in the *in vitro* time course shown in Figure 2 were sorted. The expression pattern of these genes was compared between the 3-h sample *in vitro* (left-most column) and *in vivo* from five patients (one patient was sampled twice as described in Materials and methods). This data set was clustered according to gene-expression profile, and the nodes that demonstrated similar expression profiles in the *in vitro* and *in vivo* samples are shown. Fifty-two genes were included in the node that demonstrated upregulation in response to IL-2, and 28 in the node that demonstrated downregulation. The expression of these genes was also analyzed in subsets of leukocytes (orange bar) and FNA samples (blue bar). Leukocyte subsets were separated from PBMC exposed to IL-2 for 3 h and included monocytes (1), CD4 (2), CD8 (3), total lymphocytes (4) and neutrophils (5). The FNAs (10 samples) were obtained from six patients: the first four samples were taken after one dose of IL-2 (patients C.F.1, L.F.1, K.F.1, M.F.1) and the other six samples (G.F.a4, G.F.b4, H.F.4, K.F.4, L.F.4, M.F.4) after four doses of IL-2. Pre-IL-2 samples were labeled with Cy3. Samples obtained after IL-2 administration or exposure to supernatant were labeled with Cy5 and co-hybridized on the cDNA chip with their paired pre-IL-2 sample. Red exemplifies upregulation in test samples compared with the pre-IL-2 administration reference sample and green the opposite. The dendrogram on the left depicts similarity in gene expression between PBMC *in vitro* and *in vivo*. Individual genes are shown on the right along with suggested functional signatures.

whether FNAs obtained sequentially after one or four doses of IL-2 from identical or different lesions would cluster according to time of sampling or tissue of origin. According to this data set, based on the most consistently expressed genes, samples from the same lesion clustered together independently of time, suggesting that profiles of IL-2-induced genes tend to be lesion specific throughout IL-2 therapy. The relationship among the samples independent of time of

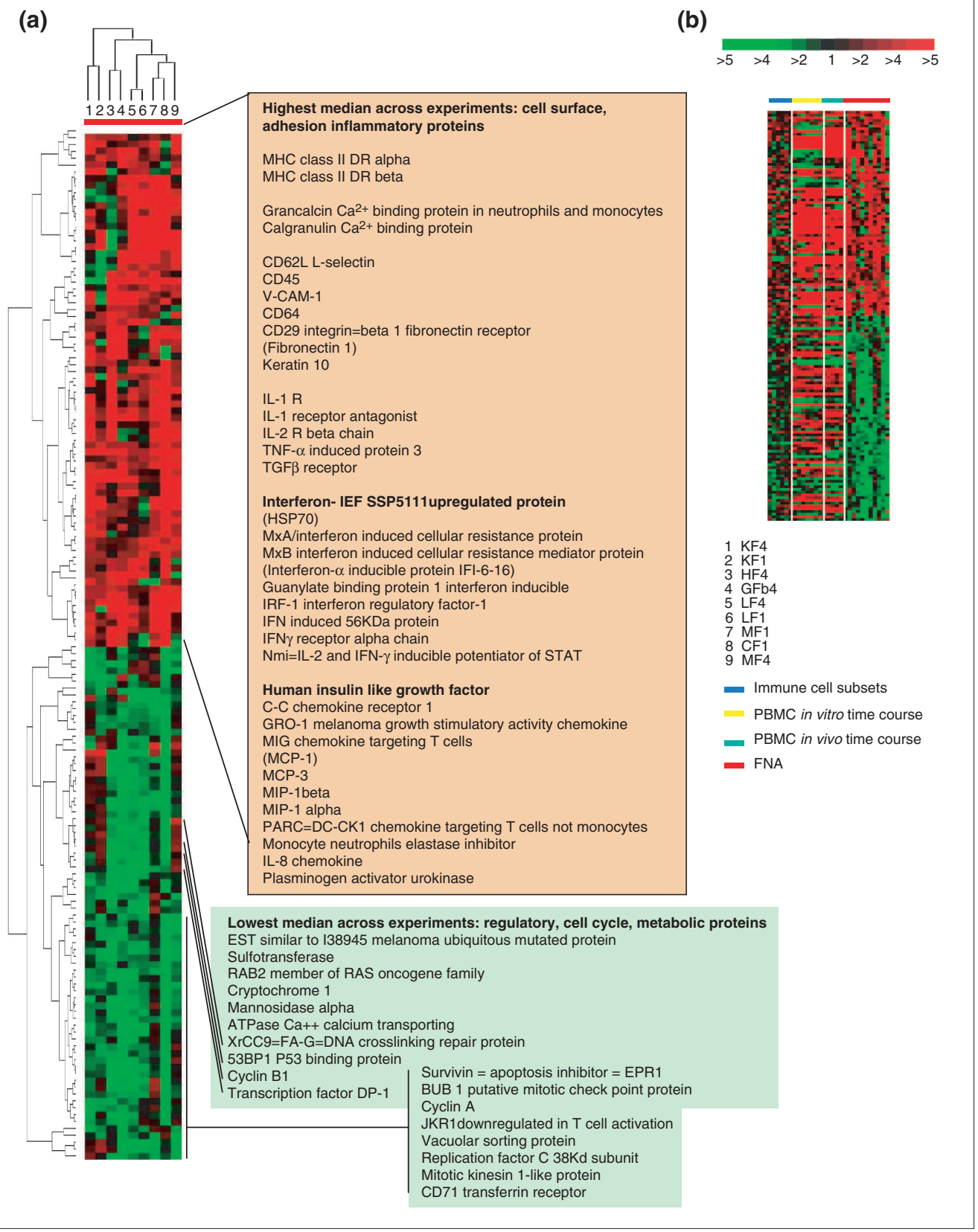

Figure 5 *(see legend on the next page)*

treatment was even more robust when the all-data set was queried (data not shown). Different functional subsets of genes were identified that included those for several chemokines (GRO-1, MIG, MCP-1, MCP-3, MIP1-α, MIP-β, PARC, IL-8), cytokine receptors (C-CR-1, IL-R, IL-1R antagonist, IL-2Rβ, transforming growth factor-β receptor (TGFβR), IFNγR α chain), adhesion molecules associated with mononuclear cell migration (CD62L, VCAM-1, CD64, CD29), cytotoxic proteins in granules produced by activated monocytes (grancalcin and calgranulin) and several genes associated with IFN-γ activity, such as HLA class II molecules and IFN-regulatory or responsive genes such as those for MxA, MxB and IRF-1. Interestingly, no genes associated with apoptosis or cell proliferation were identified. In addition, no evidence of migration of various immune-cell subsets was observed by analyzing changes in frequency of genes constitutively expressed by such cells (that is, CD8, CD4, CD14, and so on, see Figure 1). Thus, the early effects of IL-2 administration are more associated with an inflammatory modulation of the tumor microenvironment rather than with specific migration, activation and/or proliferation of immune cells at the tumor site.

