

## Effect of Indoleamine 2,3 Dioxygenase Inhibitor on the Cytotoxic Activity of Tumour-infiltrating Lymphocytes

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There have been major therapeutic advances in the treatment of melanoma in recent years (1, 2). Adoptive T-cell therapy, based on tumour-infiltrating lymphocytes (TILs) obtained from tumour tissues, has been developed in the field of immunotherapy. An objective clinical response of between 40% and 72% in the metastatic stage has been reported (3, 4) but the clinical response remains limited in time. In the metastatic stage of melanoma, the clinical response remains limited and short-term. A major current challenge is to increase the efficacy of TILs by countering the immune tolerance of the tumour microenvironment through identification of the molecules implicated in this phenomenon. Many factors have been suggested, including indoleamine 2,3 dioxygenase (IDO), an intracellular enzyme that metabolizes tryptophan to kynurenine. Tryptophan deficiency inhibits the activity and proliferation of T cells (5). A recent paper (6) has shown that IDO1 expression in metastatic melanoma lymph nodes is associated with an increased risk of relapse and death. Thus, we hypothesize that the presence of IDO in the tumour microenvironment could inhibit the activity of TILs, and that adding an IDO inhibitor to the TILs could increase their cytotoxic activity against melanoma cells.

### MATERIAL AND METHODS

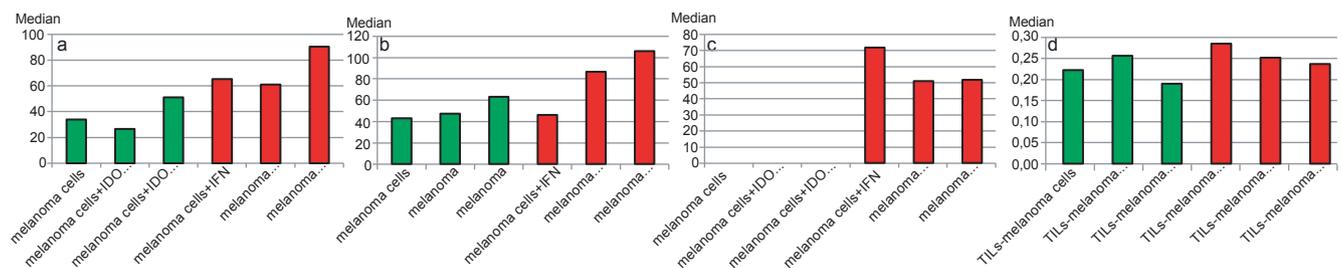
To investigate this hypothesis we used the model of co-culture of a melanoma cell-line and autologous TILs obtained from the same patient (6), with the addition of IDO inhibitor. The endpoints were the frequency of reactive autologous T cells among the TILs and the expression of molecules involved in the recognition of tumour antigens by T cells with and without IDO inhibitor. Melanoma cells were derived from patients with metastatic melanoma, who were previously included in several distinct clinical trials of TILs (7–10). Seven tumour cell-lines with associated autologous TILs were selected (4 wild-type cell-lines and 3 cell-lines with positive BRAF mutational status, but no difference according this mutational status was noted).

In a first step, the 7 melanoma cell-lines were incubated either with an IDO inhibitor, 1-methyl-L-tryptophan (1-MT) (in 2 different concentrations), alone or after incubation with interferon gamma (INF- $\gamma$ ) for mimicking the chronic inflammatory micro-environment state of a metastasis (associated with an increase in immune tolerance). In a second step, the 7 melanoma cells were distributed on a 96-well plate (300,000 cells per well) with 100,000 autologous TILs. Co-cultures were incubated for 6 h at 37°C, 5% CO<sub>2</sub>. Finally, melanoma cells were analysed by flow cytometry for expression of IDO1, MHC-I, MHC-II and PD-L1 antigens and reactivity of TILs by a double-labelling: membrane CD3 and intracellular interferon (IFN)- $\gamma$ . The data were analysed using CellQuestPro<sup>®</sup> software. Tryptophan and kynurenine levels were determined by enzyme-linked immunoassay (ELISA) (ImmuSmol (BD Biosciences, San Jose, USA)) in order to calculate kynurenine/tryptophan ratios.

