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Direct T cell–tumour interaction triggers T_H1 phenotype activation through the modification of the mesenchymal stromal cells transcriptional programme

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Background: Mesenchymal stromal cells (MSCs) are heterogeneous cells with immunoregulatory and wound-healing properties. In cancer, they are known to be an essential part of the tumour microenvironment. However, their role in tumour growth and rejection remains unclear. To investigate this, we co-cultured human MSCs, tumour infiltrating lymphocytes (TIL), and melanoma cells to investigate the role of MSCs in the tumour environment.

Methods: Mesenchymal stromal cells were co-cultured with melanoma antigen-specific TIL that were stimulated either with HLA-A*0201$^+$ melanoma cells or with a corresponding clone that had lost HLA-A*0201 expression.

Results: Activated TIL induced profound pro-inflammatory gene expression signature in MSCs. Analysis of culture supernatant found that MSCs secreted pro-inflammatory cytokines, including T_H1 cytokines that have been previously associated with immune-mediated antitumor responses. In addition, immunohistochemical analysis on selected markers revealed that the same activated MSCs secreted both the T_H1 cytokine (interleukin-12) and indoleamine 2,3 dioxygenase (IDO), a classical immunosuppressive factor.

Conclusions: This study reflected that the plasticity of MSCs is highly dependent upon microenvironment conditions. Tumour-activated TIL induced T_H1 phenotype change in MSCs that is qualitatively similar to the previously described immunologic constant of rejection signature observed during immune-mediated, tissue-specific destruction. This response may be responsible for the *in loco* amplification of antigen-specific anti-cancer immune response.

Mesenchymal stromal cells (MSCs) are multipotent cells that have a central role in tissue regeneration, wound healing, and maintenance of tissue homeostasis (Prockop, 1997; Pittenger *et al*, 1999; Jiang *et al*, 2002). MSCs are found in almost every tissue including the bone marrow, where they are known as bone marrow stromal cells (BMSCs).

Previous studies have demonstrated MSCs' immunoregulatory capabilities. They have been shown in some assays to suppress T- and NK-cell proliferation (Tse *et al*, 2003; Spaggiari *et al*, 2008), induce T regulatory differentiation (Maccario *et al*, 2005), inhibit dendritic cell maturation (Jiang *et al*, 2005), and suppress B-cell function and maturation (Corcione *et al*, 2006; Asari *et al*, 2009). Notably, MSCs are able to migrate into injured tissues to promote wound healing through the secretion of a wide variety of growth factors (Wu *et al*, 2007; Qu *et al*, 2008). In consideration of their biological properties, MSCs have been successfully employed as an

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immune modulator in pilot clinical trials to treat several immune-related diseases including graft- versus-host-disease and multiple sclerosis (Wang *et al*, 2012; Uccelli *et al*, 2013).

The immunoregulatory nature of MSCs shows significant clinical relevance, especially in the tumour microenvironment. They are likely the precursor of tumour-associated fibroblasts (Spaeth *et al*, 2009), which along with multiple types of infiltrating immune cells, are involved in shaping the tumour microenvironment (Bergfeld and Declerck, 2010). Despite extensive investigations, the impact of MSCs on tumour growth or rejection remains unclear. Thus far, investigations performed in animal models have provided inconsistent results. According to a recent literature review, the role of MSCs in promoting or suppressing tumour progression was split equally (Klopp *et al*, 2011). The inconsistency in published data could be explained, at least in part, by the complexity and plasticity of the tumour microenvironment and the variable response of MSCs to different stimuli.

The mechanism by which MSCs exert their immunomodulatory functions in the tumour microenvironment is complex, but has been shown to be dependent upon the crosstalk between MSCs and immune cells, and involves both direct contact and the release of soluble factors (English, 2013). Among immune cells, T cells may trigger changes in response to antigen-specific stimulation. Our group (Wang *et al*, 2008) and others (Boon *et al*, 2006) previously suggested that anti-tumour responses are likely the result of an *in loco* expansion of pro-inflammatory stimuli triggered by antigen-specific elicitation of T cells that ultimately result in a full-blown acute inflammatory reaction. In steady-state conditions, MSCs constitutively secrete suppressive factors such as transforming growth factor-β1, prostaglandin-E2 (PG-E2) and soluble human leukocyte antigen (HLA)-G5 (Uccelli *et al*, 2008). However, soluble factors produced by T cells can modulate MSC function. Several studies have found that the *ex vivo* incubation of MSCs with either interferon (IFN)-γ or tumor necrosis factor (TNF)-α can induce phenotype changes in MSCs (Hemeda *et al*, 2010). These cytokines can increase the constitutive production of PG-E2 (Aggarwal and Pittenger, 2005) and release of indoleamine 2,3 dioxygenase (IDO) by MSC (English, 2013). In tumours, cancer cells can modulate T-cell responses (and vice versa), and this complex interaction likely affects MSC function.

To better understand the modulation of the tumour microenvironment induced by MSCs in response to direct interaction with T cells and cancer cells, MSCs and tumour infiltrating lymphocytes (TIL) activated by cancer cells were tested in a co-culture system that modelled the tumour microenvironment. Melanoma-specific TILs were co-cultured in direct contact with relevant melanoma cells, while MSCs were co-cultured in the transwells that allowed cell to cell interaction through the secretion of soluble factors. We observed that TIL activated in an HLA-restricted fashion by melanoma cells released soluble factors that resulted in a 'T_H1-like' phenotype change in the MSCs. This induced phenotype is characterised by the coordinated expression of pro-inflammatory genes classically associated with immune-mediated cancer rejection (Wang *et al*, 2008; Galon *et al*, 2013). Overall, these results suggest that TIL-activated MSCs can prime the microenvironment to create conditions favourable for tumour regression.

MATERIALS AND METHODS

Human MSCs. MSCs were isolated from healthy donor bone marrow aspirates at the Department of Transfusion Medicine, Clinical Center, NIH. These cells were expanded and characterised as described in our previous work (Ren *et al*, 2011). Briefly, cells from the bone marrow aspirates were seeded in complete media (α-minimal essential medium, 2 mM glutamine, 10 μg ml^{-1} gentamicin and 20% fetal bovine serum) for 24 h, and the non-adherent cells were removed. The adherent cells were expanded until 70–80% confluence was reached. Cells were subcultured and kept in complete media. These BMSCs were $>70\%$ viable and expressed CD73, CD90, CD105, and CD146 but not CD11b, CD14, CD19, CD34, or CD45. BMSCs from one healthy donor were used for the studies.

