University of Genoa

School of Medical and Pharmaceutical Sciences

Master's Degree in

MEDICAL AND PHARMACEUTICAL BIOTECHNOLOGY

Immunoregulatory role of IL-27 in human malignant pleural mesothelioma

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Summary

Background Malignant pleural mesothelioma (MPM) is a rare aggressive tumor with poor prognosis and limited therapeutic approaches. IL-27 is a cytokine produced by myelomonocytic cells in the tumor microenvironment and shares many biological functions with IFN-γ. Indeed, IL-27, as well as IFN-γ, shows a dual role in cancer immune response, since it may exert antitumor functions, as HLA class I upregulation, but also promote the expression of immune-suppressive molecules as IDO and PD-L1 in different tumor models. Moreover, IL-27 is related to IL-6 cytokine, which can interfere with IL-27-mediated signals and carries out pro-tumor functions in mesothelioma microenvironment.

Aim Since there is no information regarding IL-27 in mesothelioma, here it was addressed the role of IL-27 in the immune-response against MPM. The effects of IL-27 were examined *in vitro* on mesothelioma cell lines and then its concentration and its correlation with patients' overall survival were assessed *in vivo* in MPM pleural exudates. In all the analyses, IL-27 activity was compared to that of IFN-γ, as our gold standard of activity, and of IL-6, an inflammatory cytokine and relevant component of mesothelioma secretome.

Methods The human MPM cell lines MPP89, MSTO and IST-MES1 were treated with IL-27, IFN-γ, IL-6 or the soluble IL-6R/IL-6 chimeric protein (hIL-6), at different time points, and then tested for surface molecule expression by immunofluorescence and FACS analysis, for specific mRNA expression by qRT-PCR and for STATs protein phosphorylation by Western blot. Cytokines in pleural fluids were quantified by Milliplex immunoassay and High Sensitivity ELISA. Kaplan-Meier method was used to calculate survival functions and log rank test to compare survival distributions. Student's T test was performed for the statistical analysis of experimental replicates.

Results IL-27, as well as IFN-γ, upregulated the surface expression of HLA class I molecules, which favor cytotoxic T cell response, but it also induced the expression of the immune-suppressive PD-L1 molecule on MPM cell membrane. On the contrary, IL-6 and hIL-6 were ineffective in this respect. At the mRNA level, IL-27 and IFN-γ were able to induce the expression of PD-L1, IDO1 and GAL9 in all three MPM cell lines tested, whereas hIL-6 increased PD-L1 mRNA levels only in IST-MES1 cells and IDO1 mRNA levels only is MSTO cell line. Western blot analysis revealed that, also in MPM cells, IL-27 and

IFN-γ signaled mainly through STAT1 and, to a lesser extent, STAT3 phosphorylation, while hIL-6 induced a major phosphorylation of STAT3 and a milder one of STAT1. Although TCGA-MESO dataset analysis did not show any correlation between *IL27A*, *EBI3*, *IL6* or *IFNG* gene expression and overall survival, quantification of cytokines in MPM pleural fluids revealed that IL-27 and IL-6 accumulate *in vivo*. Therefore, it was tested their possible correlation with patients' overall survival. IL-6 resulted negatively associated with survival in the entire cohort of MPM pleural exudates, whereas IL-27 significantly correlated with survival considering only the samples of epithelioid tumor subtype.

Conclusions Overall, these data suggest that IL-27 might play a role in MPM immune-resistance, since it up-regulates molecules involved in immune-suppressive circuits and it's associated with worse survival. However, further analyses on a larger and more homogeneous MPM patients' cohort will be required to confirm these results.

1 Introduction

1.1 Malignant pleural mesothelioma: incidence and prognosis

Malignant mesothelioma is a rare and aggressive tumor developing from the mesothelial cells of serous cavities (pleura, peritoneum, pericardium and vaginal tunic of the testicle). In particular, malignant pleural mesothelioma (MPM) represents the 80% of all malignant mesotheliomas and arises from the inner lining of pleural membrane, the visceral pleura (Figure 1). It is usually associated with asbestos fibers exposure, with a latency period of about 30-50 years between exposure and diagnosis (Peto et al., 1999).

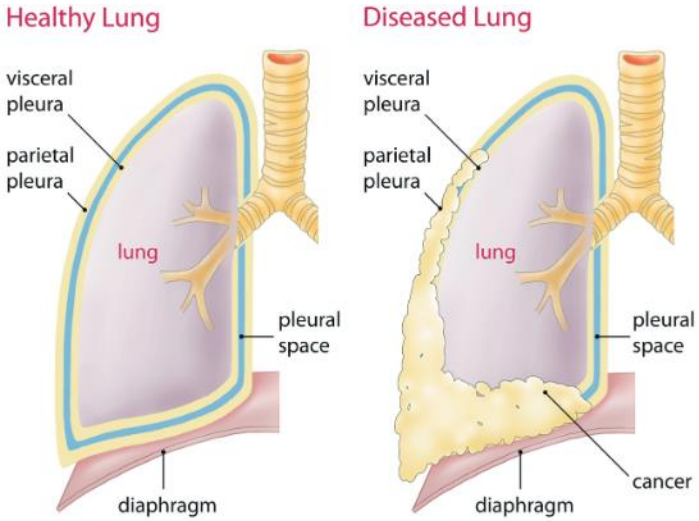

Figure 1 Normal and diseased pleura (Opitz I., 2014)

The annual incidence in Europe is about 20 cases per million people, while in Italy it reaches 3.26 and 0.87 cases per 100.000 inhabitants for males and females respectively (Alessandro Marinaccio et al., 2018; Cavone et al., 2019). Since asbestos was extensively used until 1970s and this malignancy has such a long latency period, the incidence has constantly increased in the last twenty years in the industrialized countries and is expected to peak around 2020–2025. Unfortunately, there are still countries (such as India, Brazil and Russia) in which asbestos usage isn't regulated yet, therefore an epidemic of asbestos-related diseases can be expected in the next decades (Bibby et al., 2016; Kameda et al., 2014). Moreover, there's a substantial difference in the risk fraction, attributable to occupational asbestos exposure, between men (>80%) and women (<40%), with women having a more favorable prognosis (A. Scherpereel et al., 2010).

Prognosis of MPM is still poor and the median overall survival without treatment ranges from 8 to 14 months since diagnosis, reaching about 17 to 25 months in case of chemotherapy followed by surgery (Bibby et al., 2016; Opitz et al., 2015).

1.2 Histological subtypes and pathogenesis of malignant pleural mesothelioma

There are four main histological subtypes of MPM, epithelioid (80-85%), sarcomatoid (10%), biphasic (10-15%) and desmoplastic (<2%) (Figure 2).

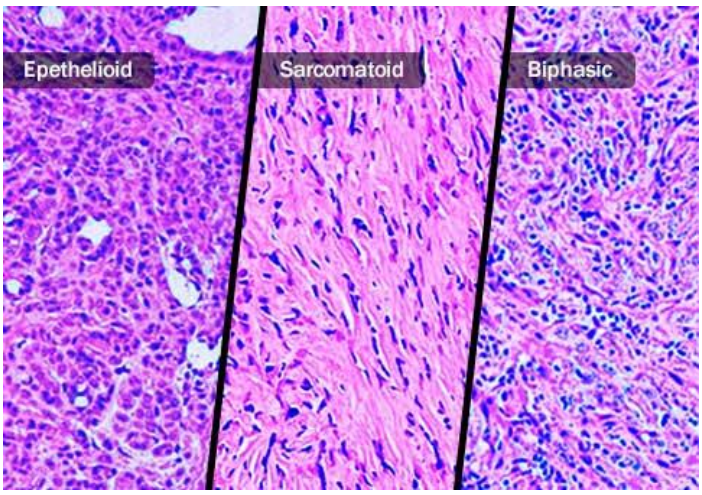

Figure 2 MPM histological subtypes (https://www.webmd.com/cancer/ss/slideshow-malignant-mesothelioma)

The epithelioid type is made of globular cells with large cytoplasm and uniform nuclei, forming solid aggregates, and its stroma is fibrous, dense and hypocellular. The sarcomatoid variant is characterized by long tapered cells organized in short fascicles that infiltrate soft tissues of pleural and pulmonary parenchyma and it can show relevant areas of tumor necrosis. The desmoplastic histotype is the most difficult to diagnose, indeed it shows mild proliferation of spindle-shaped cells arranged in a disordered fashion within a collagen stroma. Finally, the biphasic subtype has both the epithelioid and the sarcomatoid component (Scagliotti G. V. et al., 2016).

As most asbestos exposure is work-related, mesothelioma is classified as an occupational disease. However, in the last decade also end-users handling asbestos materials, people living in areas of the world where asbestos is a geological component of the soil and households of asbestos workers have developed pleural mesothelioma (Maule et al., 2007; A. Scherpereel et al., 2010). In addition, a dose-effect relationship between asbestos exposure and tumor development has been demonstrated, but it is difficult to define a threshold of cumulative exposure below which there is no increased risk of tumor growth (Maule et al., 2007; A. Scherpereel et al., 2010).

Asbestos is a natural silicate mineral that can be classified into two structural groups: the chrysotile, also called white asbestos, with curly fibers, and the amphiboles, having sharp fibers and divided into further subtypes. The risk of developing MPM depends also on the type of fiber: generally, chrysotile is less persistent in the lungs than amphiboles, the latter having a higher pleural carcinogenic potency compared to chrysotile fibers (A. Scherpereel et al., 2010; Wiggins et al., 2007).

When inhaled, asbestos fibers migrate towards the pleural space, where they interact with mesothelial cells and give rise to a chronic inflammatory response (reviewed in Bibby et al., 2016; Cavone et al., 2019).

Mesothelial cells in contact with asbestos fibers secrete CCL2 chemokine attracting macrophages, which produce high quantities of reactive oxygen and nitrogen species (ROS and RNS) trying to phagocytize fibers (Sekido, 2013). Furthermore, asbestos-exposed mesothelial cells release cytokines such as TGF-β, PDGF and VEGF (Figure 3), but also High Mobility Group Box 1 (HMGB1) protein, a damage associated molecular pattern (DAMP) that induces macrophages to produce the pro-inflammatory cytokines TNF-α and IL-1β (Figure 3) (Chu et al., 2019; Sekido, 2013; Yang et al., 2010).

Moreover, asbestos fibers themselves catalyze free radicals production, penetrate within mesothelial cells, interfering with the mitotic process and causing DNA mutations and chromosomal aberrations, and induce the phosphorylation of different MAPKs, thus increasing proto-oncogenes expression (Figure 3) (Chu et al., 2019; Robinson & Lake, 2005). An example is the constitutive upregulation of two major cell signaling cascades as Raf-MEK-ERK and PI3K-AKT pathways, which are critical for proliferation and/or survival of cells (Sekido, 2013). All these reactions due to asbestos fibers persistence contribute to create a chronic inflammatory microenvironment suitable for tumor transformation and growth.

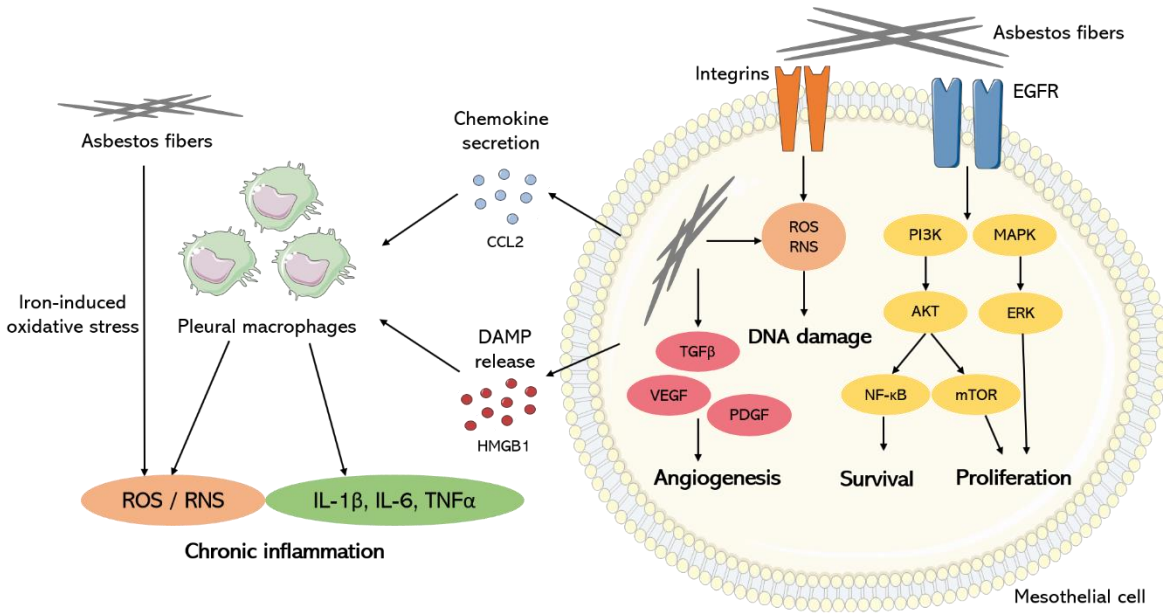

Figure 3 Asbestos-induced chronic inflammation and tumor transformation.

