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Prostaglandin E₂-mediated adenosinergic effects on CD14⁺ cells: self-amplifying immunosuppression in cancer

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Running Title: CD73 expression on human monocytes in mesothelioma

Key words: pleural effusion (PE); monocytes; CD73; adenosine; prostaglandin-E₂ (PGE₂), cAMP, STAT3.

Abbreviations:

ADP, Adenosine diphosphate; AMP, Adenosine monophosphate; ATP, Adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CBP, CREB binding protein; COX2, cyclooxygenase-2; EP, E-protein coupled prostanoid receptor; ELISA, enzyme-linked immunosorbent assay; IFN γ , interferon-gamma; FSC, forward side scatter; HGF, Hepatocyte growth factor; HLA, human leukocyte antigen; IL, interleukin; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MCP-1, monocyte chemoattractant protein-1; MPM, malignant pleural mesothelioma; NECA, N-ethylcarboxamidoadenosine; PBMC, Peripheral blood mononuclear cells; PE, pleural effusion; PDL-1, programmed death ligand-1; PGE₂, prostaglandin-E₂; PKA, protein kinase A; sPE, soluble fraction of pleural effusion; SDF-1, stroma-derived factor-1; STAT, signal-transducer and activator of transcription; TNF α , tumor necrosis factor-alpha; TGF β , transforming growth factor-beta; VEGF, vascular endothelial growth factor.

Supplementary material can be found at:

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Abstract

CD39 and CD73 are surface-expressed ectonucleotidases that hydrolyze ATP in a highly regulated, serial manner into ADP, AMP and adenosine. The end-product, adenosine, has both tumor-promoting and immunosuppressive effects. The aim of this study was to determine CD73 expression on immune cells in pleural effusion (PE) in order to have a better understanding of the immune environment in mesothelioma. PE- or blood-derived CD14⁺ cells of mesothelioma patients and healthy donors were analyzed by flow cytometry for the expression of CD39 and CD73. CD73-induction was studied by exposure of CD14⁺ cells to the soluble fraction of PE (sPE), while the signaling mechanism, responsible for CD73 induction, by phosphoflow cytometry and receptor-inhibition studies. We observed CD73 expression on CD14⁺ cells in PE but not peripheral blood of mesothelioma patients or healthy donors. CD73 expression was inducible on CD14⁺ cells with sPE, cyclic-AMP (cAMP)-inducers (forskolin and prostaglandin-E2 (PGE2)) and adenosine. Inhibition of PGE2 receptors or adenosine A2 receptors blocked CD73-induction by sPE. sPE treatment triggered protein kinase A and p38 activation. However, signal-transducer and activator of transcription 3 (STAT3)-blocking led to enhanced CD73 expression, demonstrating a hitherto unknown negative control of purinergic signaling by STAT3 in CD14⁺ cells. TNF α production by CD73⁺ CD14⁺ cells was significantly impaired in the presence of AMP, confirming immunosuppressive function. Taken together, CD73 expression can be induced by PGE2, cAMP or adenosine on human CD14⁺ cells. We suggest that targeting this autocrine loop is a valid therapeutic approach in mesothelioma that may also enhance immunotherapy.

(Words: 247)

Introduction

Solid tumors have much higher levels of extracellular ATP and adenosine than normal tissues. ¹ ATP represents a danger signal via binding to P2 purinoreceptors, such as P2X7 on leukocytes, contributing to inflammation. This signaling has been reported to have tumor suppressor effects, and, interestingly, P2X7 levels are lower in cancer than in normal tissues. ² Conversely, the ATP-metabolite adenosine has extensive immunosuppressive effects ^{3,4} via binding to a range of adenosine-receptors present on most immune cells. ⁵ ATP conversion into ADP and 5'-AMP is catalyzed by CD39, while AMP is hydrolyzed by CD73 to adenosine. In the tumor microenvironment, the CD39/CD73/adenosine axis contributes not only to immunosuppression but also directly supports tumor growth, differentiation, metastasis and angiogenesis. ^{2,6,7}

Cell surface co-expression of CD39 and CD73 ensures rapid ATP uptake from the pericellular environment leading to topical generation of adenosine. ¹ Double positive cells or extracellular vesicles, such as exosomes ⁸, can act as efficient enzyme-delivery systems reaching distant sites. The co-expression of these enzymes on immune cells has been demonstrated for activated mouse Treg and Th17 cells ^{9,10} but untreated human Tregs in the circulation only express low levels of intracellular CD73. ¹¹ Mouse peritoneal macrophages may also co-express these enzymes, ¹² while healthy non-activated human blood monocytes and macrophages have abundant expression of CD39 but not CD73 on the cell surface. ^{13,14}

Ectonucleotidase expression can be controlled by the microenvironment; e.g. CD39 expression is regulated by IL-27 in ovarian cancer, ¹⁵ while CD73 is

upregulated by hypoxia on epithelial cells¹⁶, by TGF β on murine leukocytes¹⁷ and by signal-transducer and activator of transcription-3 (STAT3) activation on murine Th17 cells.¹⁰ Endothelial cells were observed to respond to increased intracellular cAMP levels via an adenosine-mediated paracrine pathway leading to CD73 upregulation.¹⁸

The aim of this study was to reveal whether immune cells in the pleural effusion (PE) of MPM express CD73.