To identify genes whose upregulation in the tumor microenvironment was time and/or dose dependent we then queried the FNA data set by selecting genes differentially expressed between samples obtained 3 hours after one or four doses of IL-2 (unpaired sample *t*-test, p_2 value < 0.05). The cut-off p_2 value of < 0.05 was arbitrarily selected and was not corrected by the number of hypotheses tested (genes). Thus, the genes identified should be considered more as an exploratory finding than a definitive proof of differential expression according to dose number. Ratios were shown according to the central method for display using a normalization factor that emphasizes differences in expression between the experimental groups analyzed by resetting the equality parameter (ratio = 1) to the mean of all the experiments [22]. In this fashion, 54 genes were identified (Figure 6). Interestingly, among them we noted a further upregulation of MHC molecules and other interferon-inducible factors.

Among the lesions analyzed one underwent partial regression (Figure 7a). To explore possible markers associated with immune responsiveness we queried the data set for genes expressed differentially in the responder lesion after one (M.F. 1) and four (M.F. 4) doses of IL-2. Two *t*-tests (two-tail-unequal variance) were carried out comparing M.F. 1 and M.F. 4, respectively, with the rest of the FNA samples. Also in this case, a cut-off p_2 value of < 0.05 was arbitrarily selected, and was not corrected by the number of genes studied. Thus, the findings have only exploratory value. In this fashion, 68 and 102 genes were identified respectively (Figure 7b), of which four were overlapping (two expressed sequence tags (ESTs), a nuclear protein (CHD3) and a sphingosine kinase type 2 isoform). When a similar analysis was carried out by comparing each of the non-responding lesions to the rest, the average of genes differentially expressed was 2 ± 1 after one dose and 56 ± 27 after four doses. From the group of genes differentially expressed in the responder lesion after four doses of IL-2, 19 were identified with the highest ratio of expression. Among these 19 genes, those for NKG5, T-cell receptor α chain, and HLA class II related transcripts appeared of particular interest because they have been found to be upregulated in the context of acute rejection of renal allografts [23,24]. In addition, three were associated with IL-2-dependent NK-cell activation: NK4, CD62 P-selectin, and galectin 1, as recently observed when purified NK cells were exposed to IL-2 (S. Mocellin, M.C.P. and F.M.M., unpublished observations).

Discussion

The goal of this study was to dissect the events following administration of high-dose IL-2 for therapeutic purposes. In particular, we were interested in comparing the systemic effects of IL-2 as reflected by the transcriptional profile of circulating mononuclear cells with the effects observable within the tumor microenvironment. This was achieved by serial sampling of blood and tumor samples to follow the dynamic changes occurring during the course of IL-2 administration. The results suggest that IL-2 exerts a broad range of effects on circulating

Figure 5 *(see figure on the previous page)*
Genes consistently differentially expressed in FNA obtained 3 h after the first and fourth dose of IL-2. The expression profile of aRNA samples amplified from FNAs of melanoma lesions from six patients was analyzed. Four FNA (patients C.F.1, K.F.1, L.F.1, M.F.1) were obtained following one dose and five (G.F.b4, H.F.4, K.F.4, L.F.4, M.F.4) following four doses of IL-2. The FNA material was co-hybridized in pairs with reference aRNA amplified from FNA (red bar) obtained pre IL-2 therapy from each individual lesion. **(a)** Clusterogram depicts 150 genes derived from a group of 2,393 genes expressed with at least a 3-fold difference between reference and test samples in at least 90% of the experiments. In addition, genes were ranked according to their median ratio of expression among all tumor samples (75 with the highest median value of gene expression, 75 with the lowest, across nine experiments). This was done to identify those genes that, independent of sample origin or number of IL-2 doses, were more consistently affected by IL-2 treatment. An arbitrary cut-off of the 75 most representative genes in both ranges of expression was selected, which excluded genes with a median ratio of differential expression of less than 2. Red exemplifies upregulation in test samples compared with pre-IL-2 reference sample and green the opposite. Individual genes are shown on the right. To control for an influence on gene expression of the trauma of FNA, a separate control experiment included two FNAs obtained from a lesion at a 24-h interval in the absence of IL-2 administration. No direct effect of FNA alone on gene expression was observed after IL-2 administration, except for the genes reported in parentheses. Eisen's algorithm [21] was applied to this set of genes to identify proximity of different samples by applying the clustering to individual array experiments as shown by the dendrogam. Samples from the same lesion clustered together independently of number of doses received. **(b)** Clusterogram depicting the same 150 genes selected for (a) and analyzed in leukocyte subsets (blue bar) (as in Figure 4), and in PBMC *in vitro* (yellow bar) and *in vivo* (green bar) (see Figure 3).