Looking at the mutational burden of MPM, this is really poor, with less than 25 mutations per tumor, thus limiting the potential to develop effective targeted therapies. However, during a genetic profiling of MPM, some common mutations have been found mainly in tumor suppressor genes, such as p14, p16, NF2 and BAP-1(Figure 4) (Guo et al., 2015). BAP-1 is a deubiquitinating enzyme implicated in DNA damage repair, cell cycle control, chromatin modification and programmed cell death. Its mutations are found in about 50% of MPM tumors and heterozygosity for BAP1 alterations is associated with an increased risk of tumor development, therefore BAP1 is a potential marker of MPM susceptibility (Guo et al., 2015; Neviere et al., 2019).

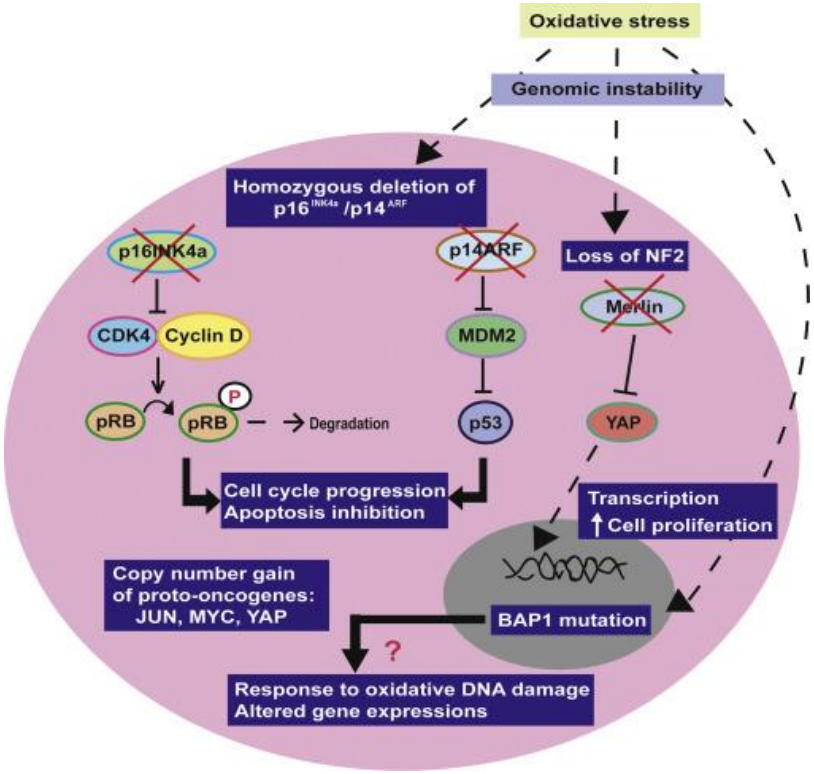

Figure 4 Common mutations in MPM (Chew S.H., Toyokuni S., 2015)

1.3 MPM microenvironment

In the context of MPM, it is essential to focus on the tumor microenvironment (Figure 5), which is involved in tumor development and resistance to therapies.

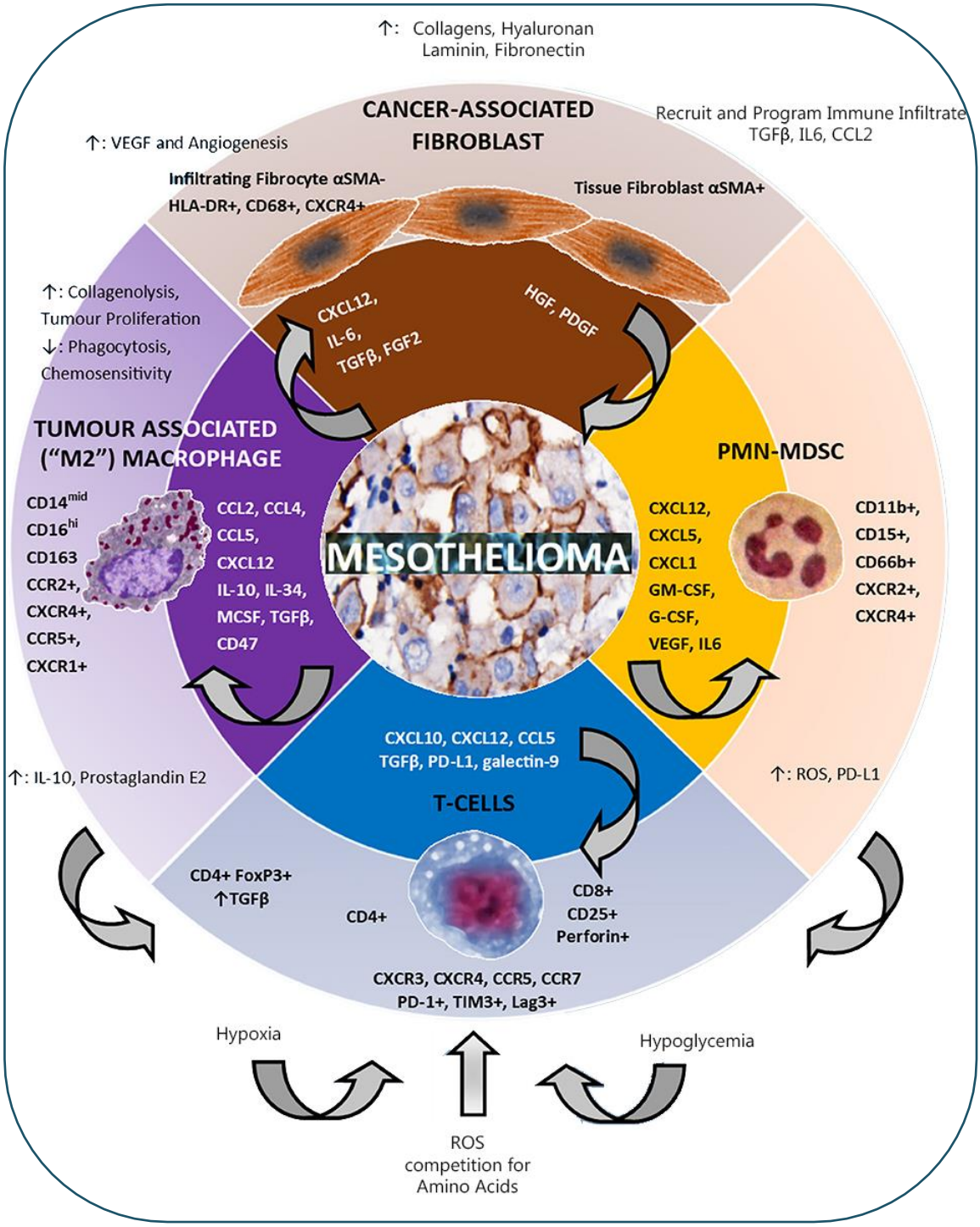

Figure 5 MPM microenvironment (adapted from Chu et al., 2019)

First of all, the extracellular matrix (ECM) plays a pivotal role in sustaining neoplastic cell growth and invasion, indeed many genes coding for ECM components (such as laminin, fibronectin and type IV collagen) are overexpressed in mesothelioma cells, as well as

metalloproteases (MMP) allowing the remodeling of the ECM for the invasion of other tissues (reviewed in Chu et al., 2019). Another important condition of MPM, and found in many other tumor types, is hypoxia, which can affect tumor growth favoring stemness, resistance to chemotherapy, epithelial to mesenchymal transition, migration and phenotypic changes, like the increased expression of Hypoxia-Inducible Factor 1α (HIF1α) (Kim et al., 2018). In particular, this last factor upregulates CXCL12 expression, thus recruiting further immune cells at the tumor site, but also stimulates the expression of VEGF, promoting angiogenesis (Bristow & Hill, 2008), and of PD-L1 on tumor cells.

For what concerns the cellular immune infiltrate of MPM microenvironment, 26-42% is made up of macrophages (Lievense et al., 2016), that are largely attracted by CCL2 and CXCL12 chemokines secreted by neoplastic cells (Chéné et al., 2016). Many Tumor Associated Macrophages (TAMs) develop an immunosuppressive M2 phenotype thanks to components of mesothelioma secretome, which include GM-CSF, M-CSF, IL-10 and TGF-β (Chéné et al., 2016; DeLong et al., 2005), thus acquiring decreased phagocytic capacity, increased IL-6, IL-10, TGF-β and IDO (indoleamine 2,3-dioxygenase) production, high collagenolytic activity and prostaglandin E2 (PGE2) production (Izzi et al., 2009).

CD3+ T lymphocytes, either CD4+ or CD8+, constitute the 20-42% of the immune cell infiltrate (Awad et al., 2016). Although high levels of CD8+ tumor-infiltrating lymphocytes (TILs) were associated with better prognosis and improved survival (Yamada et al., 2010) and despite CD8+ T cells expressing high levels of CD25 (typical sign of activation) and great perforin content were detected in pleural effusions (DeLong et al., 2005), TILs also show phenotypic markers of exhaustion such as PD-1, TIM-3 and LAG-3, which are indeed negative immune regulators (Marcq et al., 2017). As a matter of fact, the mesothelioma microenvironment contains high percentages of immune cell infiltrate and MPM expresses neoepitopes triggering T cells activation (Mansfield et al., 2019). However, MPM cells also express immune-regulatory ligands such as PD-L1 and galectin-9 (GAL-9), specific for the inhibitory receptor PD-1 and TIM-3 respectively, that are expressed on TILs and block the immune response (Bertino et al., 2019; Marcq et al., 2017).

Furthermore, also regulatory T cells are crucial players in the formation of a pro-tumor microenvironment, indeed they can be found in pleural effusions of MPM patients, even

if in lower amounts on respect to other solid tumors (DeLong et al., 2005). T-reg cells are CD4+ CD25+ T cells, whose development is induced primarily by TGF-β and COX2/PGE2 which activate the expression of FOXP3 transcription factor through STAT5 signaling (Sharma et al., 2005). Indeed, TGF-β and PGE2 are produced by cancer cells and cancer-associated cells (M2 macrophages, MDSCs, cancer-associated fibroblasts) together with IL-6, IL-10 and IDO and further sustain T-reg cells differentiation and functions (Chang et al., 2016; L. Chen et al., 2018; Najafi et al., 2019; Vera-Lozada et al., 2018). Also, Treg cells themselves secrete TGF-β, IL-10 and IL-35 and express CTLA-4, PD-L1, TIM-3 and LAG-3 on their cells surface, thus contributing to suppress the immune response (Liu et al., 2018; Maj et al., 2017; Zappasodi et al., 2018).

Another component of the immune-infiltrate in MPM are myeloid-derived suppressor cells that represent 6-9% of the immune infiltrate. Besides immune-suppressive cytokines such as IL-6, IL-10 and TGF-β, whose secretion is induced by HIF1α, these cells also produce ROS, blocking T cells proliferation and cytokine secretion (Chu et al., 2019; Khanna et al., 2018; Noman et al., 2014).

Finally, also cancer-associated fibroblasts contribute to tumor growth, thanks to the secretion of components of the extracellular matrix and of metalloproteases that model it (X. Chen & Song, 2019). Furthermore, they respond to IL-6, TGF-β and FGF2 produced by mesothelioma cells by proliferating and secreting HGF, PDGF-A, IL-6, TGF-β and VEGF (X. Chen & Song, 2019; Hegmans et al., 2006; Q. Li et al., 2011).

In summary, malignant pleural mesothelioma has a strongly immune suppressive microenvironment that is largely modeled by neoplastic cells themselves.