Human TILs. Tumour infiltrating lymphocytes were isolated from metastases of melanoma patients and maintained as previously described (Panelli *et al*, 2000). We used TIL populations from two melanoma patients: TIL-H1, which recognises antigen gp100 expressed on the surface of melanoma, and TIL-A42, which recognises MART-1, both in an HLA-restricted manner when presented by HLA-A2.

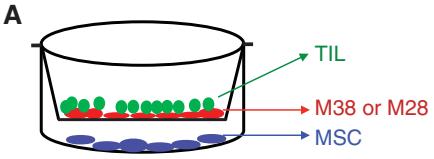
Human melanoma cell lines. Melanoma cell lines, 624.38(M38) and 624.28(M28), were generated by limiting dilution from the metastatic lesions of melanoma patients at the Surgery Branch, NCI, NIH (Rivoltini *et al*, 1995b). Cell lines M38 and M28 were from the same patient; M38 expressed HLA-A*0201$^+$ while M28 had lost its expression of HLA-A*0201 and was, hence, HLA-A*0201$^-$. They were maintained in RPMI with 10% FBS, 2 mM glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin.

Co-culture with the transwell system. MSCs were seeded at the bottom of a six-well plate; melanoma cell lines, either M38 or M28, were seeded in the six-well transwells and placed above the MSCs. The TILs were added to the same transwells containing melanoma cells. The TIL and melanoma cells were co-cultured with physical contact while physically separated from the MSCs (Figure 1A). Transwell chambers with 1.0 μm pore size membrane (Corning Costar, Cambridge, MA, USA) were used to separate the cells in co-cultures.

The MSCs used in this study were passage 4 cells. They were initially seeded at 5×10^4 cells per well. Co-cultures were put together when the MSCs reached around 80% confluence. An equal amount of TIL and melanoma cells were seeded (10^6 cells per well). Cells were cultured with RPMI complete medium supplemented with either FBS or human AB serum. MSC mono-cultures made use of the same culture conditions and served as controls. Cells and supernatant were harvested at 24, 48, and 72 h intervals for gene and protein analysis.

Total RNA purification, amplification, hybridisation, and slide processing. Total RNA from co-cultures and control samples were purified using miRNA Easy Kit (Qiagen, Valencia, CA, USA). The RNA concentration was measured using Nano Drop ND-1000 Spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and RNA quality was assessed with Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA was amplified using an Agilent LowInput QuickAmp Labeling Kit and subsequently hybridised to Universal Human Reference RNA (Stratagene, Santa Clara, CA, USA) with Agilent Chip Whole Human genome, 4×44k slides according to the provided protocol. The slides were incubated for at least 17 h at 65 °C.

Statistical and microarray data analysis. Raw images were obtained by scanning the microscope slides with Agilent Scan G2505B and Agilent Scan Control software (version 9.5). The images were extracted using the Feature Extraction Software (Agilent Technologies). Partek Genomic Suite 6.4 (Partek Inc., St Louis, MO, USA) was used for data visualisation, identification of differentially expressed transcripts (P value $\leqslant 0.05$), and hierarchical cluster analysis. We transformed the fluorescence intensity data to log2 ratios of each sample versus the universal human RNA reference (Stratagene). Then t-tests were used to identify the differentially expressed genes (both P value and false discovery rate (FDR) less than 0.05). The Ingenuity Pathway Analysis tool



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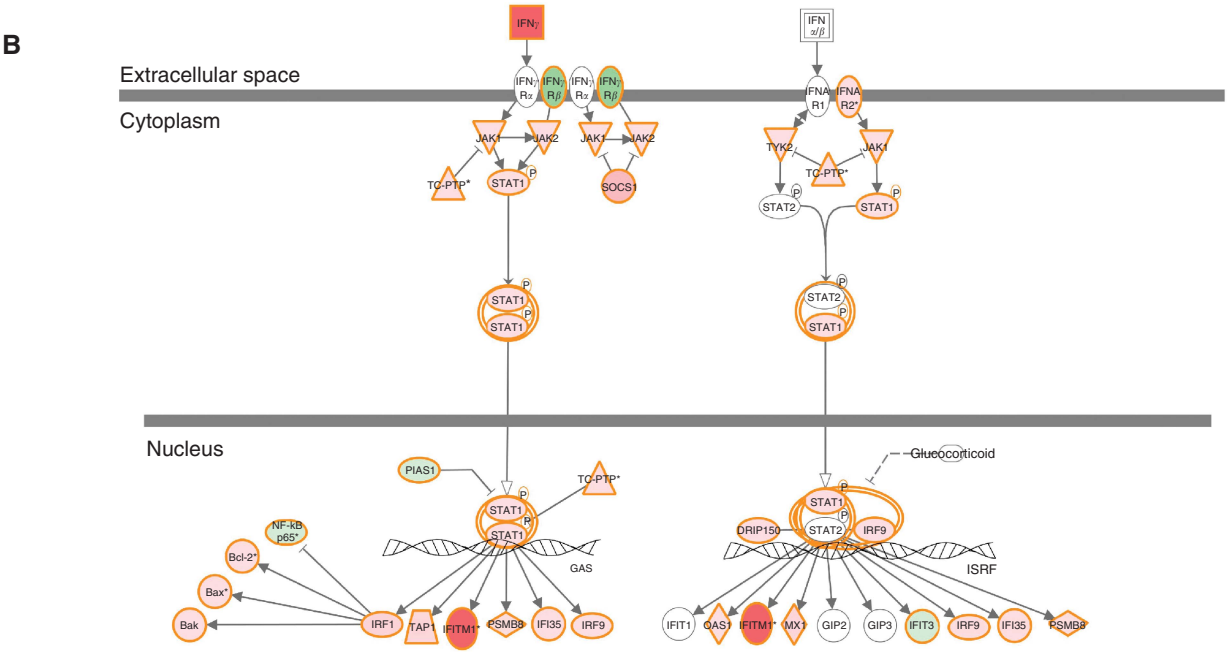
TIL+M38 MSC	TIL co-culture with M38 compared with TILs co-culture with M28			TIL+M28 MSC
Protein (gene)	Protein FC	Protein *P*-value	Gene FC	Gene *P*-value
IFNγ	473	5.17E-06	20.24	1.27E-08