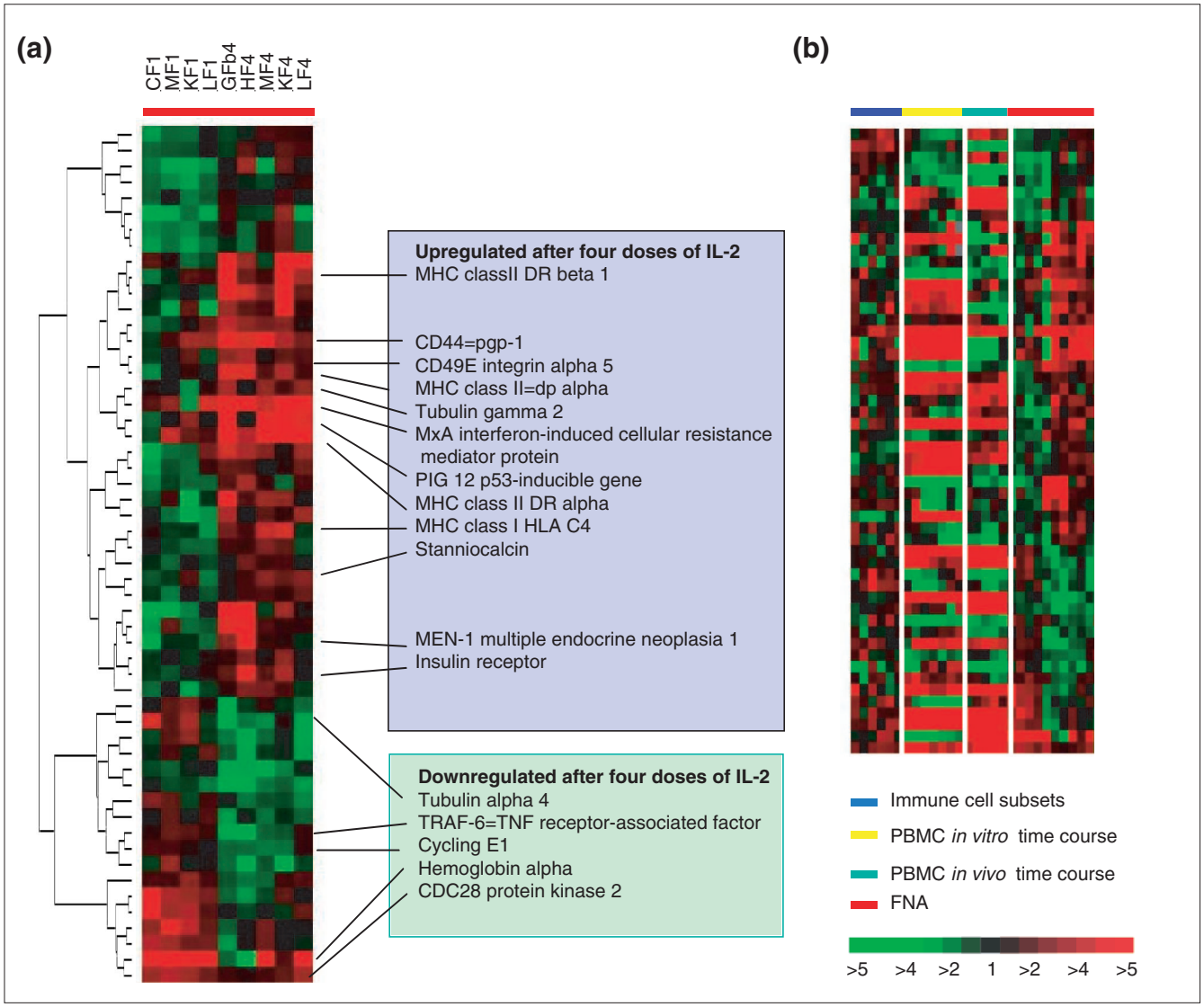

Figure 6
Dose-dependent effect of IL-2 in the tumor microenvironment. **(a)** Clusterogram of genes differentially expressed between FNA samples obtained 3 h after the first dose of IL-2 (as in Figure 5). Genes were selected using an unpaired *t*-test ($p_2 < 0.05$). In this fashion, 54 genes were identified whose modulation of expression was altered only after four doses of IL-2 (upregulated, light blue panel; downregulated, green panel). **(b)** The same gene selection as in (a) analyzed in leukocyte subsets (blue bar) as in Figure 4, and in PBMC *in vitro* (yellow bar) and *in vivo* (green bar) (see Figure 3). Ratios are displayed according to the central method for display using a normalization factor as recommended by Ross *et al.* [22].

cells. These systemic effects have downstream repercussions, only a few of which were reflected in changes in gene expression at the tumor site. Analysis of the genes identified as affected by IL-2 administration suggested that IL-2 exerts its effect at the target site by causing activation of monocytes. This could be best explained by the secondary release of pro-inflammatory substances such as IFN-γ and IL-1 by circulating or intra-tumoral lymphoid cells, as most of the genes identified are known to be responsive to the effect of either of these cytokines.

These pro-inflammatory cytokines are probably responsible for the direct activation of monocytes, as suggested by the upregulation of CD64 and IL-1R (Figure 8). In addition, gene profiling identified genes whose expression is most probably dependent upon IL-1 and IFN-γ rather than IL-2 (genes for MHC molecules, HSP70, MxA, MxB and other IFN-inducible proteins). It is possible that macrophage activation is followed by release of pro-inflammatory chemokines such as IL-8, MCP-3, MIP-1α, MIP-β and MIG. These indirect effects of IL-2 within the tumor microenvironment seem also to affect the activation of dendritic cells (DCs) through the expression of PARC/MCP4. Finally, activation of macrophages results in the upregulation of the expression of molecules with lytic activity, such as calgranulin and grancalcin. Although it is