1.4 Therapeutic approaches in MPM

The management of malignant pleural mesothelioma is difficult, primarily because of the late diagnosis in an already advanced stage, due to non-specific symptoms and to technical issues. Moreover, pleural mesotheliomas show morphological and molecular differences both within the same tumor, in space and time, and between patients. As a consequence of the inter-tumor heterogeneity, specific therapeutic approaches are not suitable for all the patients, who have to be selected according to reliable biomarkers, that are often difficult to determine (Oehl et al., 2018). On the other side, the polyclonal origin of MPM and its long latency suggest an underlying genetic variability within the

same tumor, which, together with the selective environmental pressure, leads to therapy resistance (Comertpay et al., 2014; Junttila & de Sauvage, 2013).

1.4.1 Surgery and chemotherapy

Two main therapeutic strategies are recommended in case of MPM: surgery and palliative chemotherapy (Arnaud Scherpereel et al., 2018).

Surgery is usually recommended in combination with chemotherapy or radiotherapy or both (Andujar et al., 2016). There are more radical surgical approaches, as extrapleural pneumonectomy, or more conservative ones, as extended pleurectomy with decortication and partial pleurectomy. Unfortunately, not all patients can undergo surgery, indeed they have to be selected according to their performance status, the tumor stage and the histological tumor type (Bibby et al., 2016).

Chemotherapy is the only therapeutical strategy that has been shown to improve survival of MPM patients. To date, the first-line treatment combines cisplatin with pemetrexed, a third-generation antimetabolite inhibiting the folate cycle (van Meerbeeck et al., 2005).

Radiotherapy in mainly used to relieve symptoms or during the multimodal therapeutical setting (Bibby et al., 2016).

1.4.2 Targeted therapies

Considered the importance of the angiogenic process during tumorigenesis and seen the high expression of angiogenic growth factors and their receptors in malignant pleural mesothelioma, several drugs with anti-angiogenic activity have been tested for MPM therapy (Figure 6). Among them, bevacizumab, a monoclonal antibody directed towards VEGF, provided promising results, significantly increasing the median overall survival when associated with cisplatin and pemetrexed (Gérard Zalcman et al., 2016). For this reason, the administration of bevacizumab together with first-line chemotherapy is suggested in patients with unresectable MPM.

Additional targets are now under investigation, for example EGFR tyrosine kinase inhibitors, anti-EGFR monoclonal antibodies, inhibitors of histone deacetylases (HDAC), focal adhesion kinase (FAK) inhibitors and enhancer of zeste homolog 2 (EZH2) inhibitors (Arnaud Scherpereel et al., 2018). However, good predictive biomarkers are needed to help in the selection of suitable candidates for this kind of therapies and to explain potential selection advantages (Gérard Zalcman et al., 2016).

1.4.3 Immunotherapy

Since the immune system is extensively involved in the development and progression of MPM, immunotherapy could represent a suitable therapeutic approach able to restore the immune response against transformed mesothelial cells (Figure 6). The regulation of the immune system strongly depends on intracellular pathways inhibiting the immune response, called immune checkpoints, among which we find PD-L1 and CTLA-4 molecules. CTLA-4 is expressed on primed T cells and interacts with CD80 and CD86 ligands present on antigen-presenting cells (APC), thus preventing their interaction with the CD28 co-stimulatory molecule (reviewed in (van Coillie et al., 2020)). In this way CTLA-4 blocks a second signal that, together with TCR signaling, is essential for full activation of T lymphocytes.

On the other hand, PD-L1 is a surface protein expressed by different cell types, neoplastic or not, whose interaction with PD-1 receptor on T lymphocytes suppresses the immune response, blocking activation and cytotoxicity of T cells at the tumor site. Indeed, PD-1, upon ligand engagement, recruits SHIP family tyrosine phosphatases that block activation and cytotoxicity of T cells at the tumor site (reviewed in (Ai et al., 2020)). Considering the efficacy of the immune checkpoint blockers in the treatment of melanoma and non-small cell lung cancer, monoclonal antibodies targeting CTLA-4 and PD-1/PD-L1 have been tested also for MPM therapy. Results of tremelimumab (anti-CTLA-4 mAb) administered after cisplatin chemotherapy were promising, but when patients were given tremelimumab as second or third-line therapy there wasn't any beneficial effect compared to placebo (Calabrò et al., 2013; Maio et al., 2017). On the contrary, several phase I and II clinical trials evaluating the response of MPM patients to anti-PD-1 and anti-PD-L1 monoclonal antibodies have shown encouraging results, with an objective response rate of 20-24% and a disease control rate of about 50-77% (Arnaud Scherpereel et al., 2018). The association of nivolumab (anti-PD-1 mAb) and ipilimumab (anti-CTLA-4 mAb) in MPM treatment was also evaluated, resulting in a median overall survival slightly longer for the nivolumab plus ipilimumab group (Arnaud Scherpereel et al., 2017; G. Zalcman et al., 2019), but further comparative and randomized studies are needed to assess whether the combination is better than the administration of single-agent therapy. Moreover, a critical point is to predict which patients are most likely to respond to anti-PD-1/PD-L1 immunotherapy. Indeed, in

several studies PD-L1 expression correlated with the response to PD-1/PD-L1 inhibitors (de Gooijer et al., 2020; Disselhorst et al., 2019; Nowak et al., 2018; Arnaud Scherpereel et al., 2019), but there are also results showing that PD-L1 negative tumors respond to the combination therapy similarly to PD-L1 positive ones (Arnaud Scherpereel et al., 2019). For this reason, other factors that could represent suitable and reliable biomarkers are still under investigation.

Further immunotherapy approaches rely on mesothelin, a glycoprotein physiologically expressed on mesothelial cell surface and over-expressed in MPM, that has been exploited in several ways, for example by designing monoclonal antibodies, immunotoxins, CAR-T cells or vaccines targeting mesothelin, in order to drive the immune response against tumor cells (Arnaud Scherpereel et al., 2018). Finally, recent studies evaluating the potential benefits of autologous dendritic cell vaccination provided encouraging results, demonstrating sustained tumor control and increase of the median overall survival (de Gooijer et al., 2020).

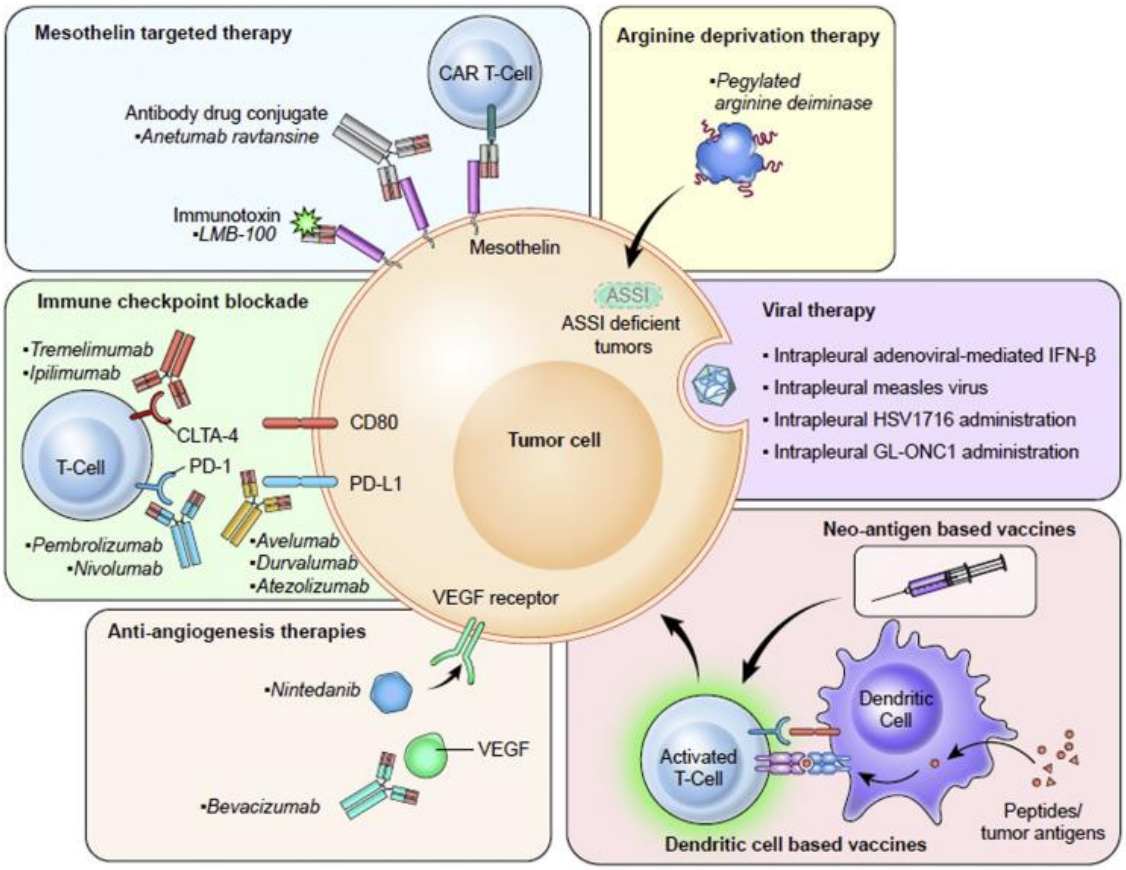

Figure 6 Innovative MPM therapeutic approaches currently in clinical trials (Mutti et al., 2018)

1.5 Background

Malignant pleural mesothelioma is characterized by a large immune infiltrate, composed of exhausted CD8+ T cells and tumor associated cells such as M2 macrophages, MDSCs and Treg cells, which secrete immune suppressive cytokines and express immune checkpoint molecules, thus promoting tumor development. For this reason, it is essential to deepen the knowledge about the tumor microenvironment, in order to conceive new therapeutic approaches effective in reactivating the immune response.

1.5.1 IL-27 biological activity

IL-27 is a heterodimeric cytokine belonging to IL-12 family, composed of p28 (IL-27A) and EBV-induced gene 3 (EBI3) chains (Figure 7). Its receptor is made up of WSX-1/IL-27Rα, which confers IL-27 signal specificity, and of gp130, shared with the other cytokines of IL-12 and IL-6 family and ubiquitously expressed (reviewed in Fabbi et al., 2017). The binding of IL-27 to its receptor triggers the JAK/STAT signaling pathway, involving mainly STAT1 and STAT3 phosphorylation, in different proportions according to the cell type (Hibbert et al., 2003). IL-27 is mainly produced by myelomonocytic cells, such as monocytes, macrophages and dendritic cells, in response to pro-inflammatory stimuli triggered by Toll-like receptors, TNF receptors and IFN-γ signaling (Fabbi et al., 2017; Molle et al., 2007). IL-27 possesses immune-enhancing activities, mediating CD4+ T cells clonal expansion and inducing the expression of Tbet transcription factor and IL-12 receptor, thus favoring Th1 polarization (Lucas et al., 2003; Pflanz et al., 2002). In addition, it stimulates CD8+ T cells proliferation and expression of IFN-γ, perforin and granzyme B, supporting CTLs cytotoxic activity (Schneider et al., 2011). In this sense, IL-27 contributes to the activation of an antitumor immune response acting on immune cells, but it also directly influences tumor cells, decreasing their survival, proliferation, angiogenic and invasive properties (reviewed in Fabbi et al., 2017; M. S. Li et al., 2015; Yoshimoto et al., 2008). Furthermore, previous studies on ovarian cancer and small cell lung cancer (SCLC) cell lines revealed that IL-27 enhances the expression of HLA class I molecules (Carbotti

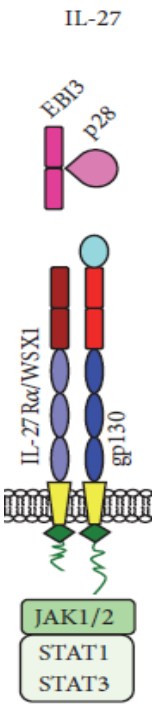

Figure 7 IL-27 cytokine, receptor and signaling JAK/STAT (adapted from Fabbi et al., 2017)

et al., 2017; Petretto et al., 2016), which play a pivotal role in triggering the cytotoxic activity of CTLs and so in tumor elimination.