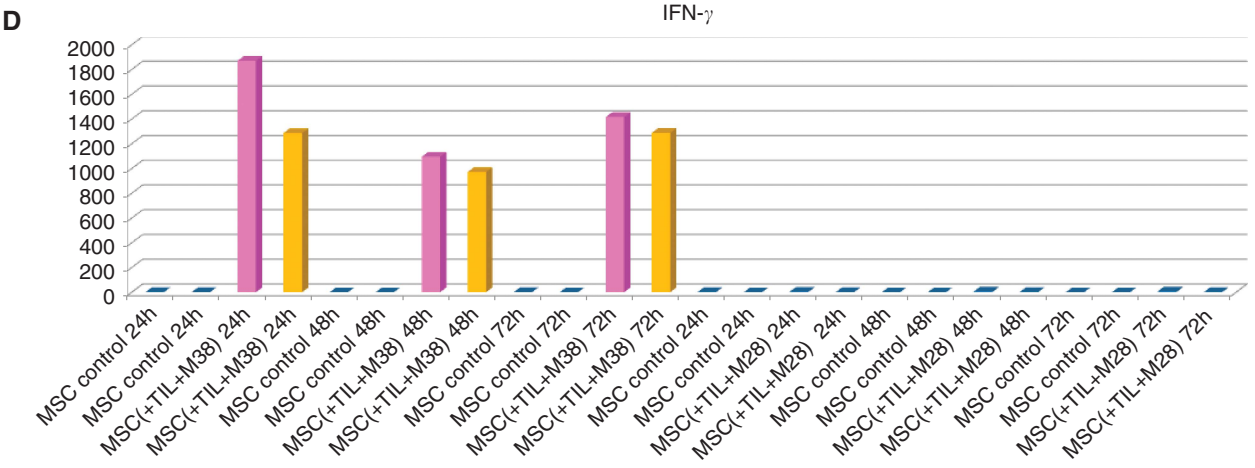


Figure 1. TIL activated by co-culture with M38 melanoma upregulate interferon signalling pathways. MSCs and HLA-A*0201+ TIL (TIL-H1 and TIL-A42) were co-cultured with HLA-A*0201$^+$ M38 melanoma ($n=12$) or M28 melanoma, which lacked HLA-A*0201 ($n=12$); control TIL and TIL gene expression and supernatant protein levels were analysed. (**A**) Experimental design: MSCs were seeded at the bottom of a six-well plate; the melanoma cell lines, either M38 or M28, were seeded in the six-well transwells and placed above the MSCs. The TILs were added to the same transwells containing melanoma cells. (**B**) Ingenuity pathway analysis (IPA) of activated differentially expressed TIL genes showed that the expression of genes in the interferon signalling pathway changed, including IFN-γ, IRF1, JAK1, and STAT1. (**C**) In M38 melanoma-activated TIL IFN-γ increased 473-fold at the protein level and 20.24-fold at the gene level compared with control TIL. (**D**) Protein analysis shows the IFN-γ concentration in the co-culture supernatants (pg ml^{-1}). The pink bars represent supernatants from MSCs co-cultured with TIL-H1 and M38 melanoma cell lines and the yellow bars represent MSCs co-cultured with TIL-A42 and M38 melanoma cell lines.

(http://www.ingenuity.com, Ingenuity System Inc., Redwood City, CA, USA) was used for analysis of functional pathways.

Supernatant cytokine and growth factor analysis using multiplex enzyme-linked immunosorbent assay (ELISA). Supernatants from the samples were evaluated using multiplex ELISA, Search-Light Protein Array Analysis (Aushon Biosystems, Billerica, MA, USA). Forty-two soluble factors were evaluated and Partek Genomic Suite 6.4 (Partek Inc., St Louis, MO, USA) was used for protein data visualisation, identification of differentially expression (P value$\leqslant 0.05$), and hierarchical cluster analysis. The data were normalised using $\log(X+\text{offset})$ and then t-tests were used to identify the differentially expressed soluble factors (both P value and FDR are <0.05).

Immunofluorescence analysis. MSCs were fixed with 4% paraformaldehyde (Electron Microscopy Science, Hatfield, PA, USA) for 15 min, washed in phosphate-buffered saline (PBS), and quenched in 50 mM NH_4Cl for 10 min. Cells were washed with PBS and treated with background buster (Innovex Biosciences, Richmond, CA, USA) for 30 min and Fc Receptor blocker (Innovex) for 15 min and permeabilised in PBS containing 0.3% saponin. Cells were subsequently immunolabelled using an indirect procedure in which all incubations (primary, secondary antibodies, and washes) were performed in solution containing 0.3% saponin. The antibodies used in this study were primary: interleukin (IL)-12 and IDO (Abcam, Cambridge, MA, USA), and secondary: AlexaFlour (Life Technologies, Grand Island, NY, USA). The images were obtained using AxioVert 200M microscope (Carl Zeiss, Oberkochen, Germany).

Melanoma tissue sample microarray analysis. Total RNA from 112 melanoma metastases from patients treated at the Surgery Branch, NCI, was extracted and processed using the same procedures as mentioned above. First- and second-strand cDNA were synthesised from 300 ng of total RNA according to the manufacturer's instructions (Ambion WT Expression Kit, Grand Island, NY, USA). cDNAs were fragmented, biotinylated, and hybridised to the GeneChip Human Gene 1.0 ST Arrays (Affymetrix WT Terminal Labeling Kit, Santa Clara, CA, USA). The arrays were washed and stained on a GeneChip Fluidics Station 450 (Affymetrix); scanning was carried out with the GeneChip Scanner 3000 and image analysis with the Affymetrix GeneChip Command Console Scan Control. Expression data were normalised, background-corrected, and summarised using the RNA algorithm. Data were log-transformed (base 2) for subsequent statistical analysis. Cluster analysis was performed using Partek software (Partek, St Louis, MO, USA).

RESULTS

Melanoma activated-TIL induce changes in MSC gene expression. To determine the factors present in the tumour microenvironment that could trigger MSCs to exert their immunomodulatory function, we utilised a TIL/melanoma