Malignancy-associated PE is a relatively easily accessible material in MPM. It is a complex milieu that reflects some of the characteristics of the tumor and represents a rich source of tumor and immune cells and soluble factors. PE is responsible for indirectly influencing tumor cells, regulating inflammation and contributing to tumor dissemination.¹⁹ The population of CD14⁺ cells in the tumor microenvironment consists of tumor-associated macrophages and monocytes. In mesothelioma, M2 macrophages with immunosuppressive features have been observed in the tumor; their ratio (CD163/CD68) having a negative prognostic value.²⁰ The effect of sPE on skewing macrophage differentiation into M2 type has recently been demonstrated,^{21,22} however, CD73 expression has not been studied on these cells.

We report here the presence of CD39/CD73 co-expressing CD14⁺ cells in the PE of patients with MPM. When healthy donor peripheral blood mononuclear cells (PBMC) were exposed to the cell-free, soluble fraction of PE (sPE), surface expression of CD73 was induced in a dose- and cell type-dependent manner on CD14⁺ but not on CD3⁺ cells. The effect of sPE on CD73 induction was mimicked by cAMP-inducing agents, such as forskolin or PGE₂, or with adenosine, in a

protein kinase A (PKA)- and p38-dependent manner. Conversely, sPE-induced CD73 expression was significantly amplified by inhibiting STAT3 phosphorylation. We demonstrate that CD73, induced on CD14+ cells, is functional, as its activity suppresses TNF α production. These observations point towards a cross-talk between cAMP/adenosine and STAT3 signaling in the regulation of CD73 expression on monocytes in the tumor microenvironment, offering novel treatment targets in MPM.

Results

CD73 is expressed on CD14+ cells in mesothelioma-associated PE. The aim of our study was to explore whether tumor-associated immune cells co-express CD39 and CD73. The cellular fraction of PE from MPM patients was phenotyped and the frequencies of those CD14+ or CD3+ cells that co-expressed CD39 and CD73 were determined and compared with frequencies found in patients' or healthy donors' PBMC. We found CD73 expression on CD14+ cells in PE but not in blood (Fig. 1A upper panel). The mean frequency of CD39+CD73+ double positive CD14+ cells was 17.4% in the PE of three donors (Fig. 1B) compared to <0.5% in PBMC (Figure 1B). CD39 was expressed at comparable levels on CD14+ cells in healthy or patient PBMC (mfi: 455 and 547), while in PE its expression was more than three-fold higher (mfi: 1,732) (Fig. 1A upper panel). CD73 expression levels and co-expression with CD39 were also analyzed on CD3+ cells: The frequency of CD3+ cells expressing CD73 was not elevated in PE compared to that in PBMC (Fig. 1A, lower panel). These results demonstrate a specific induction of CD73 in the tumor environment on myeloid (CD14+) but not T (CD3+) cells.

Only CD14+ cells in PE co-express CD73 and CD39. Tumor cells have been shown to express CD73 while some also co-express CD39.²³ In order to demonstrate that the CD39+CD73+CD14+ cells in PE are distinct from e.g. tumor or mesothelial cells, which are CD14-, CD73 expression on CD3-CD14-/FSC^{high} cells(T) vs. CD3-CD14+FSC^{intermediate} (M) cells was studied (Fig. 1C). As expected, the CD14- FSC^{high} (T; potentially tumor-containing) cell population was only present in the PE and not blood. These cells expressed CD73 but not CD39 and this pattern was similar to that we observed on established primary

mesothelioma cell lines ^{24, 25} (Fig. 1D). These results prove that CD39⁺CD73⁺ co-expression is a characteristic of CD14⁺ monocytes/macrophages in MPM-associated PE.

CD73 expression is induced selectively on CD14⁺ cells by sPE. In order to determine whether soluble factors in PE are responsible for inducing CD73 expression on CD14⁺ cells, healthy donor PBMC was exposed to increasing concentrations of sPE for 48h in vitro. Flow cytometry analysis of CD14⁺ cells demonstrated that CD73 is significantly upregulated by sPE, pooled from four patients, and this upregulation is dose-dependent (Fig. 2A, upper panel). CD73 induction was the property of 9/9 sPEs studied. While four sPE samples were pooled for the experiments carried out in this paper (individual effects shown in Fig S1 and patient details in Table 1) an additional five sPE samples (individual or pooled) have confirmed the findings (Supplementary Figure 1). One sample (sPE9) derived from a patient with sarcomatoid mesothelioma. The induction of CD73 expression was protein-specific, as CD39 expression levels were not altered. It was also cell type specific, as CD39⁺CD73⁺ cell frequencies remained unchanged in the CD3⁺ subset (Fig. 2A, lower panel). Phenotyping of sPE-treated CD14⁺ cells indicated CD14, CD16 and CD163 upregulation, compared to that on untreated cells, while CD62L, CD86 and HLA-DR were expressed at lower levels following sPE-treatment (See Supplementary Fig. 2.A). We also tested for PDL-1 antigen expression, a marker of immunosuppression, but its level was not enhanced by treatment with sPE, in fact it was downregulated. HLA-DR, CD16 and CD163 expression were lower on CD73⁺ cells than on CD73⁻ cells in the sPE-treated group (See Fig. S2.B). The results indicate that soluble factors in PE have

a significant and selective effect on inducing CD73 expression on CD14⁺ cells, however, CD73⁺CD14⁺ cells do not express typical M2 macrophage markers.