However, IL-27 shows also immune-regulatory and anti-inflammatory functions, cooperating in the physiological need to control the immune response. For example, it induces CD4+ T cells to produce IL-10 and to differentiate into PD-L1-positive Treg cells (Hirahara et al., 2012). Indeed, it has been recently demonstrated that IL-27 is able to up-regulate PD-L1 expression in SCLC (Carbotti et al., 2017) and IL-18BP, PD-L1 and IDO expression in ovarian cancer cell lines (Carbotti et al., 2015). Therefore, IL-27 shows a dual role in controlling tumor proliferation, contributing to the adaptive immune resistance. Moreover, the quantification of IL-27 in ascites from ovarian cancer patients revealed that this cytokine is present in relevant concentrations (about 2 ng/ml) (Carbotti et al., 2020). The results obtained by studying epithelial ovarian cancer models can be particularly indicative since, in some respects, it shares common features with mesothelioma: indeed, it has an epithelial origin too, it develops inside the peritoneal cavity, primarily metastasizing on mesothelial tissues, and induces ascites formation (Lengyel, 2010). The latter is a fluid that accumulates within the peritoneal cavity and that can be simply withdrawn and analyzed, similarly to pleural exudate from pleural mesothelioma.

A previous proteomics study in ovarian cancer cell lines revealed that, thanks to the common activation of STAT1 pathway, IL-27 and IFN-γ induced the expression of multiple overlapping proteins involved in IFN-γ signaling and activities, including proteins responsible for the anti-proliferative and pro-apoptotic effects of IFN-γ (Petretto et al., 2016). Indeed, IL-27, as well as IFN-γ, was able to inhibit the proliferation and favor the apoptotic cell death of ovarian cancer cell lines (Petretto et al., 2016). Interferon-γ (IFN-γ) is known to have antitumor properties by promoting M1 macrophages polarization, Th1 differentiation and the cytotoxic activity of NK cells and CD8+ T lymphocytes. However, this cytokine, similar to IL-27, is also responsible for the induction of IDO and PD-L1 expression on tumor cells, which are physiologically involved in the regulation of the immune response (Abiko et al., 2015; Carbotti et al., 2015; Ikeda et al., 2002; Spranger et al., 2013). In this respect, it has been recently demonstrated that IFN-γ enhances the expression of PD-L1 also in mesothelioma cell lines (Pistillo et al., 2020), thus contributing to the adaptive immune resistance process. In this context,

we recently measured IFN-γ concentration in the pleural fluids of a training cohort of 72 MPM patients (submitted article, Dozin et al., 2021). IFN-γ levels were low, showing a large variation (0-85 pg/ml) around a median value of 13.84 pg/ml. Nevertheless, survival analysis revealed that the median survival was 14.7 months in patients with high levels of IFN-γ and 12.8 months in patients with low levels (log rank p value=0.050) (figure 8), suggesting that IFN-γ was significantly associated with survival and that high IFN-γ concentrations might be a positive prognostic marker.

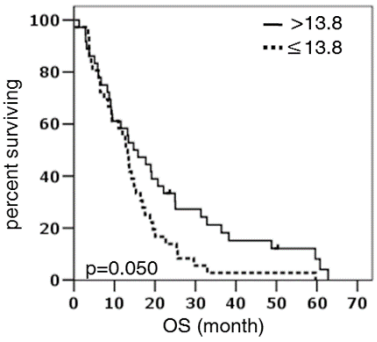

Figure 8 IFN-γ concentration in pleural exudates correlates with overall survival (submitted article, Dozin et al., 2021).

1.5.2 IL-6 and its role in MPM

IL-6 is a monomeric cytokine which signals using a heterodimeric receptor composed of IL-6Rα, specific for IL-6, and gp130, common to many cytokines of the same family, including IL-27. Besides classical signaling, IL-6 can also mediate trans-signaling in cells expressing only the gp130 chain, thanks to a soluble form of IL-6Rα to which the cytokine binds forming the sIL-6R/IL-6 complex (Figure 9) (Rose-John & Neurath, 2004). IL-6 is an extremely pleiotropic and pro-inflammatory cytokine, secreted by various activated immune cells and non-immune cells, essential for the acute phase inflammatory response, for the proliferation of B lymphocytes and their maturation into plasma cells, as well as for the promotion of CTLs differentiation and blockage of Treg cells formation (reviewed in Murakami et al., 2019). However, within the tumor microenvironment, IL-6 generally exerts pro-tumor functions, favoring survival and proliferation, inhibiting apoptosis and supporting angiogenesis, tumor escape and chemoresistance (Fisher et al., 2014; Hodge et al., 2005).

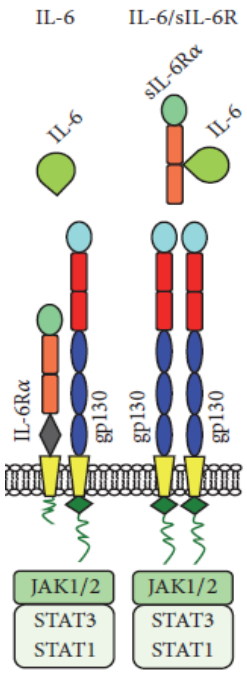

Figure 9 IL-6 cytokine, receptor and signaling JAK/STAT (adapted from Fabbi et al., 2017)

It has been observed that IL-6 plays a pivotal role in the microenvironment of MPM (reviewed in Abdul Rahim et al., 2015), since it's a relevant component of mesothelioma secretome and it accumulates in pleural exudates and sera (Hegmans et al., 2006; Nakano et al., 1998). Moreover, IL-6 shares with IL-27 the engagement of gp130, which

has been shown to be expressed in MPM cell lines (Adachi et al., 2006), and the usage of JAK/STAT signaling pathway through the activation of STAT1/STAT3 (Pflanz et al., 2004). In this respect, it was recently demonstrated that IL-6 can negatively interfere with IL-27 signaling pathway in different cancer models, likely by blocking STAT proteins phosphorylation by a SOCS3-mediated mechanism (Carbotti et al., 2017; Ginolhac et al., 2017).

2 Aim of the study

IL-27, a cytokine produced by myelomonocytic cells, carries out a biological activity largely overlapping that of IFN-γ. Indeed, it has been shown that in ovarian cancer IL-27 can trigger the same adaptive immune-resistance mechanisms induced by IFN-γ, such as the expression of IDO and PD-L1 molecules, having immune-suppressive activities. Furthermore, IL-27 is present in the microenvironment of ovarian cancer and has been measured in the ascitic fluid of the patients. However, this cytokine is not only active on ovarian cancer cells, but also on other tumor models, such as SCLC, in which it may exert an anti-tumor function. Therefore, IL-27, as IFN-γ, is able to mediate pro- or anti-tumor activities depending on the biological context. Moreover, IL-27 shares gp130 receptor chain and JAK/STAT signaling molecules with IL-6 cytokine family. IL-6 is known to perform pro-tumor functions in mesothelioma microenvironment and there are evidences suggesting that it can interfere with IL-27 signal transduction in different tumor types.

Considering these findings and since there isn't any study about IL-27 in mesothelioma, we wondered if IL-27 could play a role in the immune response against MPM, first evaluating its activity on mesothelioma cell lines *in vitro*. We tested the effects of IL-27 on cell viability and its capacity to modulate the expression of HLA-class I molecules, as well as that of molecules involved in immune-suppressive circuits, such as PD-L1, IDO1 and GAL-9. The effects of IL-27 were studied in comparison with those induced by IFN-γ, IL-6 and an IL-6/sIL-6R chimeric protein (hIL-6).

Then, in order to check if IL-27 had any relevance in tumor microenvironment also *in vivo*, we assessed its concentration in the pleural fluids of a cohort of MPM patients, together with IL-6 and IFN-γ levels, since we recently found that IFN-γ is positively

associated with patients' survival in a different cohort of MPM pleural exudates. Finally, the cytokine concentrations were related to the patients' survival data.

3 Materials and methods

3.1 Cells and treatments

Analyses were performed as described in (Carbotti et al., 2017). The human MPM cell lines MPP89, MSTO and IST-MES1 were purchased from Interlab Cell Line Collection (ICLC, Ospedale Policlinico San Martino, Genoa, Italy). Cells were grown in RPMI 1640, with 1% L-glutamine, 10% FCS, 1% Penicillin and Streptomycin (Lonza) and kept in culture at 37°C in 5% carbon dioxide. Treatments with cytokines were performed with slight differences, according to the final use of the stimulated samples. Conditions were set on the bases of preliminary titration experiments and previous reports (Carbotti et al., 2017, 2020; Petretto et al., 2016). Briefly, for immunofluorescence and QRT-PCR analyses, cells were seeded in 24-well plates in culture medium at 5×10^4 cells/well and stimulated with different cytokines: IL-27 (100 ng/ml R&D System, 2526-IL-010), IFN-γ (1000 IU/ml, PeproTech, 300–02), IL-6 (50 ng/ml R&D System 206-IL-010) or recombinant human IL-6Rα/IL-6 chimera (sIL-6R/IL-6), referred to as hyper IL-6 (hIL-6) (50 ng/ml R&D System 8954-SR-025). Treatments were carried out for 48 h.

For the analysis of tyrosine-phosphorylated STAT proteins, 1×10^5 MPM cells were incubated for 20 minutes in 0.5 ml of medium in a test tube at 37 °C with or without IL-27 (50 ng/ ml), IL-6 (20 ng/ml), sIL-6R/IL-6 (40 ng/ml), IFN-γ (1000 IU/ml). Cells were then rescued by centrifugation and immediately processed.

3.2 Immunofluorescence

For each stimulus cells were centrifuged, counted and divided into test tubes in order to have from 5 to $10x10^4$ cells for each sample. Direct immunofluorescence was performed incubating the samples with anti-PD-L1 PE or Isotype Control PE (eBioscience Bender, BMS-125983-41 and BMS-124724-41, respectively), while indirect immunofluorescence was carried out with anti-HLA class I W6/32 mAb (ATCC) and FITC-labeled goat anti-mouse (Jackson Immunoresearch, 115–096-068). Concentrations used were those indicated by the manufacturer's instructions, usually around 1µg/ml. Then the

antibodies were removed by centrifugation, after addition of serum-free medium, and cell pellet was dried and resuspended in paraformaldehyde (1% PFA). The gate was placed around cells with common physical characteristics and 10^4 gated events were acquired. Cell fluorescence was analyzed by flow cytometry with a FACScan (Becton & Dickinson) using the Cell Quest software (Beckman Coulter).

3.3 RT-PCR analysis

Cells were washed in medium without serum and antibiotics, counted and collected about $2x10^5$/sample. Total RNA was extracted by the NucleoSpin RNA kit (Macherey-Nagel, 740,955.250), based on silica membrane affinity chromatography, and reverse-transcribed to cDNA using the SuperScript III Reverse Transcriptase (Invitrogen, 18,064–071). Amplification was carried out using the iQTM SYBR® Green Supermix system (Bio-Rad Laboratories, 170–8882) by the Mastercycler® ep realplex4 instrument (Eppendorf International). In each sample were inserted cDNA, specific primers for the polymerization of the gene transcript of interest and SYBR Green Supermix (containing iTaq DNA polymerase, deoxy-nucleotides-triphosphate, MgCl2 as enzyme cofactor, TrisHCl, KCl, fluorescein and SYBR Green as a DNA intercalating fluorescent agent). Quantification of mRNAs relative to housekeeping gene was examined calculating ΔCT, representing the difference between the number of cycles required for the analyzed gene and the housekeeping gene to reach the defined threshold. Expression levels of mRNAs relative to untreated control were calculated by the ΔΔCT method, corresponding to the difference between ΔCT of the stimulated sample and ΔCT of the untreated control. Data were represented as $2^{-\Delta\Delta CT}$, expressing the fold-increase on respect to the control.

3.4 Western blot

At the end of the treatment, cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Brij97) containing 2 mM Na Orthovanadate and protease inhibitors (Roche Diagnostics, Complete Mini 04693124001), adding 100µl/10^6 cells. Sample Buffer reducing agent (Laemmli, with 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0,02% bromophenol blue, 10% β-mercaptoethanol) was added to the lysates, then samples were loaded on a 10% or 13% acrylamide gel and resolved under reducing

conditions by SDS-PAGE. Next, proteins were transferred onto a nitrocellulose membrane (Hybond C-Extra, GE Healthcare), saturated with in 5% milk/TBST and analyzed by Western blotting using the following antibodies: rabbit anti-phospho-STAT1 (pY701) and anti-STAT1 anti-sera (Cell Signaling Technology, 9167 and 9172, respectively), murine anti-phospho-STAT3 (pY705) and anti-STAT3 mAbs (BD Transduction Laboratories, 612,356 and 610,190, respectively) and murine α-tubulin mAbs (Sigma-Aldrich T6074 and A2228, respectively).