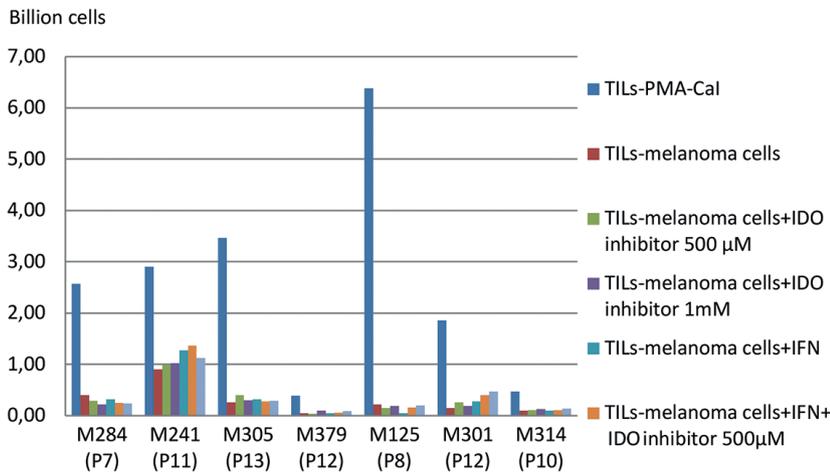
### RESULTS

In the basal state (without INF- $\gamma$ ), IDO1 expression was shown for 5 melanoma cell-lines (**Fig. 1a**). MHC-I was expressed by all the cell-lines. MHC-II was expressed by only 4 cell-lines. No expression of PD-L1 was noted in any of the 7 cell-lines. Intracytoplasmic expression of IFN- $\gamma$  by TILs was very low in the basal state. Adding the IDO inhibitor decreased the expression of IDO1 in a dose-dependent manner (**Fig. 1a**). For both MHC-I and MHC-II, IDO inhibitor increased their expression in a dose-dependent manner (**Fig. 1b, c**). No effect on the cytotoxic activity of TILs was found by adding IDO inhibitor (**Fig. 2**). The absence of expression of IDO1 by 2 melanoma cell-lines is in agreement with a recent paper showing that not all melanoma metastases express IDO (11).

In the inflammatory state (induced by INF- $\gamma$ ), IDO1 was expressed in all melanoma cell-lines: 2 melanoma cell-lines not expressing IDO1 in the basal state, and increased in the other 5 cell-lines in a dose-dependent manner (**Fig.**



**Fig. 1.** (a) Kynurenine/tryptophan ratios. (b) Mean fluorescence intensity (MFI) for MHC-I expression. (c) MFI for MHC-II expression. (d) Percentage of positive cells for PD-L1: results expressed as median, green = basal state, red = inflammatory state.



**Fig. 2. Intracytoplasmic expression of interferon gamma (IFN- $\gamma$ ) by tumour-infiltrating lymphocytes (TILs), BRAF V600E cell-lines and wild-type cell-lines; absolute number expressed in billion cells.** PMA: phorbol myristate acetate; CaI: calcium ionophore RAF V600E cell-lines (4): M284, M2421, M305 and M379 wild-type cell-lines (3): M125, M301 and M314.

1a). PD-L1 expression was induced in the 7 melanoma cell-lines (Fig. 1d). MHC-I and MHC-II were expressed by all the cell-lines after IFN- $\gamma$  induction (Fig. 1b, c).

Adding IDO inhibitor decreased expression of IDO1 and PD-L1 in a dose-dependent manner. As in the basal state, MHC-I and MHC-II expression was increased in the presence of IDO inhibitor in a dose-dependent manner. IDO inhibitor in the inflammatory state increased the reactivity of TILs in 2 cell-lines; 1 in a dose-dependent manner (M301, M125) (Fig. 2).

## DISCUSSION

The results of the current study identify 3 main targets of the IDO inhibitor implicated in lymphocyte-tumour cell interactions. The first 2 targets are MHC-I and MHC-II, whose expression is increased in both basal and inflammatory states. The third target is PD-L1, a ligand of PD-1, whose expression is decreased in the inflammatory state (not expressed at basal state). It has been confirmed that IDO inhibitor stimulates tryptophan degradation activity in a dose-dependent manner (12).

This study also demonstrated that IDO inhibitor can increase the cytotoxic activity of TILs in the inflammatory state, but for only 2 cell-lines. Given that the 2 cell-lines have the same expression profile for CMH-I, CMH-II and PD-L1 than the other 5 cell-lines, this result suggests that mechanisms other than the modulation of MHC-I, MHC-II or PD-L1 by melanoma cells are implicated for increasing the cytotoxicity of TILs by an, as-yet unidentified, IDO inhibitor. These results appear to be consistent with recent clinical trials with IDO inhibitors, in which no benefit was demonstrated, and increase interest in a strategy to define the subgroup of patients who might benefit clinically from this molecule (3).

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*The authors have no conflicts of interest to declare.*

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