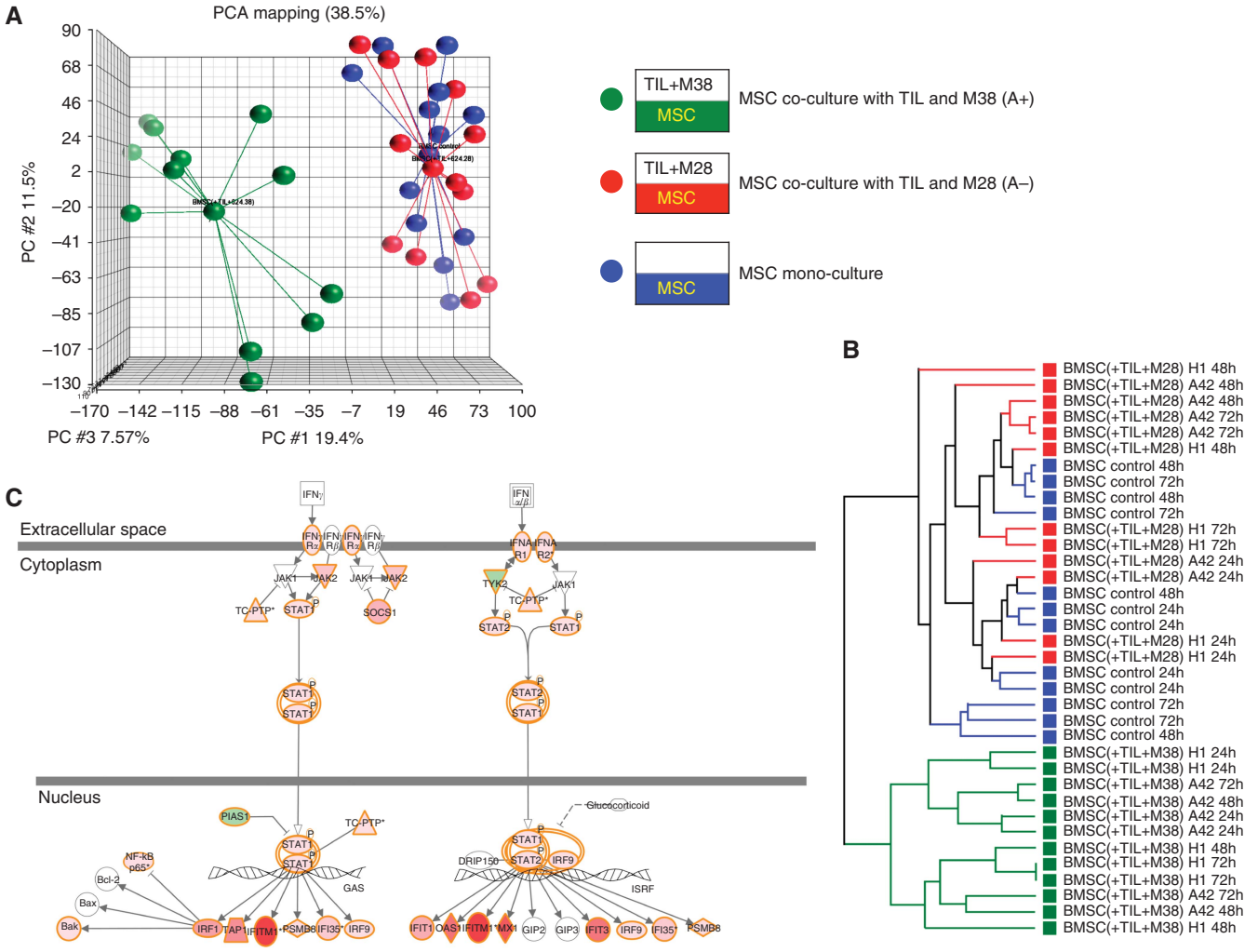


Figure 2. Global gene expression analysis of MSCs co-cultured with activated TIL. MSCs were co-cultured with HLA-A*0201 + TIL and the M38 HLA-A*0201 $^+$ melanoma cell line or with the M28 HLA-A*0201 $^-$ melanoma cell line. (**A**) Principal component analysis (PCA) shows that the gene expression signature of MSCs co-cultured with TIL and M38 melanoma (Green) is different from that of MSCs co-cultured with TIL and M28 melanoma (red) and control MSCs (blue). Each MSC sample is represented by one sphere. The sphere in the centre of each cluster represents the mean value for the group. (**B**) Unsupervised hierarchical clustering analysis of the same samples revealed that all the MSCs (green) co-cultured with TIL and M38 melanoma are in the same cluster and MSCs co-cultured with TIL and M28 melanoma are in another cluster with MSC monocultures. MSCs from one donor at 3 time points (24, 48, and 72 h) were tested twice. (**C**) Ingenuity pathway analysis (IPA) of MSC gene profiling data showed interferon pathway gene changes after co-culturing with TIL + M38.

interaction model to mimic the *in vivo* tumour microenvironment. We used two TIL cell lines from metastatic melanoma patients: TIL cells H1 and A42, which recognise melanoma antigen gp100 and MART-1, respectively, in a HLA-A*0201-restricted manner. The TILs were seeded in the absence of exogenous IL-2 with melanoma cell lines in the same transwell, and the transwells (containing TILs and melanoma in physical contact) were inserted into the six-well plate seeded with BMSCs. The final co-culture consisted of BMSCs on the bottom without physical contact with the TIL and melanoma cells in the transwell (Figure 1A). Two melanoma cell clones derived by limiting dilution from the same cell line were used: M28 (624.28) and M38 (624.38). M38 (624.38) expressed HLA-A*0201, while M28 (624.28) lacked its expression as previously described (Rivoltini *et al*, 1995a; Wang *et al*, 1999). When the TILs were co-cultured with M38, but not M28, they recognised the antigens (gp100 or Mart-1) expressed on the melanoma cell surface, destroyed the melanoma cells and released IFN-γ and other factors as previously described

After co-culture, TIL gene expression profiling was performed. The results showed a 20-fold increase in IFN-γ expression in TIL co-cultured with M38 (HLA-A*0201$^+$) compared to those co-cultured with M28 (HLA-A*0201$^-$) (Figure 1C). Ingenuity pathway analysis (IPA) also showed enhanced expression of genes downstream of interferon signalling by TIL (Figure 1B). In addition, protein profiling analysis revealed a 473-fold increase in IFN-γ levels in the supernatant (Figures 1C and D), while no IFN-γ protein was detected in the supernatant of TILs cultured with M28.

Next, we assessed MSC gene expression after co-culture with TILs and M38. The gene expression pattern of these MSCs differed significantly from MSCs co-cultured with TILs and M28, as well as control MSCs cultured alone. Principal component analysis (PCA) based on the whole transcriptome separated the MSCs co-cultured with TIL + M38 from those co-cultured with TIL + M28 and control MSCs (Figure 2A). Unsupervised hierarchical clustering analysis confirmed that MSCs co-cultured with M28 + TIL and control MSCs were mixed within the same cluster, while MSCs co-cultured with M38 + TIL formed a distinct cluster (Figure 2B). These results suggest that MSCs detected signals released by activated TILs in the melanoma microenvironment and responded by changing their gene expression patterns.

MSCs are polarised toward T_H1 phenotype by activated TIL. We then assessed the transcript activation in MSCs co-cultured with activated TIL by IPA by using the list of genes differentially expressed between MSCs co-cultured with TIL + M38 and the MSC control group (*P* value and FDR <0.05). The most over-represented pathways included many immune-related functions such as interferon signalling, antigen presentation, dendritic cell maturation, and crosstalk between dendritic cells and natural killer cells (Supplementary Table 1).