Primary antibodies were used at a concentration of 1:1000 or 1:2000, diluted in TBST (20 mM Tris-HCl pH 8, 0.9% NaCl, 0.05% Tween 20) with 0.5% milk, with the exception of anti-tubulin, used at a lower concentration (1: 20000). Incubation with primary antibodies was carried out overnight at 4°C under agitation. Then the membranes were incubated at room temperature for 1 hour under agitation with secondary antibodies (anti-mouse or anti-rabbit) diluted 1:5000 in 0.5% milk/TBST. After each incubation, membranes were washed 3 times with TBST for 10 minutes.

Proteins were then detected by ECL Prime (GE Healthcare, RPN2232), based on a chemiluminescence reaction, and visualized by a proper analysis system (MINI HD, UVITEC, Cambridge). After image acquisition, all antibodies are removed with a 20-minute incubation with Stripping Solution (Thermo Scientific).

3.5 Cytokine dosage

Levels of IL-1, IL-6, IL-10, IL-12, IL-27 and TNF-α cytokines in pleural exudates were measured by the usage of MILLIPLEX® MAP method, based on LUMINEX® xMAP® technology (MILLIPLEX® MAP Human TH17 Magnetic Bead Panel, customized HTH17MAG-8K, Merck Millipore, Darmstadt, Germany) (Figure 10). This is a bead-based multiplexed immunoassay that allows the simultaneous quantification of multiple substrates with a minimum sample volume. Fluorescent-coded magnetic beads coated each one with a specific capture antibody were incubated with the test sample. Then, biotinylated detection antibodies, specific for the analytes under investigation, and streptavidin-PE conjugate were introduced into the reaction mixture. Finally, the sample was examined by the Luminex MAGPIX® analyzer, which identifies each analyte thanks to the fluorescence of the bead and quantifies it through the reporter fluorescence. Cytokine concentrations were derived by interpolating the obtained results with the

standard curve developed at the beginning of the procedure. Samples were processed both at 1:2 and 1:10 dilution in Assay Buffer and the procedure was carried out following manufacturer's instructions.

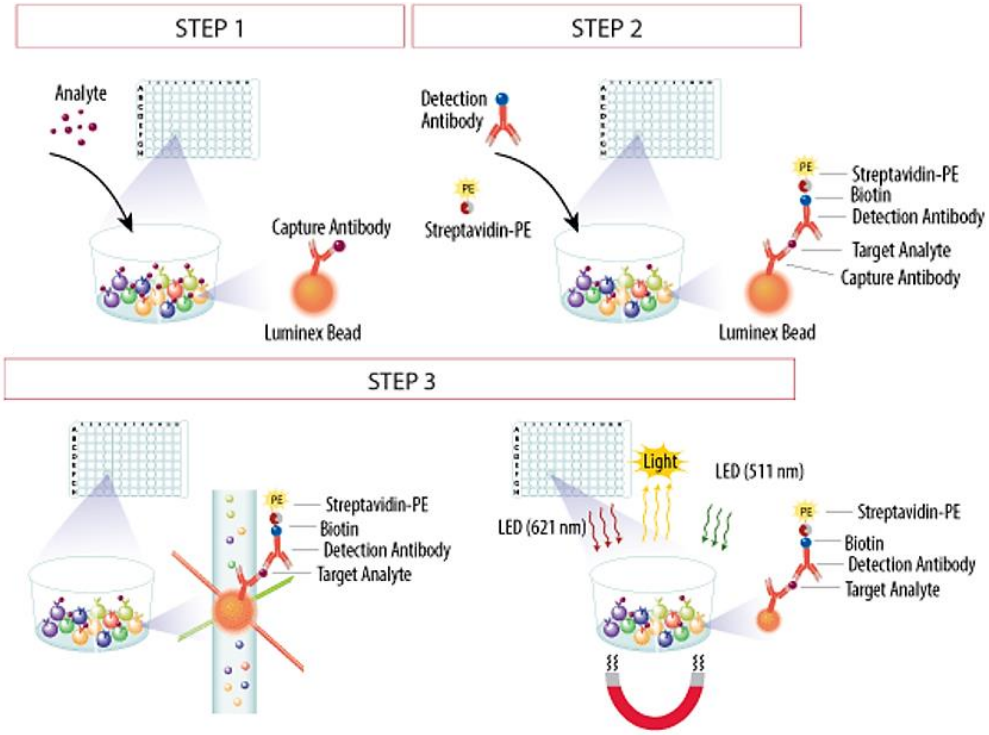

Figure 10 Schematic representation of MILLIPLEX MAP and LUMINEX xMAP technology. (https://www.rndsystems.com/resources/technical/luminex-assay-principle)

Since many samples were below the minimum detectable concentration, IFN-γ levels were evaluated by Quantikine High Sensitivity ELISA kit (R&D Systems, Inc., HSDIF0), a standard sandwich ELISA (Figure 11) with sensitivity between 0.469 pg/ml and 30 pg/ml. Quantification of the substrate was done through a colorimetric assay using TMB development solution and sulfuric acid stop solution, enabling accurate measurement of color intensity at 450 nm by a spectrophotometer.

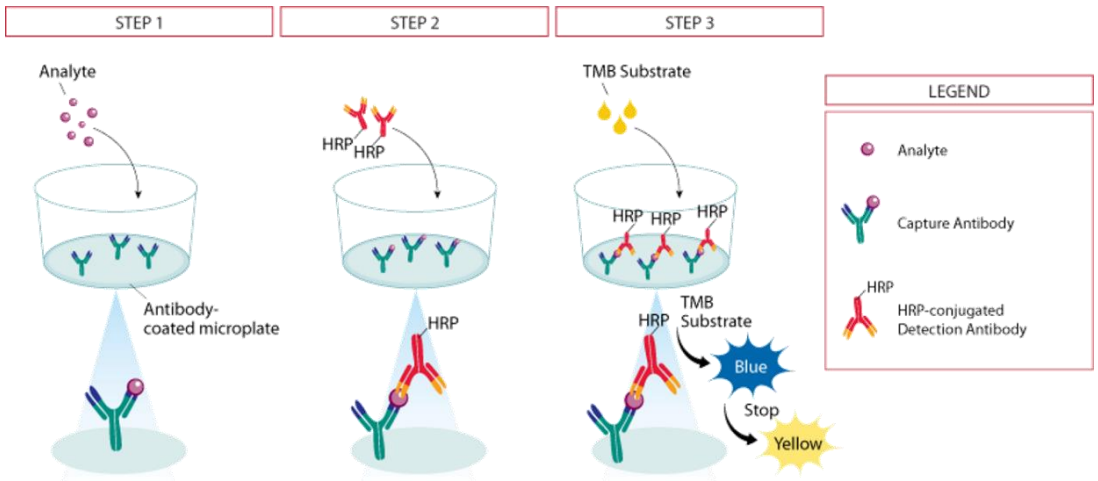

Figure 11 Schematic representation of sandwich ELISA assay (https://www.rndsystems.com/products/quantikine-colorimetric-sandwich-elisa-assay-principle)

The detection limits and sensitivity of each quantification method are reported in the tables below (Table 1).

MILLIPLEX MAP Immunoassay						
Standard curve	IFN-γ pg/ml	IL-10 pg/ml	IL-12p70 pg/ml	IL-1β pg/ml	TNF pg/ml	IL-6 pg/ml
Min	10	1	5	5	2.5	2.5
Max	40000	5000	20000	20000	10000	10000
Sensitivity	1.8	0.3	1.1	2.1	0.9	1.7

Quantikine High Sensitivity ELISA	
Standard curve	IFN-γ HS pg/ml
Min	0.469
Max	30
Sensitivity	0.025

Table 1

3.6 Patients

First analysis was performed on RNAseq expression data retrieved from the TCGA-MESO database (https://www.cancer.gov/tcga), containing 86 samples of mesothelial pleural mesothelioma. The MPM patients cohort (n=77) was obtained from the "Alessandria Biobank-Centro Raccolta Materiali Biologici", Department of Integrated Activities Research and Innovation, Azienda Ospedaliera SS. Antonio e Biagio e Cesare Arrigo, Alessandria, Italy.

3.7 Statistical analysis

The Student's T test was used for the statistical analysis of experimental replicates. Survival analysis with respect to mesothelioma gene expression was carried out on TCGA-MESO dataset by using R software (version 4.0.2 for Windows). Survival analysis on mesothelioma secretome (pleural fluids) were performed by Beatrice Dozin (Clinical Epidemiology Unit, IRCCS Ospedale Policlinico San Martino, Genoa, Italy) using SPSS software package (version 21.0 for Windows). In both cases, cytokine levels were dichotomized according to the median concentration and patients were grouped depending on whether they showed cytokine expression above or below the median value. Kaplan-Meier method was used to estimate the survival functions of the sample sets and log-rank test was performed to compare the curves. Survival time was

considered from the date of surgery to the date of the last contact or death. A value of $p<0.05$ was considered significant.

4 Results

4.1 IL-27 increases surface expression of HLA class I and PD-L1 molecules in MPM cells

Recent reports indicate that IL-27 is able to up-regulate surface HLA class-I and PD-L1 expression in different types of tumors, including ovarian cancer (Petretto et al., 2016) and small-cell lung cancer (Carbotti et al., 2017). Therefore, we may assume that IL-27 may exert a similar function in malignant pleural mesothelioma. To address this hypothesis we tested if IL-27 could mediate the same effects on a panel of three previously characterized MPM cell lines (MPP89, MSTO, IST-MES1) (Orengo et al., 1999). Cells were stimulated with IL-27 or with the related cytokine IL-6 or with the chimeric molecule hyper IL-6 (hIL-6), which mimics the biological complex of IL-6 with the soluble form of IL-6 receptor alpha (IL-6/sIL-6R). Then cells were analyzed by indirect immunofluorescence and flow cytometry using the W6/32 mAb, recognizing HLA class I heavy chains complexed with β2-microglobulin. As shown in figure 12, although HLA class I molecules were constitutively expressed on human MPM cells, IL-27 was able to further increase their expression by 2.1 to 3 folds in all the three models tested, whereas IL-6 and hIL-6 didn't show any effect.

Then, since PD-L1 is involved in the immune-suppressive circuits in the tumor microenvironment (Ai et al., 2020a), we assessed whether IL-27 could induce surface PD-L1 expression in MPM cells, as it does in other cancer cell models (Carbotti et al., 2015, 2017). We observed that IL-27, as well as IFN-γ, increased PD-L1 surface levels from 2.4 to 3.75 folds compared to the baseline (figure 13A), while IL-6 and hIL-6 were ineffective (figure 13B).

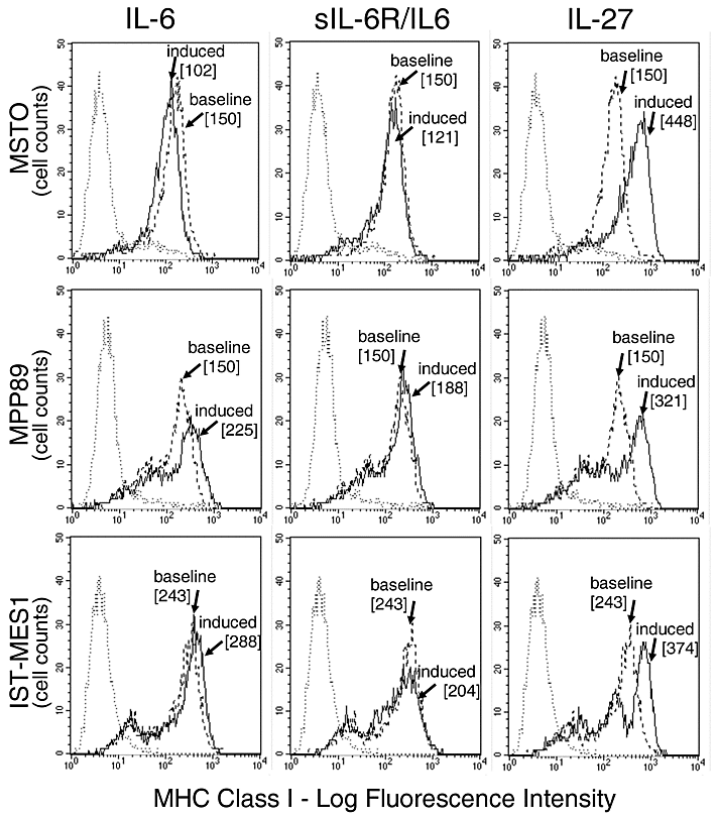

Figure 12 Effect of IL-27 on HLA class I surface expression. Flow cytometry analysis of HLA class I expression in MPM cell lines cultured with medium alone (control), IL-6, sIL-6R/IL-6 or IL-27 cytokines (treatments) for 48h. Data are indicated as Median Fluorescence Intensity (MFI), calculated as the difference between the median of anti-HLA-I mAb and the median of isotype control. One representative experiment is shown out of two with consistent data.