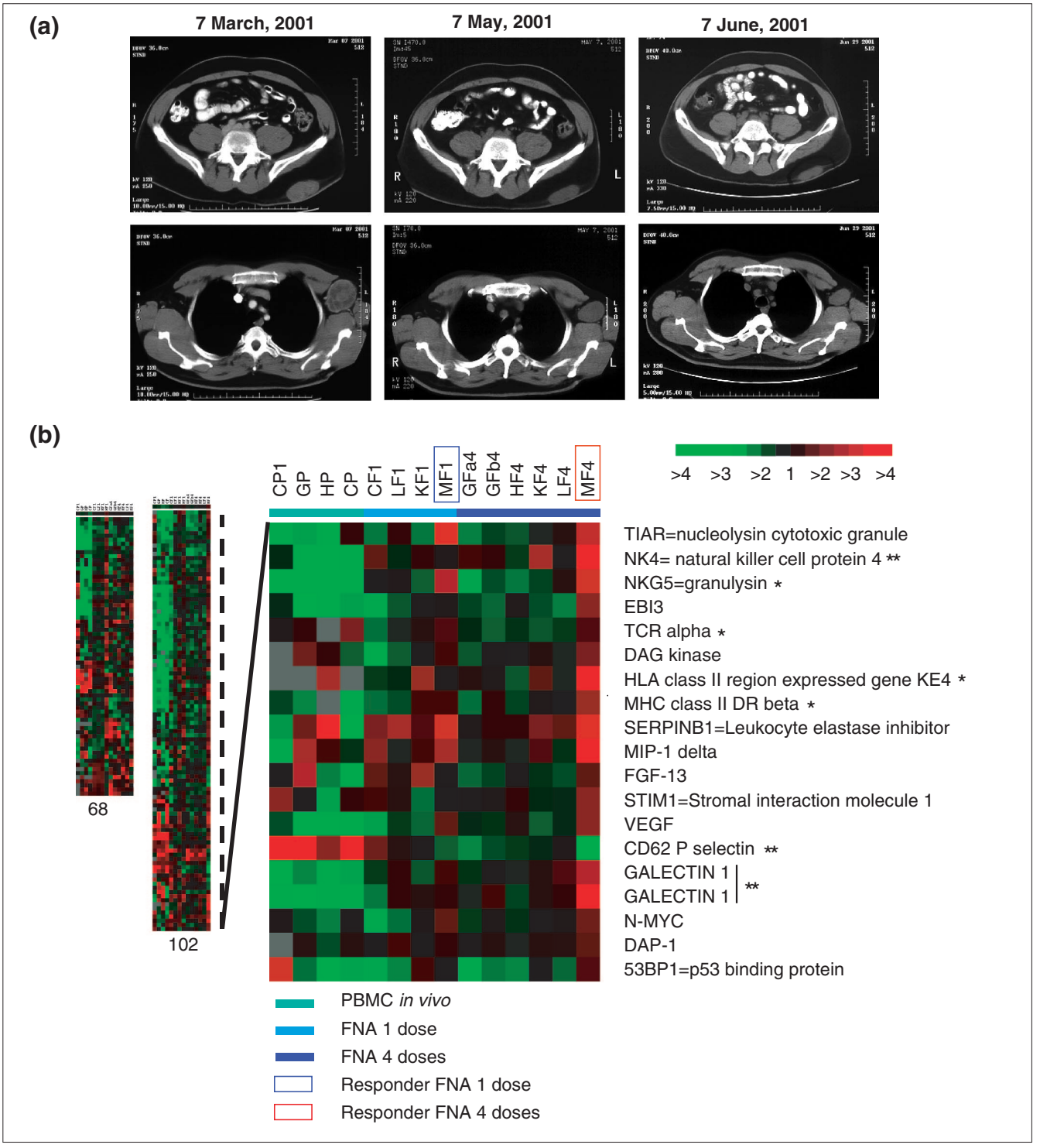

Figure 7
Gene profiling of a lesion responding to treatment. **(a)** Computer tomographic images of two lesions in the same patient (M.F.) before starting treatment (7 March, 2001) and at follow-up administration. The FNAs were obtained after the 7 March images and before the following ones. **(b)** Genes differentially expressed following the fourth IL-2 dose in M.F. compared to the rest of the FNA were selected using a two-tail *t*-test, with unequal variance ($p_2 < 0.05$). In this fashion, 102 genes were identified whose expression was specifically altered in the responding lesion. Only 19 of these are shown in the clusterogram. Red represents upregulation in test samples compared with the pre-IL-2 administration reference sample, and green the opposite. Gene identity is shown on the right. *Genes upregulated in acute rejection of kidney transplant (PBMC and renal biopsy tissue, [23]); **genes with similar expression in NK cells exposed to IL-2. Similarly, 68 genes were found to be differentially expressed between the first IL-2 treatment in M.F. and the rest of the FNA (small image on left).