The gene expression changes in MSC co-cultured with TIL + M38 appeared to be predominantly interferon signalling transcripts, notably, IFNG-Ra, Jak2, STAT1 and STAT2, and IRF1 (Figure 2C). In addition, many immune-related cytokine and chemokine genes were upregulated (Table 1 and Supplementary Tables 3 and 4). Among these were several factors associated with a T_H1 immune response, including CCL5, CCL8, CXCL9, CXCL10, CXCL11, IL-12, and IL-15. Interestingly, the expression of the T_H2-associated immunosuppressive factor IDO was also increased 114.69-fold, but other T_H2-associated factors such as IL4, IL10, and PG-E2 did not show similar increases.

Analysis of co-culture supernatants shows increased levels of cytokines and chemokines consistent with a T_H1 polarisation. We tested the levels of 42 immune-related soluble factors in co-culture supernatants including both T_H1 and T_H2 cytokines. Hierarchical clustering analysis showed that many of the 42 factors were differentially expressed among MSCs co-cultured with activated TIL and control TIL (Figure 3B). The pattern of expression of the 42 factors in the supernatant of MSCs co-cultured with TIL + M38 was different from that of MSCs co-cultured with TIL + M28 as well as the control MSCs. PCA analysis confirmed that the 42 soluble factors were differently expressed among the three groups (Figure 3A). Consistent with transcriptional results, Student's *t*-test analysis showed that in the supernatant from MSCs co-cultured with TIL + M38, the levels of CXCL10, CXCL9, IFN-γ, CXCL11, CCL8, CCL5, TNF-α, IL-12, IL-17, CCL2, IL-8, IL-15, IL-2, IL-7, IL-5, granulocyte macrophage-colony stimulating factor (GM-CSF), ICAM1, IL-6, CXCL1, CSF1, IL-1A, IL-1B, and VCAM1 were elevated (Figure 3B and Supplementary Table 2).

Table 1. Upregulated genes in MSCs co-cultured with TIL and M38 compared with MSCs cultured alone

TIL + M38 MSC	MSC co-culture with TIL plus M38 compared with MSC mono-cultures	MSC
Gene	**Fold change**	***P* value**
CXCL9	183.21	2.17E – 23
IDO1	114.69	3.27E – 29
CXCL11	64.71	1.1E – 14
CCL13	55.67	1.95E – 18
CCL8	44.32	4.34E – 21
CXCL10	35.67	1.21E – 14
ICAM1	34.60	4.71E – 13
CCL7	25.49	3.95E – 10
CCL2	24.41	6.05E – 11
TLR3	21.27	3.81E – 19
CCL5	20.93	2.39E – 10
IRF1	11.25	1.19E – 17
GZMA	9.57	9.55E – 06
HLA-G	8.30	5.56E – 14
CXCL2	8.00	1.35E – 08
IRF8	7.07	2.21E – 06
IL-15	6.82	1.86E – 18
IL-1B	6.38	0.00013
CXCL1	6.00	0.001554
CXCL16	5.03	7.51E – 12
ICAM4	4.92	2.01E – 10
IL-7	4.73	1.19E – 08
CXCL3	4.69	8.95E – 05
IL12A	4.25	3.64E – 07
STAT2	4.05	1.24E – 15
STAT1	3.85	3.86E – 19
IL17C	3.73	9.2E – 06
IRF2	3.57	1.04E – 11
IRF7	3.50	3.76E – 11
CCL22	3.37	1.8E – 08
TLR4	2.93	3.19E – 10
IL32	2.89	0.002099
IDO2	2.84	0.000233
IL-6	2.73	1.75E – 08
CXCR3	2.68	3.28E – 09
IRF9	2.53	1.63E – 16
VCAM1	2.50	4.03E – 05
GZMB	2.49	0.001454
CCR1	2.46	0.000189
CCL4	2.42	0.002967
CCL26	2.13	0.001105
VEGFC	2.05	1.58E – 07

Abbreviations: HLA = human leukocyte antigen; IDO = indoleamine 2,3 dioxygenase; IL = interleukin; MSC = mesenchymal stromal cells; TIL = tumour infiltrating lymphocytes.