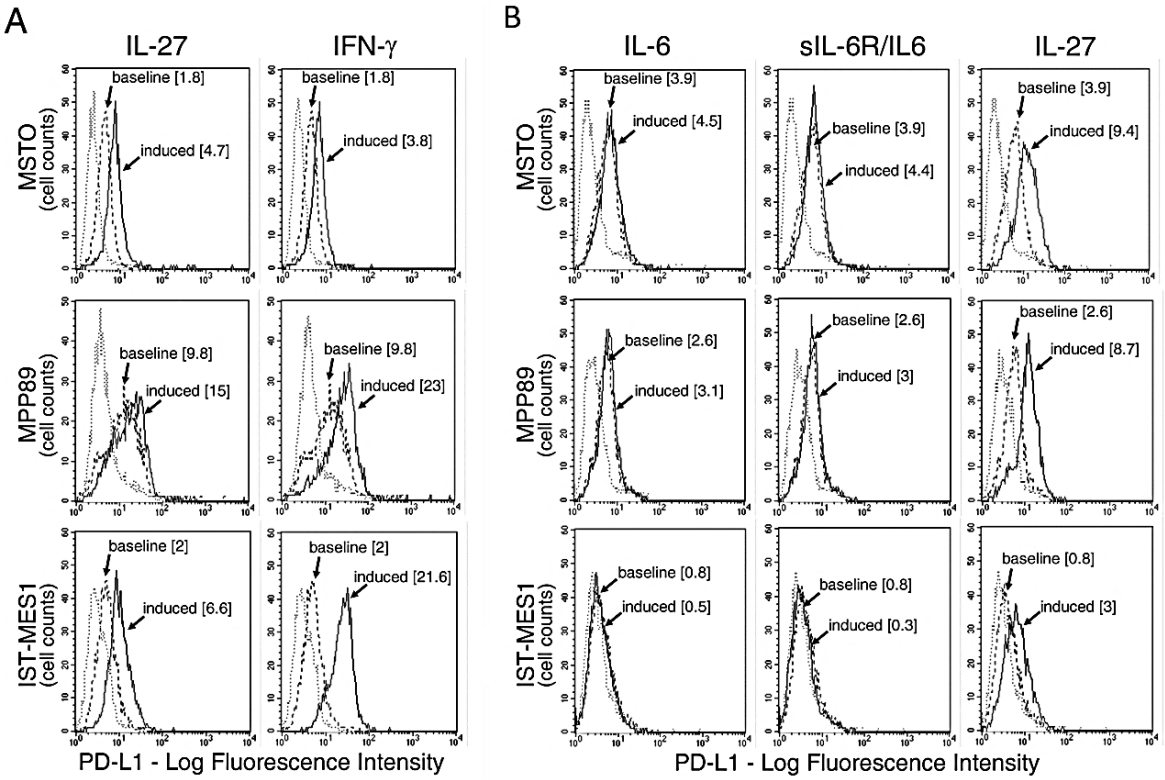

Figure 13 Effect of IL-27 on PD-L1 surface expression. Flow cytometry analysis of PD-L1 expression in MPM cell lines cultured with medium alone (control), IL-27 or IFN-γ (treatments) (**A**) and with medium, IL-6, sIL-6R/IL-6 or IL-27 (**B**) for 48h. Data are indicated as Median Fluorescence Intensity (MFI), calculated as the difference between the median of anti-PD-L1 mAb and the median of isotype control. Here we show one representative experiment out of two with consistent data.

4.2 IL-27 up-regulates the mRNA expression of immune-suppressive molecules

To confirm that the treatment with cytokines was indeed able to trigger gene expression in target cells, we analyzed by quantitative PCR the mRNA levels of several molecules in MPM cells stimulated for 48 hours with IL-27, IFN-γ, IL-6 or IL-6/sIL-6R chimeric molecule (hIL-6). The analysis of *PDL1* mRNA expression by QRT-PCR showed an 8 fold-increase with IL-27 stimulus and an even higher effect with IFN-γ treatment, with respect to the untreated control (figure 14). On the contrary, hIL-6 could slightly up-regulate *PDL1* mRNA levels only in IST-MES1 but not in the other MPM cell lines.

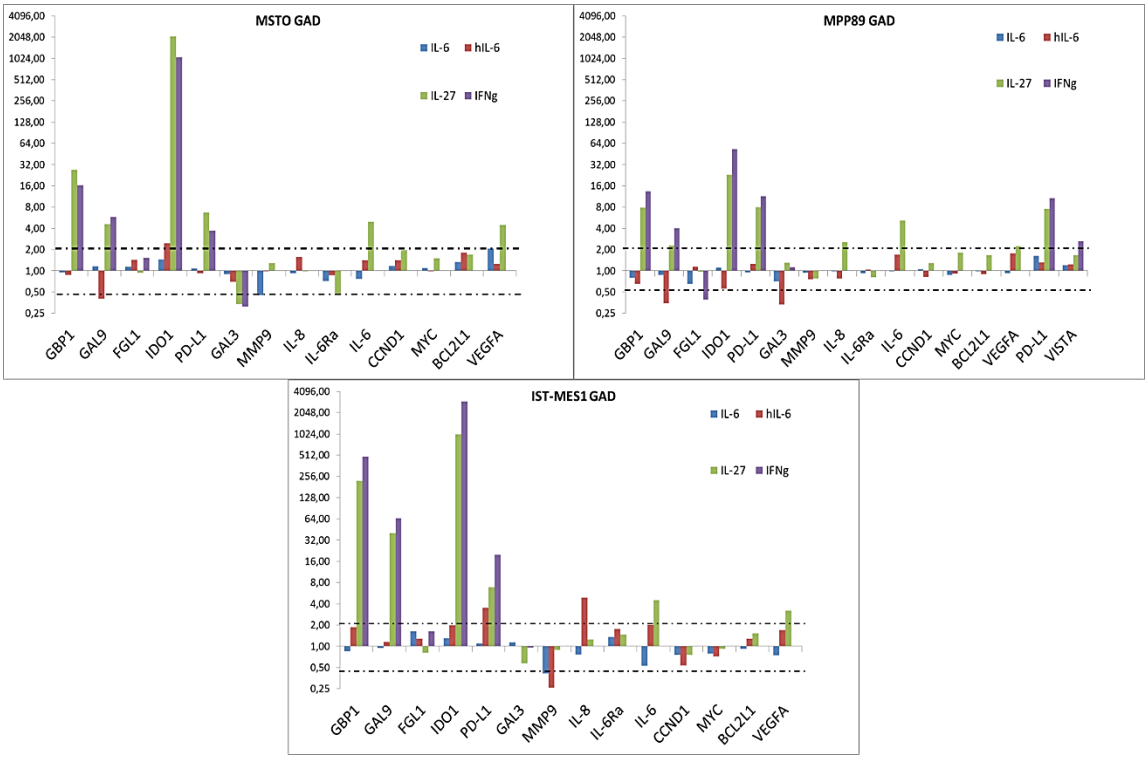

Figure 14 IL-27 increases mRNA expression of PD-L1, IDO1 and GAL-9. QRT-PCR analysis of PD-L1, IDO1, Galectins and GBP1 mRNA expression in three MPM cell lines stimulated with IL-6, hIL-6, IL-27 or IFN-γ relatively to untreated controls. Cells were cultured in the presence of medium, IL-6 (blue histograms), hIL-6 (red histograms), IL-27 (green histograms) or IFN-γ (violet histograms) for 48 h. Data were normalized to *GAD* housekeeping gene and are expressed as ΔΔCT-fold change relative to control. Each sample was analyzed in technical triplicate and SD was lower than 10%.

Besides PD-L1, we evaluated, at the mRNA level, the expression of additional molecules potentially involved in tumor progression and immune suppression, including, for example, the enzyme indoleamine 2,3-dioxygenase (IDO)-1 and galectins (GAL). In addition, we also tested the effect of IL-27 on guanylate-binding protein (GBP)-1 mRNA, since it is potently up-regulated by IFN-γ and by IL-27 in other experimental models (Carbotti et al., 2020). After IL-27 treatment, *IDO1* mRNA transcription was remarkably

induced in MSTO and IST-MES1 cells (2048 and 1024 fold-increase respectively), while MPP89 cell line showed a milder but considerable effect (24 fold-increase) (figure 14). Moreover, *LGALS9* (GAL-9) mRNA expression was enhanced by IL-27 treatment in IST-MES1 and MSTO (34 and 4 fold-increase respectively), and weakly in MPP89 cells, whereas *LGALS3* (GAL-3) gene expression was not induced. Differently, hIL-6 increased *IDO1* mRNA levels only in MSTO cells and wasn't effective in inducing *LGALS9* (GAL-9). Overall, these data indicate that IL-27 may up-regulate the expression of different immune-suppressive circuits involving PD-L1, IDO and GAL-9 in MPM cells.

4.3 IL-27 triggers STAT1 and STAT3 phosphorylation in MPM cells

Next, we investigated the signal cascade triggered by IL-27 in MPM cells, compared to IL-6, hIL-6 and IFN-γ. To this end we studied by western blot analysis the presence of the phosphorylated form of protein kinases in MPM cell lysates following short-time stimulation with cytokines. Our results, showed in figure 15, revealed that IST-MES1 cell line responded to IL-27 through STAT1 activation, while hIL-6 induced phosphorylation of both STAT1 and STAT3 (figure 15A). Moreover, IST-MES1 could also weakly respond to IL-6 by STAT3 activation, while the other two MPM cell lines tested were unresponsive to IL-6. Overall, our western blot analysis indicated that hIL-6 mainly signals through STAT3 activation but also through a weaker phosphorylation of STAT1, unlike IL-27 that predominantly induces the phosphorylation of STAT1 and to a lesser extent of STAT3. Similarly to IL-27, MSTO and MPP89 cell lines respond to IFN-γ stimulation with a major phosphorylation of STAT1 and a slight one of STAT3 (figure 15B).

Furthermore, we examined if IL-27, IL-6, hIL-6 and IFN-γ could influence by themselves the cell viability of three MPM cell lines. The MTT analysis revealed that only MPP89 cell viability was slightly reduced by IL-6, hIL-6, IL-27 and IFN-γ treatment (respectively 4.8% $p=0.02$, 4.2% $p=0.002$, 7.7% $p=0.06$ and 15.7% $p=0.01$) (figure 15C). However, the same cytokines didn't affect MSTO and IST-MES1 cell viability. Therefore, in general these cytokines do not significantly impact on MPM cell viability/proliferation.