PGE₂ induces CD73 expression on CD14⁺ cells. As CD73 was only expressed on MPM patients' CD14⁺ cells in the PE but not in blood, we analyzed the differences in cytokine composition of PE vs. plasma. A cytokine array revealed that sPE contained elevated levels of IL-6, IL-8, IL-13, IP-10 and MCP-1 (Fig. S3) compared to plasma, while ELISA experiments demonstrated that hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) levels were also higher in sPE than in plasma (Fig. 2B). Next we tested the concentration of PGE₂ in the pooled sPE used in the experiments: PGE₂ concentration was more than two-fold higher in the sPE than in MPM plasma (Fig. 2B last panel). On the other hand, we did not find elevated TGFβ levels in PE compared to that in plasma by TGFβ ELISA (Fig 2B). We then screened a variety of cytokines for their ability to induce CD73-expression on monocytes. We found that IL-6, IL-8, HGF, VEGF and SDF, alone or in combination (Fig 2C), or TGFβ alone (not shown) or in combination with IL-6 and IL-10 failed to induce CD73 expression (Fig. 2D). However, when CD14⁺ cells were treated with 100 ng/ml PGE₂ for 48h, significant induction of CD73 expression was observed (Fig. 2E). In order to demonstrate that PGE₂ in sPE is a relevant factor that can drive CD73 induction, G-protein coupled E-type prostanoid receptors, EP2 and EP4, that have been demonstrated to be the main PGE₂ receptors present on human monocytic cells²⁶, were blocked before exposing CD14⁺ cells to sPE. The near complete inhibition of CD73 expression (Fig 2E) confirmed that PGE₂ in sPE plays a dominant role in the induction of CD73 on human CD14⁺ cells. PGE₂ is a known inducer of intracellular cAMP.²⁷

Thus, next we tested if cAMP is involved in CD73 induction, by stimulation with forskolin, which is a receptor-independent, pharmacological inducer of intracellular cAMP. Indeed, forskolin proved efficient in inducing CD73 expression on healthy donor CD14⁺ cells (Fig. 2F), confirming the role of cAMP signaling in CD73 upregulation.

Adenosine can also induce CD73 expression on CD14⁺ cells.

Extracellular adenosine is a relevant factor in supporting mesothelioma, as we show it in a tumor cell growth support experiment, where NECA significantly enhanced primary mesothelioma cell line proliferation (Fig. S4). As adenosine, signaling via the A_{2A} receptor, has also been shown to induce intracellular cAMP²⁸, we tested the effect of the adenosine analogue NECA on CD73 expression. We found significant CD73 induction on CD14⁺ cells by NECA (Fig. 3A). To confirm the relevance of this observation in our PE study, we blocked A_{2A} adenosine receptor before CD14⁺ cells were exposed to sPE. A partial, but significant prevention of CD73 induction was observed both at 24h and 48h (Fig. 3B). The results show that the adenosine pathway triggers the induction of CD73 surface expression on CD14⁺ cells, generating a potentially self-amplifying autocrine loop.

CD73 is induced on CD14⁺ cells by sPE via PKA and p38 signaling. As cAMP is known to signal via PKA and p38, we tested whether exposure of CD14⁺ cells to sPE activates this pathway. First we pre-treated the cells with the PKA inhibitor H-89. Upon sPE- or PGE₂-treatment, CD73 upregulation was significantly inhibited (Fig. 3C) by the PKA inhibitor. The phosphorylation of p38 after exposure of monocytes to sPE was also tested. p38 activation was elevated as early as 10 min, compared to untreated cells, and gradually decreased by 4h

(Fig. 3D). Anisomycin (antibiotic, known to activate p38), used as a positive control, displayed slightly slower kinetics of p38 phosphorylation (Fig. 3D). Inhibition of p38 phosphorylation with the small molecule inhibitor SB203580 was also carried out, and it completely abolished CD73 upregulation either by sPE or NECA (Fig. 3E). These results demonstrate that CD73 expression can be induced on CD14⁺ cells by PGE₂ and adenosine, soluble factors relevant in cancer, and this induction can be mimicked by forskolin via the cAMP/PKA/p38-signaling pathway.