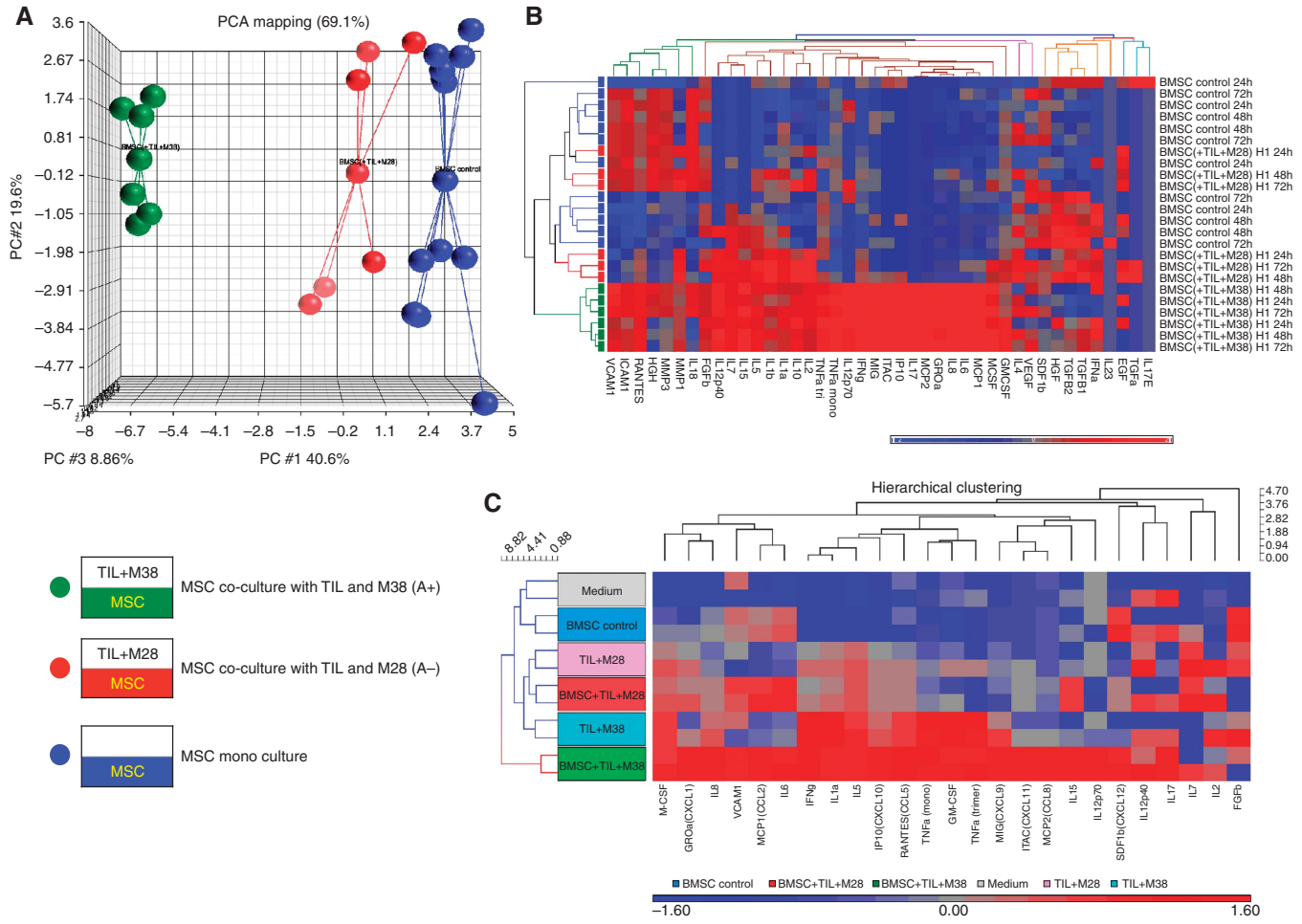


Figure 3. Analysis of proteins in the supernatant of co-cultures of MSCs and activated TIL. The levels of selected proteins from culture supernatant of MSCs co-cultured with TIL and M38 melanoma, compared with MSCs co-cultured with control TIL and M28 melanoma. (**A**) Principal component analysis (PCA) shows that the expression pattern of MSCs co-cultured with TIL and M38 melanoma (green) is different from that of MSCs co-cultured with TIL and M28 melanoma (red) and MSC monocultures (blue). (**B**) Unsupervised hierarchical clustering analysis of the protein levels revealed that all of the MSCs co-cultured with TIL and M38 melanoma are in one cluster (green), and MSCs co-cultured with TIL and M28 melanoma (red) clustered together with MSC monoculture (blue). (**C**) The heat map of supernatant protein levels shows that M-CSF, IFNγ, IL-1α, IL-5, TNFα, GM-CSF have almost same-level expression in the co-culture supernatants of TIL + M38 and BMSC + TIL + M38, implying those factors were secreted by activated TIL, while CXCL1, IL-8, VCAM1, CCL2, IL-6, CXCL10, CCL5, CXCL9, CXCL11, CCL8, IL-12, and CXCL10 were secreted by MSCs. MSCs from one subject were tested at three time points and were tested twice.

To evaluate the origin of these cytokines and chemokines, we tested the protein expression in supernatants of each culture: MSC/TIL + M28, TIL + M28, MSC/TIL + M28, TIL + M28, and MSCs alone. The results showed that IFN-γ, TNF-α, M-CSF, GM-CSF, IL-1a, and IL-5 were produced by the activated TIL, while CCL2 (MCP1), CCL8 (MCP2), CCL5 (RANTES), CXCL1 (GROa), CXCL9 (MIG), CXCL10 (IP10), CXCL11 (ITAC), IL-6, IL-8, IL-12, IL-15, IL-17, and VCAM1 were produced specifically by MSCs stimulated by active TIL (Figure 3C and Supplementary Table 5).

Immunofluorescence analysis of stimulated MSCs reveals that one cell population expresses both IL-12 and IDO. Since MSCs are a heterogeneous population of cells, we wondered if two MSCs subpopulations had emerged from the co-cultures with activated TIL: one producing T_H1 factors and another producing the T_H2-associated immunosuppressive factor IDO, or if a single population produced both T_H1 factors and T_H2-associated factor IDO. Immunofluorescence staining of MSC for IL-12 and IDO revealed that after co-culture with activated TIL the expression of both IL-12 and IDO increased and that a single population of MSCs expressed both factors (Figure 4).

MSC genes whose expression was induced by active TIL are more likely to be expressed by melanoma samples from patients with a good prognosis. To determine if MSCs within the tumour microenvironment showed T_H1 polarisation while concurrently expressing IDO, we assessed the expression of 276 immune-related genes selected from MSC genes differentially expressed after co-culture with activated TIL in 112 melanoma metastases. These 112 melanoma metastases have been previously characterised and found to belong to three phenotypes (Spivey *et al*, 2012). The first phenotype was characterised by prevalent expression of cancer testes antigens, the WTN signalling gene WNT5A, and a T_H17 immune phenotype, ascribed to be a more aggressive cancer (Class A). The second phenotype (Class B) was characterised by the expression of melanoma differentiation antigens and a T_H1 immune phenotype. Class B is considered to have better prognosis and be more likely to respond to immunotherapy. Lastly, Class C lies between the two polar groups, Classes A and B. Hierarchical clustering analysis of the 122 samples based on 276 transcripts clustered the metastases into several clusters. Specifically, one of the clusters contained 21 samples that included 9 Class B and 12 Class C samples and was

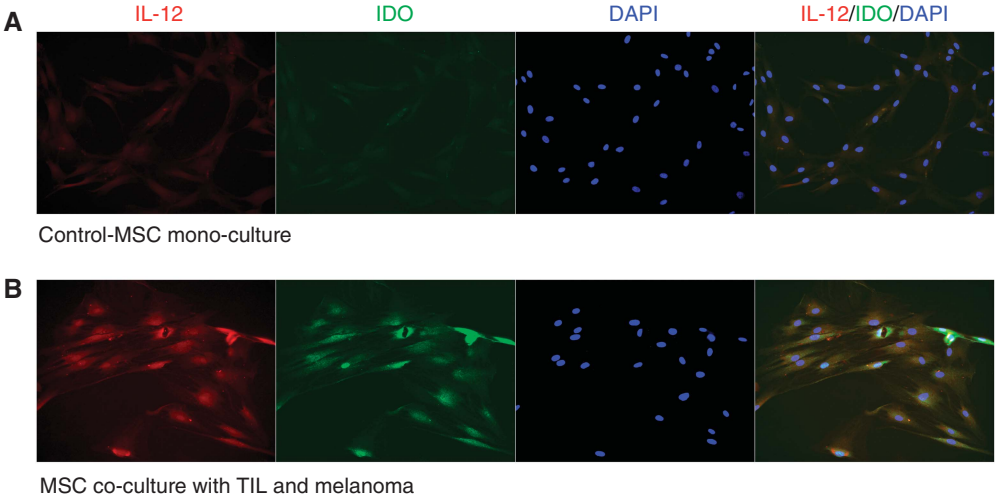


Figure 4. Indirect immunofluorescence of IL-12 and IDO on MSCs. (**A**) Immunostaining of control MSCs. (**B**) Immunofluorescence of MSCs co-cultured with TIL and M38 melanoma cells. Staining with anti-IL-12 is shown in red, anti-IDO in green and DAPI in blue.

characterised by the expression of T_H1 factors, CXCR3 ligands, CCL5 ligands, and IDO (Figure 5A). The expression of T_H1 factors, CXCR3 ligands, and CCL5 ligands was enhanced in most of the other samples, but to a lesser extent. In addition, another group with 12 Class A samples and 7 Class B samples was characterised by the expression of T_H17 genes in addition to T_H1 factors, CXCR ligands, and CCL5 ligands (Figure 5A). The genes that characterise Class A and Class B phenotypes and their expression by MSCs co-cultured with activated TIL are summarised in Supplementary Tables 3 and 4. A more focused analysis showed that in some melanoma samples both pro-inflammatory molecules and IDO are expressed and this concordant expression of divergent function may influence the overall behaviour of such tumours (Figure 5B).