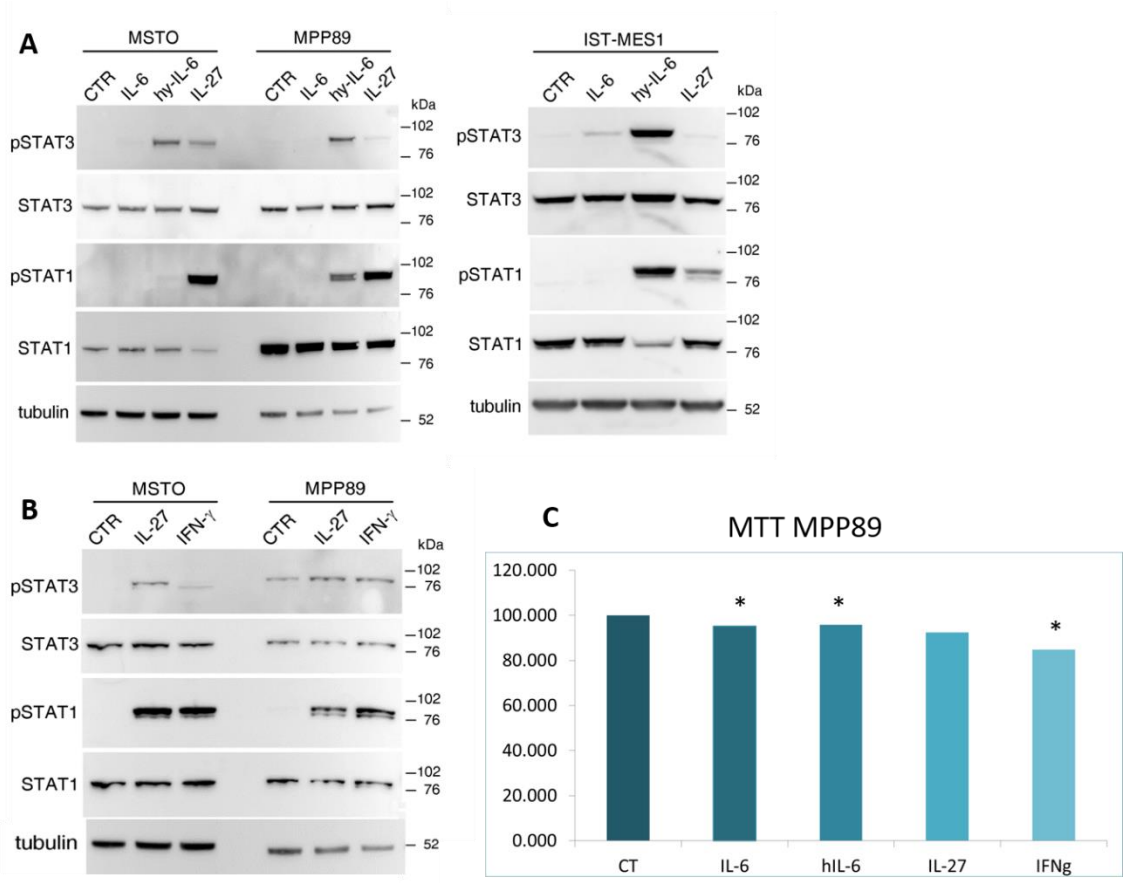

Figure 15 IL-27 mediates STAT1 and STAT3 phosphorylation in MPM cell lines. A. Western blot analysis of tyrosine phosphorylated (P)-STAT1, P-STAT3, total STAT1 and total STAT3 proteins in MSTO, MPP89 and IST-MES1 cells stimulated for 20 min with medium (CTR), IL-6, hIL-6 and IL-27. B. Analysis of P-STAT1, P-STAT3, total STAT1 and total STAT3 in MSTO and MPP89 cell lines treated with medium (CTR), IL-27 or IFN-γ for 20 min. Total STATs and α-tubulin were used as loading controls. C. MTT analysis of cell viability in MPP89 cells stimulated with medium (CT), IL-6, hIL-6, IL-27 and IFN-γ for 48h. Histogram bars display the mean percentage of cells still viable out of three replicates. (*) indicates $p<0.05$.

4.4 Evaluation of IL-27, IL-6 and IFN-γ accumulation in pleural exudates and association with patients' survival

Overall, the data from the *in vitro* experiments indicate that IL-27 and related cytokines exert a range of activities on MPM cells and thus may play a role in MPM progression. However, to verify their potential impact *in vivo* it is necessary to study the presence of the cytokines in the mesothelioma microenvironment, through the analysis of specimens from clinical cases, such as tumor and pleural fluid samples.

To evaluate the possible impact of the expression of these cytokines on MPM clinical outcome, we first analyzed MPM gene expression data, available online on the TCGA database, in particular the TCGA-MESO dataset (The Cancer Genome Atlas). *IFNG* gene expression didn't show any association with patients' overall survival, neither with respect to the median (log rank p=0.93) nor with respect to the third quartile of

expression (log rank p=0.63). Since IL-27 is a heterodimeric cytokine composed of EBI3 and IL-27A, the possible correlation of *IL27A, EBI3* and *IL6* expression with overall survival was explored, but also in this case no significant result was obtained. However, it should be noted that the TCGA-MESO dataset has been generated by samples enriched for the tumor cell component as compared to the inflammatory cell infiltrate. Therefore, it may under-evaluate pro-inflammatory cytokines. This consideration led us to undertake the measurement of cytokines in the MPM pleural exudates.

To this end, we obtained a cohort of pleural fluids (n=77) from the "Alessandria Biobank", in which we measured the concentration of IL-27 and IFN-γ together with that of other cytokines involved in the chronic inflammatory response and macrophage polarization (IL-6, TNFα, IL-1β, IL-10, IL-12) (figure 16A, B). The clinical-pathological characteristics of cases are summarized in Table 2.

Patients' characteristic Table 2

	n (%)
Age (around median)	
≤74y	39 (50.6)
>74y	38 (49.4)
Gender	
Male	56 (72.7)
Female	21 (27.3)
Histology	
Epithelioid	55 (71.4)
Sarcomatoid	12 (15.6)
Biphasic	10 (13.0)
Stage	
I	1 (1.3)
II	17 (22.0)
III-IV	35 (45.5)
Unknown	24 (31.2)
ECOG	
0	11 (14.3)
1	6 (7.8)
≥2	5 (6.5)
Unknown	55 (71.4)
Exposure to asbestos	
Environmental	29 (37.7)
Professional	29 (37.7)
Unknown	19 (24.6)
Smoking history	
Never smoked	28 (36.4)
Current smoker	5 (6.5)
Ex smoker	25 (32.4)
Unknown	19 (24.7)
Therapy	
No	22 (28.6)
At least 1 line	48 (62.3)
Unknown	7 (9.1)

First of all we found that IL-27 is present in considerable amounts in pleural exudates of MPM patients, with a median concentration of 358 pg/ml and a contained distribution of values (figure 16B). This new finding supports the idea that IL-27 participates in the physiopathology of mesothelioma tumors. We then analyzed the association between the levels of IL-27 and patients' overall survival (OS). Interestingly, we did not observe any correlation between IL-27 and survival when considering all the 77 samples together (figure 16E), but, narrowing the analysis to the epithelioid histological subtypes only (n=55), IL-27 resulted significantly and negatively associated with overall survival. Indeed, median survival was 16.6 months in

patients having IL-27 under 358 pg/ml with respect to 9.1 months in those with IL-27 over 358 pg/ml (log rank p=0.009) (figure 16F). This observation may suggest that IL-27 plays a pro-tumor role in MPM, possibly in relationship to its immune-regulatory functions.

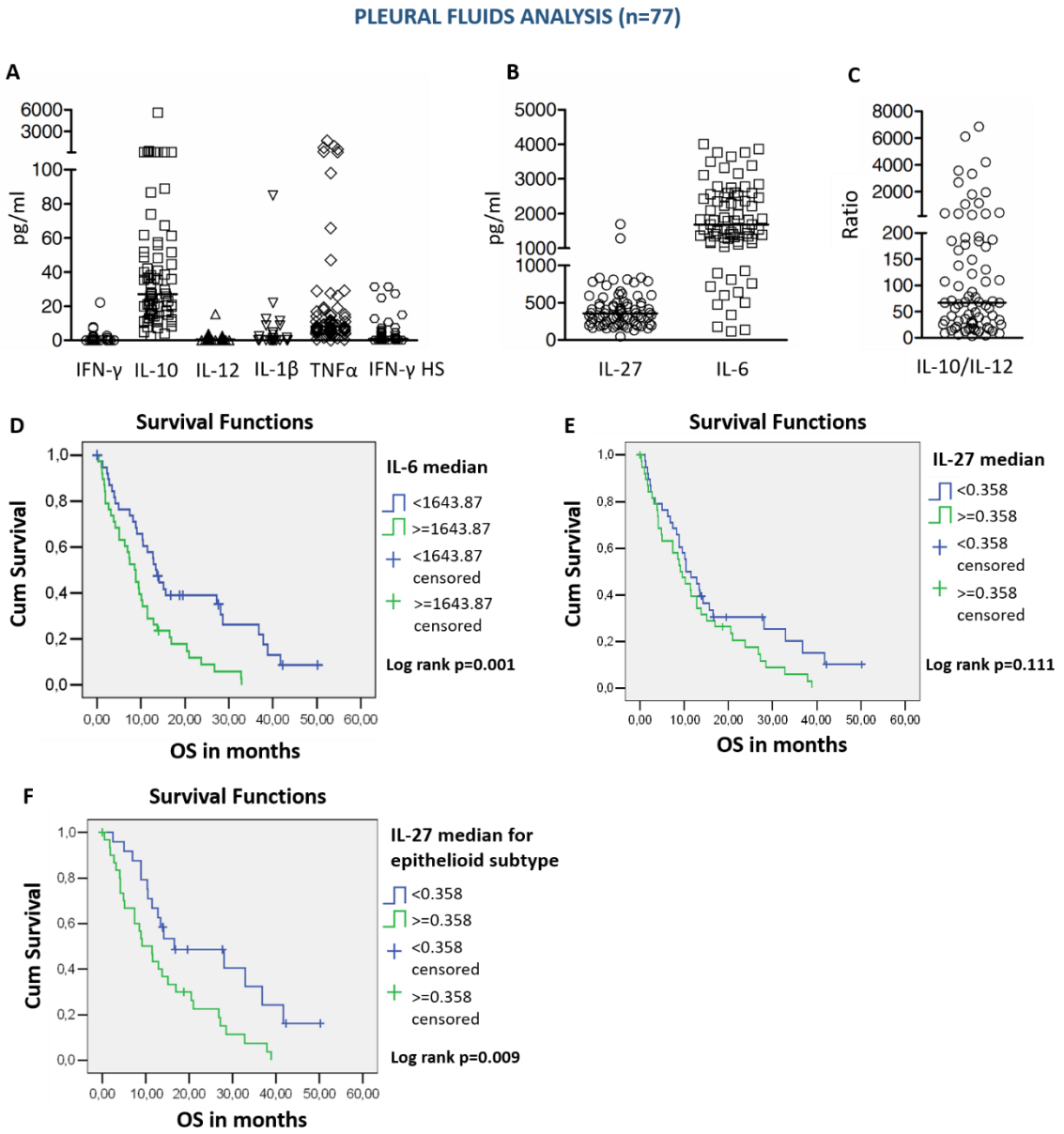

Figure 16 Cytokine quantification and survival analysis in a MPM pleural fluid cohort. **A.** Concentration (pg/ml) of IFN-γ, IL-10, IL-12, IL-1β, TNFα (detected by Milliplex MAP analysis) and IFN-γ HS (measured by Quantikine High Sensitivity Immunoassay) in pleural fluids of 77 MPM patients. **B.** Levels (pg/ml) of IL-27 and IL-6 in 77 MPM pleural exudates. Median distribution value is represented for each cytokine. **C.** Ratio IL10/IL12 calculated to estimate the relative quantity of regulatory macrophages compared to M1 macrophages. **D.** Kaplan-Meier survival analysis with respect to IL-6 median level. Patients with low IL-6 content in pleural fluid lived longer than patients with high IL-6 concentration (log rank p=0.001). **E.** Kaplan-Meier survival analysis as compared to IL-27 median concentration, considering the entire sample group (n=77) (log rank p=0.111) **F.** Kaplan-Meier survival analysis with respect to IL-27 median concentration in pleural exudates for epithelioid tumor subtype only (n=55). Survival was significantly longer in patients with low amount of IL-27 compared to those with high levels of IL-27 (log rank p=0.009). Overall survival was calculated in months, from the date of surgery to the date of the last visit or death.

Secondly, we analyzed our cohort for the presence of IL-6, which is notoriously detected at high concentration in MPM pleural fluids (Abdul Rahim et al., 2015; Hegmans et al., 2006; Nakano et al., 1998; Schmitter et al., 1992) and is associated with established poor prognostic factors of MPM (reviewed in Abdul Rahim et al., 2015).

We found that IL-6 was present with a median concentration of 1683.47 pg/ml, although its values were also very dispersed (figure 16B). Strikingly, IL-6 displayed a highly significant and negative association with survival, showing a median overall survival of 13.4 months if IL-6 was under 1683.47 pg/ml as compared to 8.6 months if IL-6 was over 1683.47 pg/ml (log rank p=0.001) (figure 16D). There was no significant correlation between the levels of IL-6 and IL-27 (Spearman's coefficient *r*=0.193, p value =0.093).