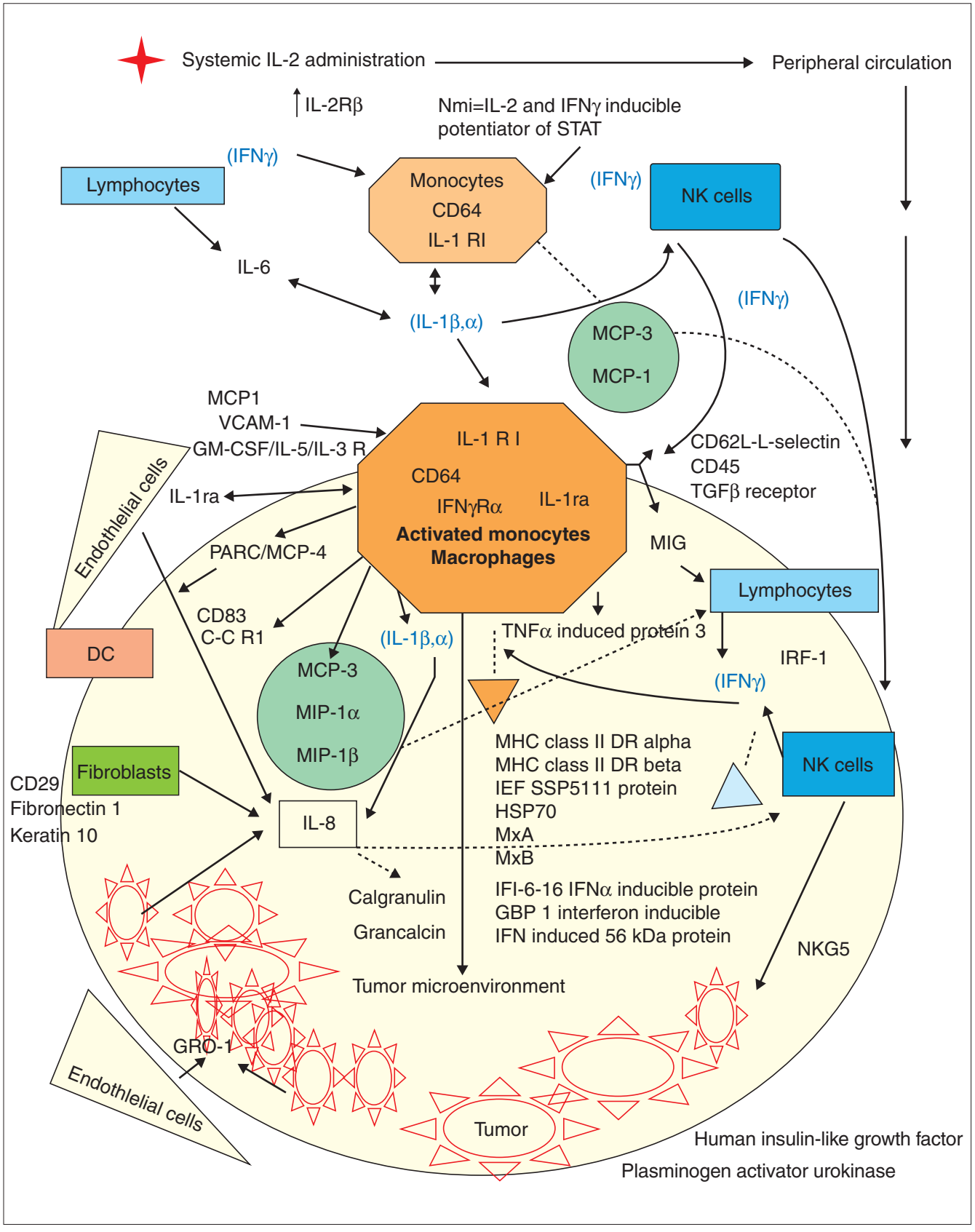

Figure 8 *(see legend on the next page)*

possible that the downstream effects noted after IL-2 administration could be attributed to stimulation of tumor cells, exposure of three melanoma cell lines (LF1TU, 624.38 and A375) to supernatant from IL-2-conditioned PBMC identified transcriptional changes only in a small proportion of these genes (for IFI-16, IFNγR2 and TGF-β-inducible protein, data not shown). The results, therefore, suggest that IL-2 exerts its primary role at tumor sites through the release of pro-inflammatory cytokines. On the basis of the transcriptional profile identified, we postulate that IL-1 and IFN-γ are the most likely cytokines responsible for this phenomenon. These in turn activate a local inflammation, probably through induction of monocytes and macrophages. This inflammatory process can subsequently help recruit immune cells from the periphery and/or activate locally infiltrating cells. This hypothesis is appealing because it converges on IFN-γ as a central mediator of immune regulation within the tumor microenvironment. Recent murine experiments have shown that IFN-γ may be the central mediator of immune surveillance in the immunocompetent host [25]. This cytokine could possibly also have a central role in therapeutic settings. It may have broad downstream effects that according to our findings can be best summarized as activation and maturation of monocytes/DCs in concert with the release by these cells of chemokines capable of attracting lymphoid cells to the tumor. These, in turn, may complete the activation of cells of the monocytic lineage, which can then switch their function from cross-tolerization to cross-priming [26].

A particular subset of genes whose expression appeared to be associated with a clinical response was noted. Although this observation was based on a single lesion, we were surprised to observe among them the genes for NKG5, MHC class II (which was upregulated beyond the level noted in non-responding samples) and T-cell receptor, which have all been recently associated with acute rejection of renal allografts ([23,24] and M. Sarwal, unpublished data). The phenomenon of allograft rejection and IL-2-induced tumor rejection may not only be characterized by the induction of a vigorous inflammatory response but share a common cytotoxic effector mechanism (NK/T-cell degranulation) that is ultimately responsible for direct tissue destruction. This observation agrees with the suggested synergistic role of IFN-γ and effector T cells in controlling tumor growth in experimental models [25]. *In vitro* studies are presently ongoing, testing whether the changes in expression profile noted in tumor samples following IL-2 administration can be directly ascribed to the effects of IFN-γ and/or IL-1 on monocytes.

Conclusions

The results of this study suggest that IL-2 administration induces inflammation at the tumor site, with three predominant secondary effects: activation of antigen-presenting monocytes; a massive production of chemoattractants that may recruit other immune cells to the tumor site, among which are the chemokines MIG and PARC specific for T cells; and the activation of lytic mechanisms ascribable to monocytes (calgranulin, grancalcin) and NK cells (for example, NKG5, NK4) (Figure 8). In practical terms, these results suggest that systemic IL-2 administration may facilitate T-cell effector function in the target organ not by sustaining their proliferation, as presently believed, but rather by promoting their migration and by providing a milieu conducive to their activation *in situ* through activation of antigen-presenting cells. If this hypothesis is correct, then adoptive transfer of effector T cells should follow, rather than precede as at present, administration of systemic IL-2 [27,28].

Materials and methods

Selection of patients, treatment, and sampling of PBMC and FNAs

PMBCs and FNA biopsies of melanoma metastases were obtained from patients with metastatic melanoma undergoing immunotherapy with high-dose IL-2 at the Surgery Branch, NCI (Bethesda, MD). IL-2 (720,000 IU/kg) was administered by 15 min intravenous infusion every 8 h for up to 14 consecutive doses, over 5 days, as clinically tolerated

Figure 8 *(see figure on the previous page)*
Schematic representation of the hypothesized chain of events that follows high-dose IL-2 administration deduced by serial gene profiling of PBMC and of FNA of melanoma metastases. Administration of IL-2 first affects leukocytes in the peripheral circulation where the IL-2 receptor is immediately upregulated on key effector mononuclear cells (lymphocytes, monocytes and NK cells). Lymphocytes and NK cells directly respond to IL-2 by releasing IFN-γ, whereas monocytes upregulate CD64, a typical marker of phagocytic activity, the receptor for the inflammatory cytokine IL-1RI, and most probably begin to release IL-1α/β in a stimulatory autocrine feedback loop. Further amplification of the biological effects of IL-1 is achieved by the ability of IL-1 to upregulate receptor expression for itself, IL-2R, IFNγR and GM-CSF/IL-3/IL-5R. The circulating pro-inflammatory cytokines IL-6 and IFN-γ further increase monocyte activation by triggering the release of MCP-3 and MCP-1 and/or inducing monocyte mobilization to the tumor microenvironment. Macrophages expressing IL-1RI and IFNγR that reach the tumor microenvironment, along with resident macrophages, release additional chemokines and cytokines (MCP-3, MIP-1α and β, MIG, PARC/MCP-4, IL-8), affecting the trafficking and/or activation of monocytes themselves, lymphocytes/NK cells, dendritic cells and fibroblasts, which in turn can release inflammatory factors. Migration to the tumor microenvironment is also reflected by the active transcription of molecules involved in adhesion of mononuclear cells (CD62L selectin) to vascular endothelial cells (V-CAM). In addition, upregulation of mRNA expression for the cytolytic granule proteins calgranulin/grancalcin and NKG5 underlies the induction of a cytotoxic response by monocytes/macrophages and NK cells, respectively. The net effect of the IL-2-induced inflammatory cascade in the tumor microenvironment is the dominant expression of IFN-γ-dependent genes (MHC class II, HSP70, MxA, MxB) and/or IFN-γ regulatory genes (IRF-1, IF1616, GBP1 interferon inducible, IEF protein).