DISCUSSION

This study shows that MSCs are highly plastic, their phenotype is dependent on the state of their microenvironment and they can profoundly affect the tumour microenvironment. We found that activated TIL induce marked pro-inflammatory changes in MSCs, polarising them towards a T_H1-like phenotype. These changes were induced by soluble factors produced by activated TIL. While IFN-γ is likely responsible for many of these changes, we found that melanoma-activated TIL produced other pro-inflammatory cytokines, including TNF-α, IL-1α, and GM-CSF, which may contribute to these changes. The polarisation of MSCs differs from that of mononuclear phagocytes in that some factors considered characteristic of T_H2 responses were also produced, but activated MSCs produced far more T_H1 factors. These findings suggest that MSC have a more critical role in the tumour microenvironment than previously appreciated.

The tumour microenvironment has an important role in the natural evolution of cancer and its response to immunotherapy. Several investigations conducted in ovarian (Zhang *et al*, 2003), colorectal (Tosolini *et al*, 2011), and breast (Ascierto *et al*, 2012) cancers by evaluating thousands of patients have consistently shown that a T_H1 cancer phenotype, characterised by the expression of interferon-stimulated genes and immune-effector mechanisms, is associated with favourable prognosis (Ascierto *et al*, 2011; Galon *et al*, 2013). Recently, similar signatures have been associated with favourable prognosis in melanoma (Messina *et al*, 2012), and they were also observed in pre-treatment lesions as more likely to respond to immunotherapy (Ji *et al*, 2012; Bedognetti *et al*, 2013; Ulloa-Montoya *et al*, 2013; Wang *et al*, 2013). These signatures overlap with those observed in tumour lesions experiencing rejection following immunotherapy and during other immune-mediated tissue destruction phenomena such as graft-versus-host disease, allograft rejection, and clearance of pathogens (Mantovani *et al*, 2008; Wang *et al*, 2008, 2013; Spivey *et al*, 2011; Galon *et al*, 2013). These molecular requirements, also called immunologic constant of rejection (ICR), underlie a phenomenon that consists of coordinated activation of the IL-15/IFN-γ/IRF1/STAT1 molecular pathway, the recruitment of cytotoxic cells through the production of specific chemokine ligands (in particular CXCR3 and CCR5 ligands) and the activation of immune-effector function genes (e.g. granzyme A/B, perforin, etc.) (Wang *et al*, 2008; Bedognetti *et al*, 2010; Spivey *et al*, 2011). While there is a growing consensus on the importance of these immune signatures in immune-mediated rejection and in identifying responsiveness to immunotherapy, the exact factors responsible for their coordinated activation are unknown. It is reasonable to conclude that cancer cells, immune cells, and stromal cells collaborate to develop this favourable inflammatory phenotype (Harlin *et al*, 2009), but the degree of contribution of each is unclear.

In view of the critical role of the ICR pathways, the identification of strategies capable of inducing their enhancement is of great interest. By combining genome-wide analysis and proteomic analysis, we showed that the main consequence of the stimulation of MSCs by melanoma-activated TIL is the potent activation of the IFN signalling (Figures 2 and 3), resulting in the production of several interferon-inducible pro-inflammatory T_H1 cytokines and chemokines, including IL-8, CCR5, and CXCR3 ligands (CCL5 and CXCL9-11, respectively). Furthermore, this interaction induced the release of several other pro-inflammatory molecules by MSCs, including the T_H1 cytokines IL-15 and IL-12. In addition, gene expression data also showed that TIL-stimulated MSCs overexpress several pro-inflammatory cytokine and chemokine genes (e.g., IL-1B, CCL2, CCL7, CCL8, CCL13, CXCL11, CXCL16), which could in turn lead to the amplification of the inflammatory process at the tumour site and cause a shift toward a T_H1-flavoured acute inflammation. It is likely that when T cells within the microenvironment encounter tumour cells, they become active and release IFN-γ and other soluble factors that stimulate MSCs. Interestingly, TIL-stimulated MSCs also overexpress