Last but not least, we evaluated the IFN-γ concentration in this set of pleural fluids by Milliplex immunoassay. The vast majority of the samples were below the detection limit, therefore measures were unreliable. We thus decided to test the samples with a different and high-sensitivity IFN-γ detection kit, that allowed the reliable measurement of the samples, which were all within the detection limits of the standard curve. As shown in figure 16A, the detected levels of IFN-γ were very low, with a median value of 0.884 pg/ml (range 0.001-31.477). Survival analysis didn't reveal any significant correlation with patients' survival (log rank p=0.858) in this cohort.

Regulatory macrophages are an important macrophage subpopulation whose function is to reduce inflammation and depress the immune response. IL-10 production and IL-12 down-regulation are the most relevant characteristics of regulatory macrophages, therefore the ratio IL-10/IL-12 is useful to determine their presence in the tumor microenvironment, as reviewed in (Mosser & Edwards, 2008). Accordingly, we measured IL-10 and IL-12 levels and calculated their ratio in the cohort of MPM pleural fluids from the "Alessandria Biobank", obtaining the results shown in figure 16C. It must be considered that IL-12 levels were most of the times under the detection threshold and were extrapolated by the analytical software according to the standard curve. Together with high IL-10 levels, these data suggest that regulatory macrophages are part of the MPM immune suppressive microenvironment, although the ratio IL-10/IL-12 did not show any correlation with survival.

5 Discussion

It is well known that progression, angiogenesis and resistance to therapy of malignant pleural mesothelioma are largely due to a tumor microenvironment made up with pro-inflammatory cytokines and molecules downregulating the adaptive immune response. We recently observed that IFN-γ could be a positive prognostic factor for MPM patients and previous studies highlighted many common functions between IL-27 and IFN-γ, as well as their dual role in tumor immune response, in different cancer types (reviewed in Fabbi et al., 2017; Hibbert et al., 2003; Petretto et al., 2016). Moreover, IL-6, which is usually highly concentrated in pleural exudates (Adachi et al., 2006; Ginolhac et al., 2017; Hegmans et al., 2006; Nakano et al., 1998), shares with IL-27 the gp130 receptor chain and has been shown to interfere with IL-27 signal transduction (Carbotti et al., 2017; Ginolhac et al., 2017). On these bases, we decided to examine IL-27 activity on MPM cell lines and compare it to that of IFN-γ and IL-6. Gp130 molecule is expressed by MPM cell lines (Ginolhac et al., 2017), while there isn't any available data about WSX1 (IL-27Rα) expression. Considering that often receptors are poorly expressed on cell surface or they're below the detection threshold of a given antibody, but they work as well, we directly tested the MPM cells response to recombinant IL-27. In this study we demonstrate for the first time that MPM cells respond to IL-27 by inducing STAT1 phosphorylation and, to a lesser extent, STAT3 activation. On the contrary, IL-6 isn't able to trigger a signaling cascade in the cell panel tested. However, the chimeric protein hIL-6 (mimicking IL-6/IL-6Rα complex) mediates the trans-signaling mainly through STAT3 phosphorylation and a weaker STAT1 activation. These data are in agreement with a previous report showing that IL-6 is able to mediate signaling in human MPM cells only in the presence of soluble IL-6Ra, as MPM cell lack surface expression of IL-6Ra chain (Adachi et al., 2006). Looking at IL-27 effects on the expression of molecules involved in immune response, it was shown that this cytokine upregulates HLA class I molecules, although they're already abundantly expressed on MPM cells. Differently from IL-6 and similarly to IFN-γ, IL-27 increased PD-L1 surface expression, but also *PDL1*, *IDO1* and *LGALS9* (Galectin 9) mRNA production, suggesting that it may promote at least three different immune suppressive circuits limiting the adaptive immune response against the tumor.

To examine whether IL-27 and related cytokines may play a role in MPM progression *in vivo*, a first analysis was carried out on the TCGA-MESO dataset, which unfortunately didn't reveal any correlation between *IL27A*, *EBI3* or *IFNG* expression and overall survival. Actually, this dataset is enriched for tumor cells (at least 70% of the samples), while these cytokines are mainly produced by tumor infiltrating cells constituting the tumor microenvironment. Therefore, it may not represent the ideal sample collection on which to perform the analysis.

We then performed direct cytokine quantification in a cohort of 77 MPM pleural fluids in order to verify their potential impact *in vivo* in the mesothelioma microenvironment. IL-27 and IL-6 were present in considerable amounts, indicating that it may play a role in the immune-regulation of the antitumor response, as is already known for IL-6 (reviewed in Abdul Rahim et al., 2015). As a matter of fact, analysis of this cohort revealed that high levels of IL-6 within pleural exudates are associated with decreased survival time and the same result was obtained with IL-27, even if specifically for the epithelioid MPM subtype only. Despite this common outcome, concentrations of the two cytokines varied independently of each other: the absence of correlation can be explained considering that IL-6 and IL-27 are released by different cell types, indeed IL-27 is mainly produced by myelomonocytic cells (reviewed in Fabbi et al., 2017; Molle et al., 2007), while IL-6 can be secreted by several other cell types, including T lymphocytes but also tumor cells (reviewed in Murakami et al., 2019).

Unfortunately, data about IFN-γ didn't allow us to validate our previous result of relationship between IFN-γ and better prognosis. However, it should be considered that differences in handling and processing of the samples may justify such a difference of results, indeed IFN-γ concentrations were much lower in the cohort from the "Alessandria Biobank" with respect to the training cohort. Furthermore, it was noted that the training cohort was highly enriched with stage I and II MPM (83.3%), while the "Alessandria Biobank" cohort was primarily composed of stage III and IV tumors (43.8%) and of a further 30% of unknown stage, which is most likely attributable to the advanced stage. Therefore, the unbalance of the samples as regards the tumor stage could explain the discrepancy of results obtained in the two different sample groups, suggesting that IFN-γ might play a positive role mainly in the first stages of tumor development, while in more advanced stages its contribution is much less. On the other hand, IL-27 might

be more involved in later stages of tumor progression carrying out pro-tumor functions, at least in the epithelioid MPM subtype.

6 Conclusions

In conclusion, our results reveal that IL-27 is involved in immune suppressive processes associated to MPM progression, possibly through the induction of the immune checkpoint ligand PD-L1 and/or induction of Galectin 9 or IDO1 (figure 17). Indeed, IDO1 causes Trp depletion and then T cell dysfunction and death (Zhai et al., 2020), whereas PD-L1 and Galectin 9 on cancer cells mediate the association of their receptors with tyrosine phosphatase proteins, which dephosphorylate key molecules involved in TCR signaling pathway, thus inhibiting T cell activity and eventually causing apoptotic cell death (Ai et al., 2020b; reviewed in Yang & Hung, 2017). Furthermore, based on the survival data and the characteristics of the two MPM cohorts that we could compare, it seems possible that IL-27 plays a pro-tumor role mainly in the epithelioid mesothelioma and in the advanced stage of the disease. However, in order to ascertain this hypothesis, it will be necessary to validate these data in a larger cohort of patients with a homogeneous tissue subtype and tumor stage distribution.

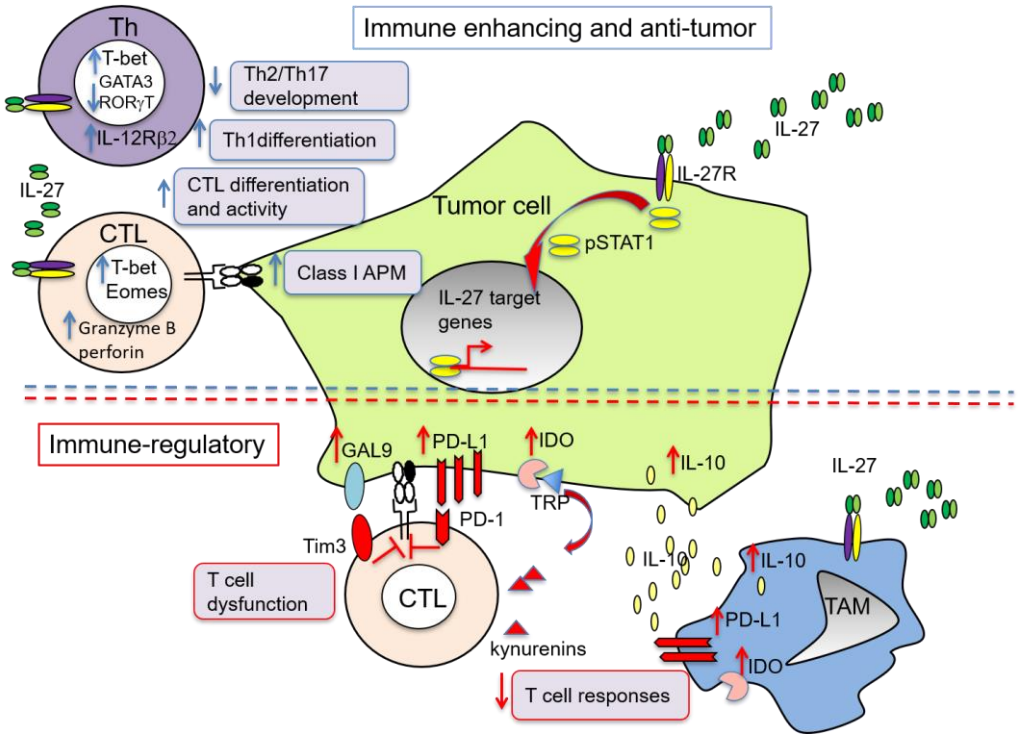

Figure 17 Representation of the immune-stimulatory and immune-regulatory functions of IL-27. The upper half of the figure shows the immune enhancing and anti-tumor activities (indicated with blue arrows), whereas lower half represents the immune regulatory and pro-tumor effects (indicated with red arrows) (adapted from Fabbi et al., 2017).

Abbreviations and acronyms

APCs: Antigen Presenting Cells; BAP-1: BRCA1-Associated Protein 1; CAFs: Cancer-Associated Fibroblasts; CAR-T: Chimeric Antigen Receptor-T cells; COX2: Cyclooxygenase 2; CTLA-4: Cytotoxic T-Lymphocyte Antigen 4; CTLs: Cytotoxic T Lymphocytes; EBI3: Epstein-Barr virus-induced Gene 3; ECM: Extracellular Matrix; EGFR: Epidermal Growth Factor Receptor; EZH2: Enhancer of Zeste Homologue 2; FAK: Focal Adhesion Kinase; GAL-3/9: Galectin 3/9; GBP-1: Guanylate Binding Protein 1; GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor; gp130: glycoprotein 130; HDAC: Histone Deacetylase; HGF: Hepatocyte Growth Factor; HIF1α: Hypoxia-Inducible Factor 1; HLA-I: Human Leukocyte Antigen-class I; HMGB1: High Mobility Group Box 1; IDO: Indoleamine 2,3-Dioxygenase; IFN-γ: Interferon- gamma; IL-1β: Interleukin 1 beta; IL-6: Interleukin 6; IL-10: Interleukin 10; IL-12: Interleukin 12; IL-18BP: Interleukin 18 Binding Protein; IL-27Rα: Interleukin 27 Receptor alpha; IL-6Rα: Interleukin 6 Receptor alpha; JAK: Janus Kinase; LAG-3: Lymphocyte-Activation Gene 3; mAb: monoclonal Antibody; MAPK: Mitogen-Activated Protein Kinase; M-CSF: Macrophage Colony Stimulating-Factor; MDSCs: Myeloid-Derived Suppressor Cells; MPM: Malignant Pleural Mesothelioma; NK: Natural Killer; PD-1: Programmed Death-1; PDGF: Platelet-Derived Growth Factor; PD-L1: Programmed Death-Ligand 1; PGE2: Prostaglandin E2; PI3K: Phosphoinositide 3-Kinase; RNS: Reactive Nitrogen Species; ROS: Reactive Oxygen Species; SCLC: Small Cell Lung Cancer; sIL-6R: soluble Interleukin 6 Receptor; STAT1/3/5: Signal Transducer and Activator of Transcription; TAMs: Tumor-Associated Macrophages; TGF-β: Transforming Growth Factor; TILs: Tumor-Infiltrating Lymphocytes; TIM-3: T-cell Immunoglobulin and Mucin-domain containing 3; TNF-α: Tumor Necrosis Factor alpha; T-reg: regulatory T cells; VEGF: Vascular Endothelial Growth Factor.

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