[29]. The effect of IL-2 was analyzed in four patients' PBMC. PBMC were obtained from each patient by apheresis before IL-2 infusion and at 3 h after the fourth dose of IL-2 (patients H.P., G.P., C.P.), with the exception of one patient who was sampled twice at 3 h after the first and the second dose of IL-2 (C.P.1, C.P.3). Additionally, PBMC were obtained from one patient (C.B.) 1, 2, 3, 4, 6 and 8 h after the first dose of IL-2 (*in vivo* time course). Serial FNA of selected melanoma metastases were obtained from six patients (three of whom also had paired PBMC samples) before IL-2 infusion (C.F.0, G.F.0, H.F.0, K.F.0, L.F.0, M.F.0) and at 3 h after the first (C.F.1, K.F.1, L.F.1, M.F.1) and/or fourth dose of IL-2 (G.F.a4, G.F.b4, H.F.4, K.F.4, L.F.4, M.F.4). PBMC and FNA samples obtained before IL-2 administration were considered references, and those obtained after IL-2 administration were used as targets for microarray analysis

Selection of the time window and cytokine concentration for *in vitro* studies

Selection of the time window and cytokine concentration for *in vitro* IL-2 studies was based on the pharmacokinetics of IL-2 [19], measuring biologically active serum IL-2 levels after administration of different doses of IL-2. Patients receiving 720,000 IU/kg IV bolus recombinant IL-2 showed a mean peak IL-2 level of 260 ± 66 ng/ml = 4,680 ± 1,188 IU/ml (18 IU = 1 ng), which then decayed with time.

To match as closely as possible the *in vitro* conditions with those occurring *in vivo*, lymphocyte/monocyte-enriched PBMC preparations were stimulated with 6,000 IU/ml IL-2 in serum-free medium.

In vitro studies

PBMC obtained from one patient (C.B.) by apheresis before administration of IL-2 were seeded at 6 x 10^6 per well of a 24-well plate in OPTI-MEM medium without serum supplementation and were incubated at 37°C in the presence or absence of 6,000 IU/ml IL-2 for 0 or 5 min, or 1, 2, 3, 4, 6 and 8 h. At each time point adherent monocytes were harvested with trypsin and combined with non-adherent cells. Adherent and non-adherent cells were washed three times with PBS, differential counts were carried out by automated counting (Cell Dyn 4000, Abbott Diagnostics, Santa Clara, CA) and cells were resuspended in lysis buffer for RNA isolation (RNeasy, Qiagen, Valencia, CA). RNA was amplified using the protocol for mRNA amplification and probe preparation previously published [17]. Each probe/test sample (antisense RNA (aRNA) obtained at each time point) was labeled with Cy5 and mixed with Cy3-labeled reference sample (non-stimulated PBMC obtained from apheresis before IL-2 administration).

In vivo studies

Peripheral blood cells were sampled from patient C.B. during course 1, cycle 1, of high-dose IV bolus IL-2 therapy. A 10-ml blood sample was drawn at 1, 2 , 3, 4, 6 and 8 h directly into a BD vacutainer™ CPT™/sodium heparin tube (BD, Franklin Lakes, NJ). Fresh blood was similarly drawn and processed from patients H.P., G.P. and C.P. at the 3-h time point during IL-2 administration. In all cases, automated differential counts were made before density-gradient separation of mononuclear cells in CPT tubes according to the manufacturer's protocol. The recovered mononuclear cells were washed three times with PBS and resuspended in lysis buffer for RNA isolation (RNeasy, Qiagen). RNA was amplified using the protocol for mRNA amplification and probe preparation according to a previously described procedure [17], with some additional modifications described below. Each probe/test PBMC sample was labeled with Cy5 and mixed with Cy3-labeled reference sample obtained from apheresis before IL-2 administration. FNA material was processed similarly and hybridized to microarrays using as reference sample FNA material obtained before IL-2 administration. In brief, FNA of selected metastases were carried out before and 3 h after IL-2 administration as previously described [30]. The FNA material was immediately resuspended in ice-cold Iscove's medium without serum supplementation, and subsequently washed in PBS before RNA isolation.

Stimulation of PBMC with IL-2 and separation of single leukocyte subsets

Monocytes, CD8 and CD4 T-cell subsets were separated from patient C.B.'s PBMC (pre-IL-2 treatment) after exposure of PBMC to IL-2. In brief, 2 x 10^8 PBMC were thawed, washed twice in a 50:50 mixture of OPTI-MEM and Plasbumin (Bayer, West Heven, CT) and incubated for 3 h in a T175 flask in 50 ml serum-free OPTI-MEM in the presence or absence of 6,000 IU IL-2 per ml OPTI-MEM. After incubation, non-adherent cells were collected, washed in PBS, and CD4 and CD8 T-cell subsets were separated by high-affinity negative selection using CD4 and CD8 subset columns (R&D System, Minneapolis, MN) according to the manufacturer's instructions. Purified IL-2-stimulated and unstimulated CD4 and CD8 T cells were washed twice in PBS and resuspended in 350 ml RNeasy lysis buffer (Qiagen) for RNA isolation and probe preparation as previously described. The purity of the recovered T-cell subsets was assessed by FACS analysis and ranged between 79 and 83% for CD4 T cells and 89 and 90% for CD8 T cells. The remaining adherent monocytes were washed twice in PBS and simultaneously harvested and lysed for RNA isolation using 2.1 ml RNeasy lysis buffer per T175 flask, according to the manufacturer's protocol.