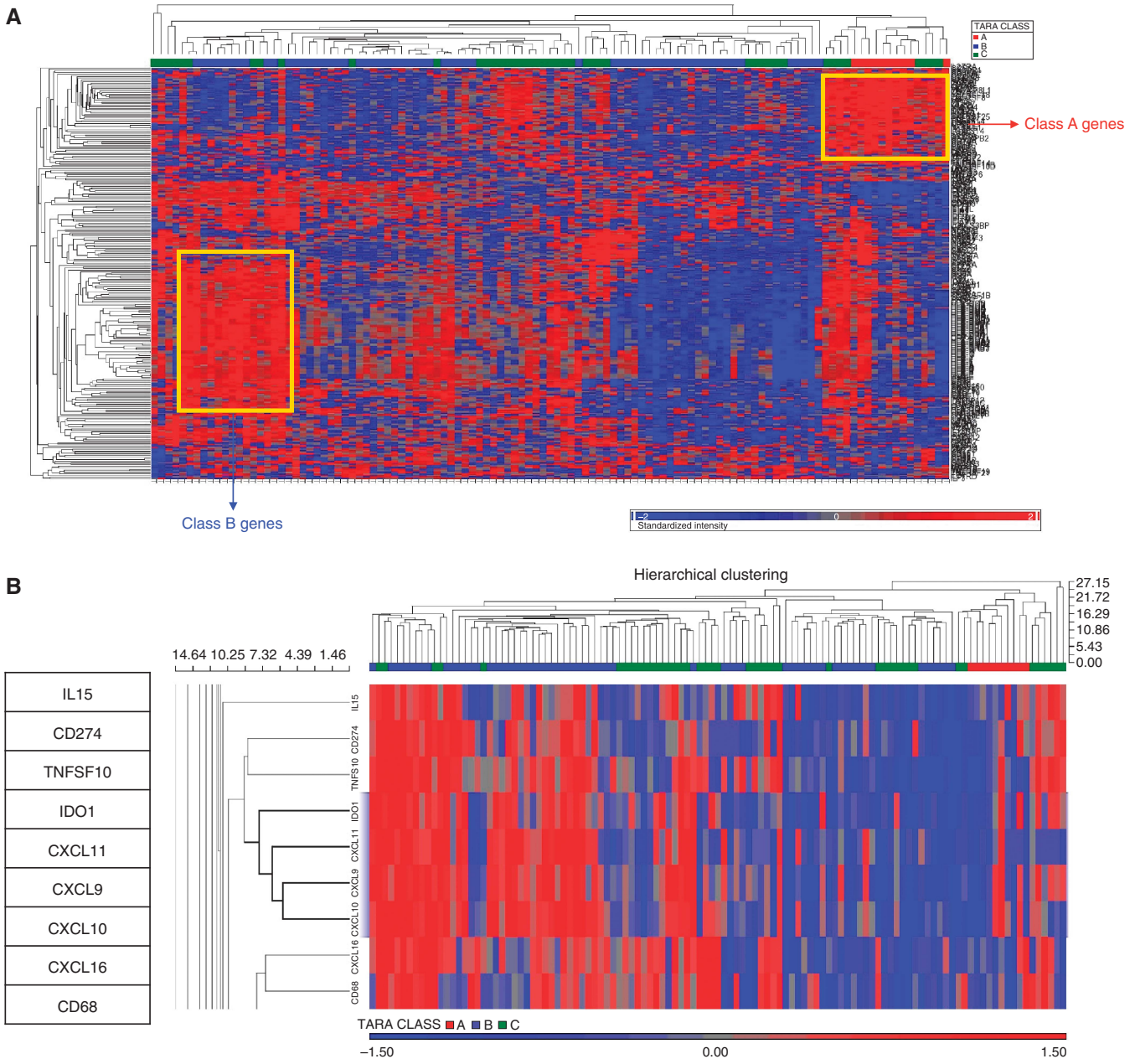


Figure 5. Analysis of the expression of 276 MSC immune-related genes by 112 melanoma samples. Among the immune-related genes whose expression changed in MSCs co-cultured with activated TIL, 276 genes were identified and the expression of these genes was analysed among 112 melanoma samples. (**A**) Hierarchical clustering analysis showed that the 276 genes separated the melanoma tissues into the three previously described phenotypes: TARA Class A, Class B, and Class C. The Class A and Class B genes that characterise these phenotypes are highlighted in the figure and are listed in Supplementary Tables 3 and 4. (**B**) Analysis of the expression of the CXCR3 ligands, CXCL9, CXCL10 and CXCL11, and IDO1 among the 112 melanoma samples shows that these factors were co-expressed.

immune-effector genes (e.g., GZMA, GZMB). The functional relevance of this unexpected and not yet described modulation of the immune-effector genes should be assessed in future studies.

Concomitantly with the induction of T_H1 pro-inflammatory response, we observed that the overexpression of T_H17-related cytokine gene IL-17, the T_H2-related genes IL-6, IL-5, and IDO1 by TIL activated MSC. Data from ELISA assays together with data from immunofluorescence confirmed the microarray data. MSCs are a heterogeneous population of cells and one recent study has suggested that they can be polarised toward either a MSC1 (secretes T_H1 pro-inflammatory factors) or an MSC2 phenotype (secretes immunosuppressive factors such as IDO) (Waterman *et al*, 2010). We hypothesised that activated TIL may polarise some MSCs to a T_H1 phenotype and others to an immunosuppressive type. However, by using immunofluorescence we observed that, remarkably, a single population of MSCs was producing the T_H1 pro-inflammatory cytokine IL-12 and the immune-suppressive factor IDO1. This mixed response suggests that activated TILs induce a complex transcriptional programme in MSC that induces individual cells to produce functionally divergent effects. In future studies, it would be interesting to explore whether MSCs from different individuals are prone to favour the expression of one or the other functional phenotype under identical stimulatory conditions.

The expression of IDO1 by TIL-activated MSCs deserves further discussion. IDO1 (or IDO) is an enzyme that catabolises tryptophan to kynurenine and it represents the first step to the

de novo synthesis of nicotinamide adenine dinucleotide. It is inducible by viruses, lipopolysaccharide, and pro-inflammatory cytokines such as IL-1, interferon-γ, and tumour necrosis factor-α (Mohib *et al*, 2007). Based on experimental studies *in vitro* and in animal models, IDO has been showed to have a crucial role in the induction of immune tolerance during pregnancy, infection, and autoimmunity (Brown *et al*, 1991; Schrocksnadel *et al*, 2003; Popov and Schultze, 2008). The induction of IDO is thought to be a central mediator in several aspects of MSC's interactions with cells of the human immune system, including the induction of immune tolerance. However, in the last few years the role of IDO in suppressing inflammation has been consistently reconsidered. Experiments in models of inflammation-associated arthritis and allergic airway disease have shown that IDO impairment can alleviate disease severity, suggesting that IDO1 is critical in promoting/sustaining inflammatory response (Prendergast *et al*, 2011). Similarly, investigations employing a classical model of skin cancer have shown that IDO1 can promote cancer-associated inflammation (Prendergast *et al*, 2011). Interestingly, data from clinical trials assessing the effectiveness of anti-CTLA4 therapy in metastatic melanoma have shown that the overexpression of IDO by infiltrating immune cells in pre-treatment lesions strongly correlates with treatment responsiveness (Hamid *et al*, 2011). Concordantly, in the same setting, Ji *et al* (2012) found that the expression of IDO1 in pre-treatment lesions is associated with the activation of the ICR pathways, which in turn predict the responsiveness to ipilimimumab treatment. In line with this observation in the current studies we found that Class B melanoma metastases bearing the favourable T_H1 phenotype also overexpress IDO1. The expression of IDO1 could reflect the activation of a counter-regulatory mechanism in the tumour microenvironment and the balance between the divergent effects that determine individual outcomes in natural conditions or in response to therapy. Alternatively it is also possible that IDO1 is directly involved in promoting a cancer-related T_H1 inflammation. For instance, it has been recently observed that IDO can induce apoptosis of melanoma tumour cells (Cetindere *et al*, 2010).

In conclusion, we have observed that the behaviour of MSC is highly dynamic and dependent upon the nature of the micro-environment. For the first time, we show that TIL activated by melanoma cells can trigger MSCs to produce a wide range of pro-inflammatory modulators including T_H1 factors that have been invariably associated with immune-mediated antitumor response. This pro-inflammatory activity is, however, not disjointed with the concomitant induction of some factors (e.g., IDO) classically considered as immunosuppressive.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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