CD73 induction on CD14⁺ cells by sPE is enhanced by STAT3

inhibition. In mouse Treg cells, STAT3 activation contributed to the upregulation of CD73.¹⁰ In order to explore the role of this pathway in the induction of CD73 expression on human monocytes, we tested if sPE-treatment triggers STAT3 phosphorylation. Exposure of CD14⁺ cells to sPE indeed resulted in significant STAT3 phosphorylation at 30 min, with sustained kinetics, as it was still significantly elevated at 2h and 4h compared to that in untreated cells (Fig. 4A). In order to explore the consequences of STAT3 activation, we inhibited STAT3 phosphorylation with the small molecule inhibitor Cpd188 in cells exposed to sPE, at a concentration we have established previously.²⁹ Unexpectedly, in the presence of the inhibitor, sPE-mediated induction of CD73 expression became significantly enhanced (Fig. 4B). sPE did not activate STAT1 and Cpd188 had no off-target effects on this transcription factor (Fig 4C, left panel). These results indicate that STAT3 exerts a negative effect on CD73 induction and thus it may have the ability to curb excessive adenosine production. This was confirmed by an experiment where IL-6 was added together with sPE or NECA to CD14⁺ cells. In combination with sPE, IL-6 had no

effect on CD73 induction (Figure 4D), presumably because IL-6 was already present at elevated levels in the sPE (as shown on Fig S3). However, when added with NECA, CD73 induction was significantly inhibited by IL-6. The negative regulation of adenosinergic signaling in human monocytes/macrophages by STAT3 represents a novel (i.e. a non-immunosuppressive) role for STAT3 in the immune regulation of cancer.

sPE-induced CD73 on CD14⁺ cells is functional. Finally, we determined if CD73, upregulated by sPE on CD14⁺ cells, is able to impair the function of these cells. Highly purified, healthy peripheral blood CD14⁺ cells were treated with sPE overnight. Adenosine A_{2A} and A_{2B} receptor-inhibitors were added to the cells, followed by AMP and LPS. TNF α production was assessed by intracellular cytokine staining (Fig. 4E). Adding AMP to these cells resulted in a nearly 50% inhibition of LPS-induced TNF α production. In the presence of adenosine receptor inhibitors, AMP's negative effect on cytokine production was prevented, proving that the inhibition is adenosine-mediated. Adenosine receptor inhibitors, added to CD14⁺ cells in the presence of LPS but without AMP, resulted in a small but significant increase in TNF α production, presumably due to a low level of adenosine being intrinsically present in the cultures. These experiments provide evidence that CD73, induced by sPE, partakes in the generation of local immunosuppression in mesothelioma in an adenosine receptor-mediated manner. The suggested mechanism of positive and negative regulation of CD73 expression is summarized on Fig. 5.

Discussion

This study shows that mesothelioma-associated PE has a regulatory role in the purinergic pathway by inducing the expression of CD73 on myeloid cells. CD73 is a rate-limiting enzyme in the hydrolysis of ATP to adenosine. We demonstrate its induction on CD14⁺ cells by SPE in a PGE₂- or adenosine-dependent manner, via cAMP, PKA and p38 signaling. As a functional consequence, pro-inflammatory cytokine production is impaired by CD73-expressing CD14⁺ cells in the presence of AMP; this impairment is reversible by A₂ receptor-inhibitors.

Human monocytes and macrophages are largely CD73 negative but express CD39.¹³ Although CD73 upregulation has been reported on other cell types, such as on T cells, epithelial cells³⁰, endothelial cells¹⁸ and murine leukocytes,¹⁷ such an observation has only recently been made on human macrophages in ovarian cancer.³¹ We have reported earlier that tumor cells may be present in mesothelioma PE²⁴ and here we demonstrate that these tumor cells in PE may express CD73 but not CD39. Co-expression of CD73 and CD39, which is only observed on CD14⁺ cells in PE but not in blood, makes these tumor-associated macrophages (TAM) a key cell type in purinergic regulation. CD39/CD73 double positive TAM have the potential of reducing extracellular ATP levels by hydrolysis, leading to the generation of adenosine, which then helps in maintaining a tumor-supporting and immunosuppressive microenvironment. We observed that SPE skews monocytic differentiation towards M2-like macrophages, as detected via CD14 and CD163 upregulation and downregulation of HLA-DR. This confirms recent findings on the effect of MPM-PE on macrophages^{21,22}. However, CD14⁺ cells, which are also positive for

CD73, are phenotypically distinct from M2-like cells as their CD163 expression level is significantly lower than that on CD73⁻ cells and there are no differences in CD14 expression levels. Thus we conclude that CD73 induction is distinct from CD14⁺ cell differentiation towards M2-like macrophages. PDL-1 expression was also downregulated or remained low on these cells, which may not be surprising as PDL-1 induction is the consequence of type I and II interferon stimulation³², while in the tumor milieu, myeloid cells predominantly encounter anti-inflammatory factors.

Extracellular adenosine is elevated in numerous cancer tissues¹ with direct tumor-promoting effects, and indirect, immunosuppressive functions, by inhibiting effector T cells or promoting regulatory T cell activity.^{6,33} In mesothelioma, high concentration of extracellular adenosine (3 mM) has been reported to cause apoptosis of tumor cells.³⁴ However, we show here that physiological concentrations support tumor growth, indicating the relevance of adenosine in the progression of this disease.