Direct separation of neutrophils from normal donor buffy coats

A 20-ml buffy coat suspension was overlayed onto 20 ml HESPAN (B. Broun Medical Inc, Melsunger, Germany), mixed rapidly, and incubated for 30 min at room temperature to separate red blood cells. After incubation, the supernatant was carefully removed and washed in Hanks's

balanced salt solution. The cell suspension was then overlayed onto 15 ml of lymphocyte-separation medium (LSM; Organon Teknica Corp., Durham, NC) for separation of a monocyte/lymphocyte interphase and a granulocyte/red blood cell pellet. Granulocytes were separated from red blood cells by ACK lysis (Biofluids International, Rockville, MD) at 4°C for 10-15 min. Purified granulocytes were then resuspended in 700 μl RNeasy lysis buffer for probe preparation as described above. The purity of the granulocyte preparation and the neutrophil content was assessed by immunocytochemistry using DIFF quick staining according to the manufacturer's protocol (American Scientific Products, McGraw Park, IL).

Permeability assay

Permeability was assayed using 6-well Falcon permeability chamber inserts positioned in 6-well plates (Falcon-BD Biosciences, Bedford, MA) according to the manufacturer's instructions. HUVEC were plated in the luminal chamber at 8 x 10^5/well/2 ml EGM-2 medium (Clonetics, San Diego, CA) on day 0 and incubated at 37°C in 5% CO_2 for 48 h. On day 2, 4 x 10^6 PBMC/well were added on top of the HUVEC monolayer in the presence or absence of IL-2 (6,000 IU/ml) and incubated for 3 h. Control wells consisted of media supplemented with or without IL-2 in the absence of PBMC. After incubation, the content of the luminal chamber was cleared and the HUVEC monolayer was washed once with 1x Dulbecco PBS (DPBS) (Biofluids). Two milliliters of EBM-2 medium + 1% human plasma were then added to the monolayer and the cells were incubated at 37°C in 5% CO_2 for 1 h. After incubation, the EBM medium was then aspirated from the luminal chamber and 1.5 ml Evans Blue dye in DPBS/0.1 BSA was added. DPBS (2 ml) was added into the abluminal (bottom) chamber followed by 1 h incubation. The insert containing the HUVEC monolayer was then discarded, and the optical density (OD) of the fluid in the abluminal chamber was measured at 620 nm using DPBS alone as blank control. Permeability was measured as increase in OD of abluminal chamber medium due to migration of Evans Blue dye from the luminal chamber. Each experimental condition was run in triplicate and repeated in three independent experiments. Changes in permeability with $p < 0.01$ were considered significant (Student *t*-test).

Differential stimulation of PBMC with IL-2 for supernatant collection

PBMC collected from apheresis before IL-2 treatment were obtained from one patient (L.B.), washed twice in a 50:50 mixture of OPTI-MEM and Plasbumin and incubated for 3 and 24 h at 1 x 10^8 cells/T175 flask in 50 ml serum-free OPTI-MEM in the presence or absence of 6,000 IU IL-2 per ml OPTI-MEM. After each incubation, supernatants containing non-adherent cells were collected and centrifuged twice to separate the non-adherent leukocyte populations and frozen at -30°C for stimulation of HUVEC monolayers.

Exposure of HUVEC to supernatant obtained from PBMC stimulated with IL-2

Supernatants obtained from PBMC exposed to IL-2 or OPTI-MEM (control) for 3 and 24 h were overlayed on confluent monolayers of HUVEC in T175 flasks (Falcon) at 37°C in 5% CO_2 for 3 h. After incubation, 1.4-2.1 ml of RNeasy lysis buffer was added to each flask to lyse the adherent cell monolayers and isolate RNA for microarray probe preparation as described below.

Probe preparation and hybridization to microarrays

Total RNA was isolated using RNeasy minikits (Qiagen). aRNA was prepared from total RNA (0.5-3 μg) according the protocol previously described by us [17]. In brief, two rounds of amplification were carried out, which consisted of a combination of *in vitro* transcription and template-switching PCR. The first round combined reverse transcription using oligo-dT (15)-T7 (5′-AAACGACGGCCAGTGAATTGTAATACGACTCACTATAGGCGC-3′) primer and template-switch primer (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′) (Clontech, Palo Alto, CA) catalyzed by the Superscript-II reverse transcriptase (Gibco-BRL) and Advantage cDNA polymerase (Clontech) for single- and double-strand cDNA synthesis respectively, with *in vitro* transcription (Megascript kit, Ambion, Austin, TX). The second round of amplification utilized 0.5 to 1 μg aRNA as starting material and resembled the first round, except for random hexamer pd(N)6 (Boehringer Mannheim, Igelheim, Germany) used in the reverse-transcription reaction in place of the template-switch primer. aRNA (3.5-5 μg) obtained after two rounds of amplification was labeled in a reverse-transcription reaction in the presence of Cy3-labeled (reference sample) or Cy5 (test sample)-labeled dUTP (Amersham, Piscataway, NJ). Test-reference sample pairs were mixed and co-hybridized to 6,500-spot microarrays.

Microarray analysis tools

Hybridized arrays were scanned at 10-μm resolution on a GenePix 4000 scanner (Axon Instruments) 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). Data was analyzed using Cluster and Tree View software by Michael Eisen [21].

Additional data files

An additional file containing the identity of the genes differentially expressed in the M.F. responding lesion after 1 (68 genes) and four doses (102 genes) of IL-2 is available with the online version of this paper.

Acknowledgements

We are grateful to David Stroncek and Joseph Even for helpful discussion. We also thank Donald White for his input on the management of array data.