We demonstrate in this study that PGE₂ induces the expression of CD73 on monocytes. PGE₂ is the main product of COX₂ and COX₂-overexpression is a negative prognostic factor in MPM.³⁵ PGE₂ has recently been identified at elevated concentrations in MPM-PE.²² It has been suggested that PGE₂ is a potential factor to induce immunosuppressive M2 macrophages, however, mechanistic data have been lacking to support this. PGE₂ has been shown to increase cAMP levels via prostanoid receptors EP2/EP4. We demonstrate here that CD73 upregulation on CD14⁺ cells by sPE is blocked by inhibiting its receptors, EP2 and EP4, confirming the role of PGE₂ in the regulation of the

adenosinergic pathway in mesothelioma. PGE₂ has also been shown to impair purinergic signaling by ATP in macrophages³⁶, which suggests that in the presence of PGE₂, extracellular ATP is preferentially undergoing hydrolysis by myeloid cells.

cAMP signals via PKA and p38, and this signaling has been shown to suppress the production of TNF α and IFN γ in keratinocytes.³⁷ We demonstrate here that sPE activation of the cAMP-PKA-p38 pathway leads to CD73 induction on the surface of human CD14⁺ cells, as small molecule inhibitors of PKA and p38 prevent CD73 induction. As CD73 enzyme activity results in the generation of adenosine, which induces further CD73 expression on myeloid cells, we describe a self-amplifying loop here which may be similar to the immunosuppressive autocrine adenosinergic loop described for Treg cells.⁹

When testing STAT3 activation in CD14⁺ cells exposed to sPE, we found simultaneous phosphorylation of p38 and STAT3. STAT3 can be activated by IL-6, IL-10 or VEGF; adenosine can mediate STAT3 activation via IL-10, as demonstrated in mouse macrophages³⁸, while PGE₂ itself has also been shown to phosphorylate STAT3 in cardiomyocytes.³⁹ Signaling via STAT3 can upregulate CD73 in Th17 cells.¹⁰ However, when STAT3 was blocked in our model, instead of inhibition of CD73 upregulation, we observed significantly enhanced CD73 expression. There is no known inhibitory influence of STAT3 on cAMP signaling; however, a possible explanation is that there may be a competition for CREB binding protein (CBP) between cAMP-activated CREB and STAT3. Thus, inhibition of STAT3 may favor CREB binding, similar to what has been shown in

the human myeloid THP cell line.⁴⁰ We are currently investigating this regulatory effect of STAT3 on CD73 expression.

Taken together, we demonstrate here that PGE₂ in mesothelioma-associated PE regulates CD73 expression on CD14⁺ cells in a cAMP-mediated manner, while STAT3 acts as a negative regulator of this process. A_{2A}-receptor inhibition has been shown to synergize with the effect of PDL-1 immune checkpoint inhibitors in preclinical tumor models⁴¹. This treatment combination is currently undergoing clinical trials in numerous advanced solid tumors (ClinicalTrials.gov: NCT02655822; Corvus Pharmaceuticals; Novartis NIR 178). Humanized CD73 blocking antibodies are also under development or in clinical trials, following promising results in combination with checkpoint inhibitors in pre-clinical models.⁴² (Innate Pharma Inc.: AACR 2016 Poster #2344; MedImmune, MEDI9447: Phase I in combination with anti-PDL1; Bristol Myers Squibb: in the pipeline). Our findings provide new mechanistic information about potential beneficial effects of targeting CD73 or the A_{2A}-receptor in mesothelioma.

Materials and Methods

Blood, PE and cell lines. Ethical approval for the study was granted by the South-East Wales Research Ethics Committee. Venous blood was collected from healthy donors or MPM patients. PE samples were collected into sterile containers attached to indwelling catheters. Patient characteristics are described in Table 1. sPE stock was prepared from four donors and used throughout these experiments. The sPE fraction was prepared by depleting cells (centrifugation at 2,000 rpm for 20min), filtering the supernatant (0.22 μ m) and storing aliquots at -80°C. Human peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Histopaque density gradient centrifugation. CD14⁺ cells were enriched by magnetic separation (19058; StemCell Technologies), according to the manufacturer's instructions. Primary mesothelioma cell lines were developed as described before.²⁴ Met5A mesothelial cells were purchased from the ATCC (Manassas, VA).

Reagents. Human recombinant IL-6 (200-06), IL-8 (200-08), IL-10 (200-10), VEGF (100-20A), HGF (100-39), TGF β (100-39C) and SDF-1 (300-28A) were purchased from Peprotech. NECA (1691), PGE₂ (2296), Forskolin (1099), SB203580 (1202; p38-inhibitor); H-89 (2910; PKA-inhibitor); SCH58261 (2270; adenosine A_{2A} receptor-inhibitor) and PSB0788 (3199; adenosine A_{2B} receptor-inhibitor) were obtained from Tocris. Cpd188 (573125; STAT3-inhibitor) was obtained from Calbiochem and Anisomycin (A9789) from Sigma. AH-6809 and AH-23848 (EP2 and EP4 PGE₂-receptor inhibitors) were obtained from Cayman Chemicals (CAY-14050 and CAY-19023). The inhibitor concentrations and treatment details are described in the figure legends. Antibodies against CD14

(25-0149 or 11-0149), CD3 (47-0036), CD39 (17-0399), TNF α (22222-22222) and pSTAT1 (12-9008-42) were purchased from Affymetrix and those for CD73 (550257), phosphorylated p38-MAPK (612565) and STAT3 phosphorylation sites pY705 and pS727 (612569 and 558557 respectively) were obtained from BD Biosciences.

sPE treatment and flow cytometry. CD14⁺ cells at 5x10⁵/500 μ l were treated with 12.5% sPE for 48h unless otherwise stated. Cell surface labeling was carried out on ice for 30 min using antibodies specific for CD14, CD3, CD39 and CD73. For phosphoprotein detection, cells were first fixed as above, permeabilized (ice-cold 80% methanol for 30 min), then labeled with PhosphoFlow antibodies for p38-MAPK, pSTAT3 (pY705/pS727 antibodies applied together), pSTAT1 or relevant isotype controls. Staining for CD14 and CD3 was then carried out for 45 min at room temperature. FACSCanto or FACSVerse cytometers (BD-Biosciences) were used for acquisition and FACSDiva software for data analysis.

ELISA. PGE₂ (KGE004B), HGF (DHG00), VEGF (DVE00), TGF β (DY240-05) and SDF-1 (DSA00) levels from sPE samples or MPM and healthy plasma samples were determined by ELISA (R&D Systems), according to the manufacturer's instructions.

TNF α release by sPE-treated CD14⁺ cells. CD14⁺ cells were isolated from healthy PBMC (StemCell Technologies) with 94% purity. 2.5x10⁶ cells were incubated in 2ml media overnight in the presence or absence of 12.5% sPE. Adenosine A_{2A} and A_{2B} receptor-inhibitors (200 nM each) were added to cells for 30min followed by 200 μ M AMP (01930, Sigma) for 30 min, and 200 ng/ml lipopolysaccharide (LPS; L4391 Sigma) for 1h. Intracellular cytokine staining

was carried out as described ⁴³ following 4h incubation with Golgi Plug (55509) and Golgi Stop (554724, both BD-Biosciences). Fixed (00-82249) and permeabilized (00-8333-56, both Affymetix) cells were labeled with CD14 and TNF α antibodies at room temperature for 30 min. Flow cytometry was carried out as above.

Statistical analysis. Results are presented as means+SD of triplicate measurements unless it is stated otherwise. Statistically significant differences were determined using Student's t-test or ANOVA with Tukey *post hoc* test (GraphPad InStat 3.06). A p-value of <0.05 was considered statistically significant.

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Figure Legends

Figure 1. CD73 is expressed on tumor-associated CD14⁺ cells. (A) CD73 (y-axis) and CD39 (x-axis) fluorescent intensity are shown on CD14⁺ (upper panel) or CD3⁺ cells (lower panel) from PE, or patient (PBMC-MPM) and healthy donor PBMC (PBMC-HD). The first dot plot shows CD3 and CD14 gating, while in the second column CD39 isotype labeling is shown. The numbers represent % double positive cells in the first row and single+double positive cells in the second row. (B) Summary of results, as in (A), from multiple donors. Means+SD of results are shown, *p<0.05, **p<0.01. (C) Upper panel: gating on CD14^{low}FSC^{high} cells (T) and on CD14^{high}FSC^{intermediate} cells (M) in PE, MPM and healthy donor PBMC. Lower panel: CD73 and CD39 expression on gated cells. (D) CD73 and CD39 expression on primary mesothelioma cell lines and non-malignant Met5A mesothelial cells.

Figure 2. sPE, PGE₂ and forskolin induce CD73 expression on CD14⁺ cells. (A) CD73 induction on CD14⁺ cells (upper panel) or on CD3⁺ cells (lower panel) of healthy donor PBMC, 48h after treatment with increasing doses of sPE. The first panel shows staining with control isotype antibodies. The numbers represent % of CD14⁺ or CD3⁺ cells, co-expressing CD73 and CD39. A representative of 12 experiments is shown. (B) ELISA measurements of growth factors, from PE or plasma, are shown as indicated (n=3-6). The boxes (panels 1-4) represent the 25th and 75th percentile values, the lines represent the means. The last panel shows the mean and SD of PGE₂ concentrations from four MPM plasma donors and the sPE mix, which has been described earlier. (C) CD73 expression on healthy donor CD14⁺ cells following treatment with 10ng/ml IL-6,

10ng/ml IL-8, 50ng/ml HGF, 50ng/ml VEGF, 10ng/ml SDF-1 or the mix of these factors (Mix) or sPE at 50%, or (D) with 12.5% PE or a mix of 10ng/ml TGF β , 10 ng/ml IL-6 and 2 ng/ml IL-10, as indicated. (E) Treatment of healthy donor CD14⁺ cells with 12.5% sPE or 100nM PGE₂ in the presence or absence of PGE₂-receptor inhibitors (PGE₂i) AH-6809 and AH-23848 (5 μ M each). (F) 250 μ M forskolin treatment of healthy donor CD14⁺ cells. C-F: Means+SD of CD73 mfi values from triplicate cultures are shown. C: FACS Canto, D-F: FACS Verse flow cytometer. *p<0.05, **p<0.01, ***p<0.001.