References

1. Margolin KA: **Interleukin-2 in the treatment of renal cancer.** *Semin Oncol* 2000, **27:**194-203.
2. Atkins MB, Lotze MT, Dutcher JP, Fisher RI, Weiss G, Margolin K, Abrams J, Sznol M, Parkinson DR, Hawkins M, *et al.*: **High-dose recombinant interleukin-2 therapy for patients with metastatic melanoma: analysis of 270 patients treated between 1985 and 1993.** *J Clin Oncol* 1999, **17:**2105-2116.
3. Rosenberg SA, Yang JC, Schwartzentruber D, Hwu P, Marincola FM, Topalian SL, Restifo NP, Dufour E, Schwartzberg L, Spiess P, *et al.*: **Immunologic and therapeutic evaluation of a synthetic tumor associated peptide vaccine for the treatment of patients with metastatic melanoma.** *Nat Med* 1998, **4:**321-327.
4. Cotran RS, Pober JS, Gimbrone MA Jr, Springer TA, Wiebke EA, Gaspari AA, Rosenberg SA, Lotze MT: **Endothelial activation during interleukin 2 immunotherapy. A possible mechanism for the vascular leak syndrome.** *J Immunol* 1988, **140:**1883-1888.
5. Kasid A, Director EP, Rosenberg SA: **Induction of endogenous cytokine-mRNA in circulating peripheral blood mononuclear cells by IL-2 administration to cancer patients.** *J Immunol* 1989, **143:**736-739.
6. Marks-Konczalik J, Dubois S, Losi JM, Sabzevari H, Yamada N, Feigenbaum L, Waldmann TA, Tagaya Y: **IL-2 induced activation-induced cell death is inhibited in IL-15 transgenic mice.** *Proc Natl Acad Sci USA* 2000, **97:**11445-11450.
7. Waldmann TA, Dubois S, Tagaya Y: **Contrasting roles of IL-2 and IL-15 in the life and death of lymphocytes: implications for immunotherapy.** *Immunity* 2001, **14:**105-110.
8. Lotze MT, Matory YL, Rayner AA, Ettinghausen SE, Vetto JT, Seipp CA, Rosenberg SA: **Clinical effects and toxicity of interleukin-2 in patients with cancer.** *Cancer* 1986, **58:**2764-2772.
9. White RLJ, Schwartzentruber D, Guleria AS, McFarlane MP, White DE, Ucker E, Rosenberg SA: **Cardiopulmonary toxicity of treatment with high-dose interleukin-2 in 199 consecutive patients with metastatic melanoma or renal cell carcinoma.** *Cancer* 1994, **74:**3212-3222.
10. Phan GQ, Attia P, Steinberg SM, White DE, Rosenberg SA: **Factors associated with response to high-dose interleukin-2 in patients with metastatic melanoma.** *J Clin Oncol* 2001, **19:**3477-3482.
11. Papa MZ, Vetto JT, Ettinghausen SE, Mule JJ, Rosenberg SA: **Effect of corticosteroid on the antitumor activity of lymphokine-activated killer cells and interleukin 2 in mice.** *Cancer Res* 1986, **46:**5618-5623.
12. Fraker DL, Langstein HN, Norton JA: **Passive immunization against tumor necrosis factor partially abrogates interleukin 2 toxicity.** *J Exp Med* 1989, **170:**1015-1020.
13. Brown PO, Botstein D: **Exploring the new world of the genome with DNA microarrays.** *Nat Genet* 1999, **21:**33-37.
14. Staudt LM, Brown PO: **Genomic views of the immune system.** *Annu Rev Immunol* 2000, **18:**829-859.
15. Alizadeh AA, Eisen MB, Botstein D, Brown PO, Staudt LM. **Probing lymphocyte biology by genomic-scale gene expression analysis.** *J Clin Immunol* 1998, **18:**373-379.
16. Rosenberg SA, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE: **Treatment of 283 consecutive patients with metastatic melanoma or renal cell cancer using high-dose bolus interleukin 2.** *J Am Med Assoc* 1994, **271:**907-913.
17. Wang E, Miller L, Ohnmacht GA, Liu E, Marincola FM: **High fidelity mRNA amplification for gene profiling using cDNA microarrays.** *Nature Biotechnol* 2000, **17:**457-459.
18. Ohnmacht GA, Wang E, Mocellin S, Abati A, Filie A, Fetsch PA, Riker A, Kammula US, Rosenberg SA, Marincola FM: **Short term kinetics of tumor antigen expression in response to vaccination.** *J Immunol* 2001, **167:**1809-1820.
19. Yang JC, Rosenberg SA: **An ongoing prospective randomized comparison of interleukin-2 regimens for the treatment of metastatic renal cell cancer.** *Cancer J Sci Am* 1997, **3 Suppl 1:**S79-S84.
20. Kammula US, White DE, Rosenberg SA: **Trends in the safety of high dose bolus interleukin-2 administration in patients with metastatic cancer.** *Cancer* 1998, **83:**797-805.
21. 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.
22. Ross DT, Scherf U, Eisen MB, Perou CM, Rees CA, Spellman PT, Iyer V, Jeffrey SS, van de Rijn M, Waltham M, *et al.*: **Systematic variation in gene expression patterns in human cancer cell lines.** *Nat Genet* 2000, **24:**227-235.
23. Sarwal MM, Jani A, Chang S, Huie P, Wang Z, Salvatierra O, Clayberger C, Sibley R, Krensky AM, Pavlakis M: **Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation.** *Hum Immunol* 2001, **62:**21-31.
24. Sarwal M, Chang S, Barry C, Alizadeh AA, Salvatierra O, Brown PO: **Genomic analysis of renal allograft dysfunction using cDNA microarrays.** *Transplant Proc* 2001, **33:**297-298.
25. Shankaran V, Ikeda H, Bruce AT, White JM, Swanson PE, Old LJ, Schreiber RD: **IFN-γ and lymphocytes prevent primary tumour development and shape tumour immunogenicity.** *Nature* 2001, **410:**1107-1111.
26. Albert ML, Jegathesan M, Darnell B: **Dendritic cell maturation is required for the cross-tolerization of CD8$^+$ T cells.** *Nature Immunol* 2001, **2:**1010-1016.
27. Rosenberg SA, Lotze MT, Muul LM, Leitman S, Chang AE, Ettinghausen SE, Matory YL, Skibber JM, Shiloni E, Vetto JT, *et al.*: **Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer.** *N Engl J Med* 1985, **313:**1485-1492.
28. Rosenberg SA, Yannelli JR, Yang JC, Topalian SL, Schwartzentruber DJ, Weber JS, Parkinson DR, Seipp CA, Einhorn JH, White DE: **Treatment of patients with metastatic melanoma with autologous tumor-infiltrating lymphocytes and interleukin 2.** *J Natl Cancer Inst* 1994, **86:**1159-1166.
29. Rosenberg SA: **Interleukin-2 and the development of immunotherapy for the treatment of patients with cancer.** *Cancer J Sci Am* 2000, **6:**S2-S7.
30. Panelli MC, Riker A, Kammula US, Lee K-H, Wang E, Rosenberg SA, Marincola FM: **Expansion of tumor/T cell pairs from fine needle aspirates (FNA) of melanoma metastases.** *J Immunol* 2000, **164:**495-504.