Figure 3. CD73 expression is also induced by adenosine; PKA- and p38-dependent pathway. (A) CD73 induction by NECA (100nM) and sPE or (B) with sPE at 4, 24 and 48h in the presence or absence of adenosine A_{2A} receptor inhibitor (SCH58261, 200nM). (C) CD73 expression on sPE- or PGE₂ (250 μ M)-treated CD14⁺ cells in the presence or absence of a PKA inhibitor (PKAi) H-89 (10 μ M). (D) p38 phosphorylation by sPE in CD14⁺ cells. Means+SD of phosphorylated p38 mfi from triplicate samples for sPE treated cells are shown while positive control anisomycin (35 μ M) and untreated control are represented by single samples. (E) CD73 expression on sPE- or NECA (250nM)-treated CD14⁺ cells in the presence or absence of p38 inhibitor (p38i) SB203580 (10 μ M). (A,B,C,E) Means+SD of CD73 mfi values from triplicate cultures are shown *p<0.05, **p<0.01, ***p<0.001.

Figure 4. pSTAT3-mediated enhancement of CD73 induction and the function of CD73-expressing CD14⁺ cells. (A) Means+SD of pSTAT3 or isotype mfi values are shown from triplicate CD14⁺ cell samples at different times after sPE-treatment. (B) CD73 expression on CD14⁺ cells 48h after treatment with sPE

in the presence or absence of pSTAT3-inhibitor (STAT3i) Cpd188 (10 μ M). Means+SD of CD73 mfi from triplicate samples are shown. (C) pSTAT3 (left panel) or pSTAT1 (right panel) or isotype control mfi values are shown 30 min after treatment with sPE in the presence or absence of Cpd188 (STAT3i) as described above. Means+SD of triplicates. (D) CD14⁺ healthy donor cells were treated with sPE or NECA (250ng/ml) in the presence or absence of 10ng/ml IL-6. Mfi of CD73 expression 48h later is shown as mean+SD of triplicate samples. (E) CD14⁺ cells were treated with sPE overnight. LPS-induced TNF α production was measured by intracellular cytokine staining in the presence or absence of AMP (200 μ M) or adenosine receptor A_{2A}- and A_{2B}-inhibitors (A2Ri; 200nM each), as indicated under the graph. Means+SD of the mfi of TNF α from triplicate samples are shown. *p<0.05, **p<0.01, ***p<0.001.

Figure 5. The mechanism of CD73 induction on CD14⁺ cells. This cartoon summarizes the results of CD73-induction by adenosine, forskolin and PGE₂, the inhibition of induction (dotted lines) by (1) PGE₂ receptor inhibitors, (2) adenosine receptor inhibitors, (3) a PKA-inhibitor and (4) a p38-inhibitor. Negative regulation by pSTAT3 was demonstrated by a STAT3-inhibitor (5).

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Table 1. Details of PE donor MPM patients

Patients	Age	Gender	Pathology	PE Collection (time post diagnosis)	Treatment history (time before PE collection)
PE4	81	M	Epithelioid	12 months	Palliative RT (3 weeks pre-collection)
PE5	67	M	Epithelioid	8 months	None
PE6	63	F	NOS*	3 months	None
PE7	66	M	Epithelioid	4 years 5 months	Pleurectomy (4 years 3 months pre-collection). Pem/Cis** (11 months pre-collection)

* Not otherwise specified

** Pemetrexed and Cisplatin, 4 cycles

Figure 1

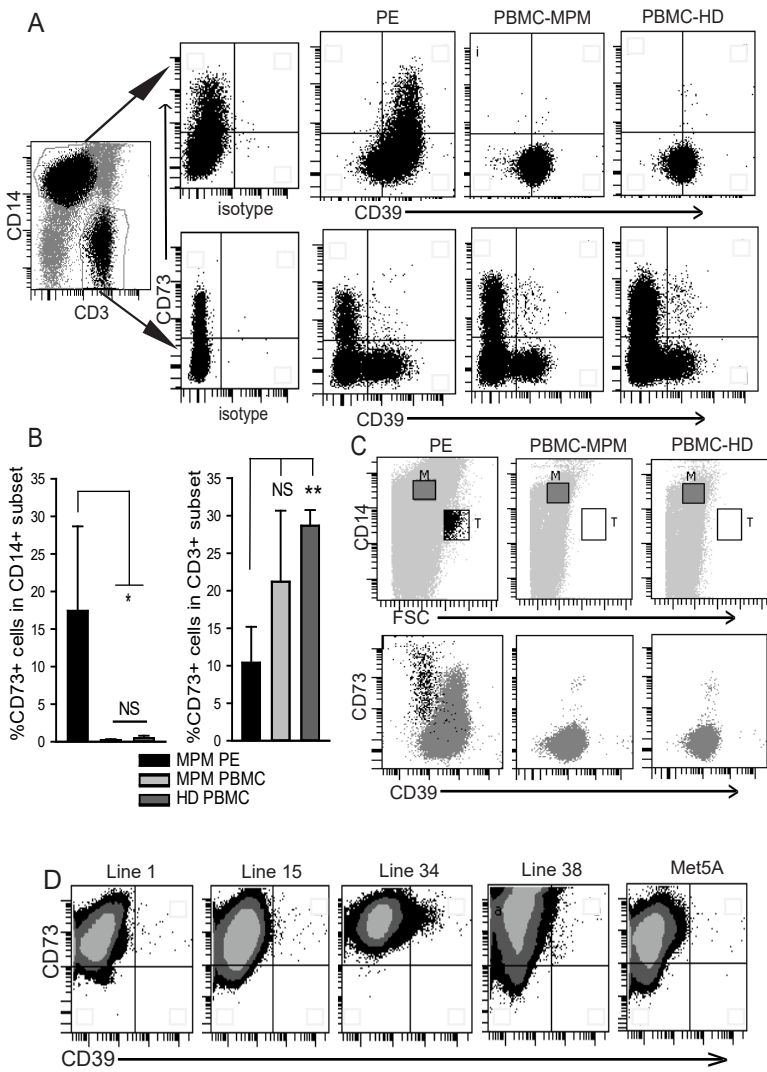


Figure 2

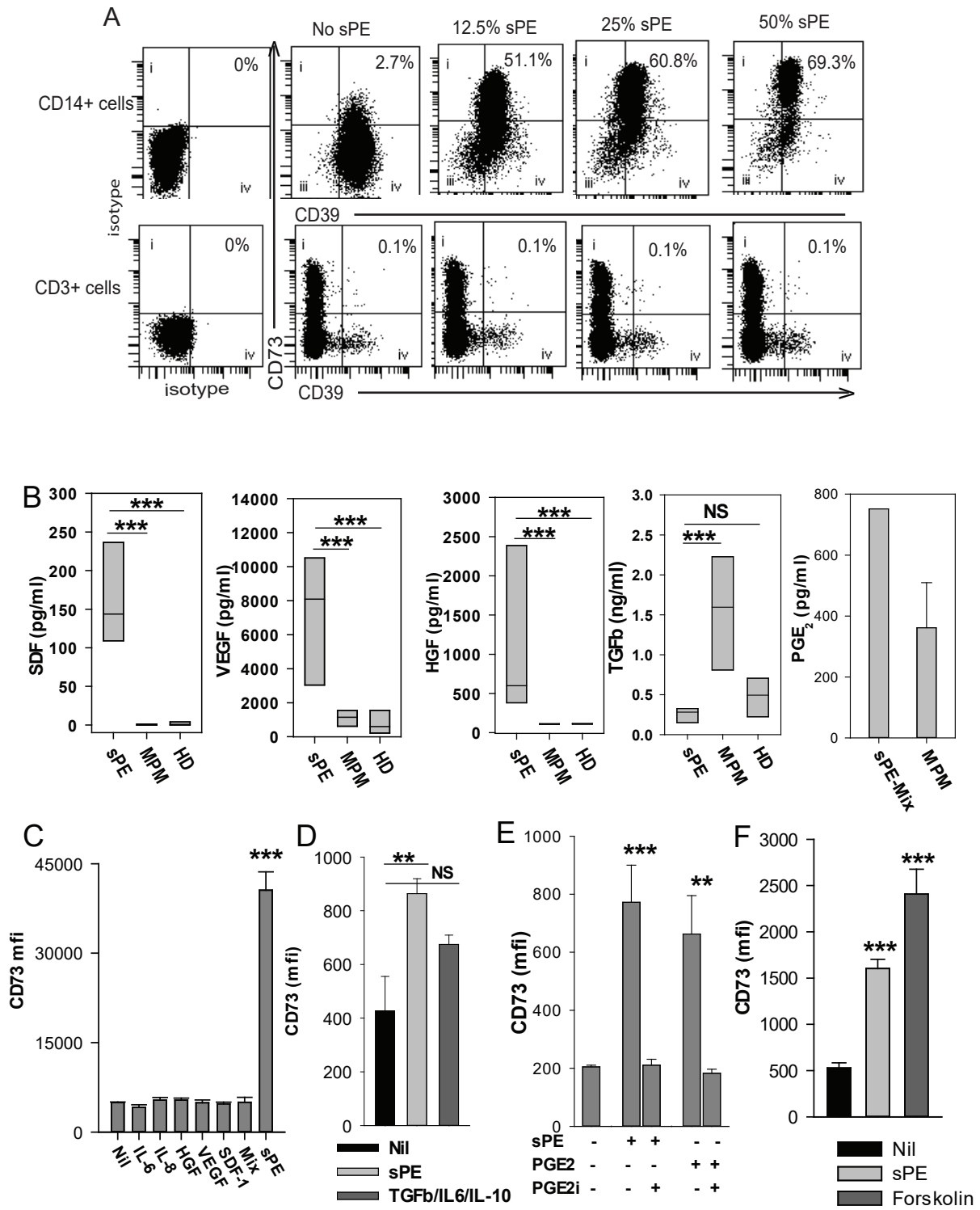


Figure 3

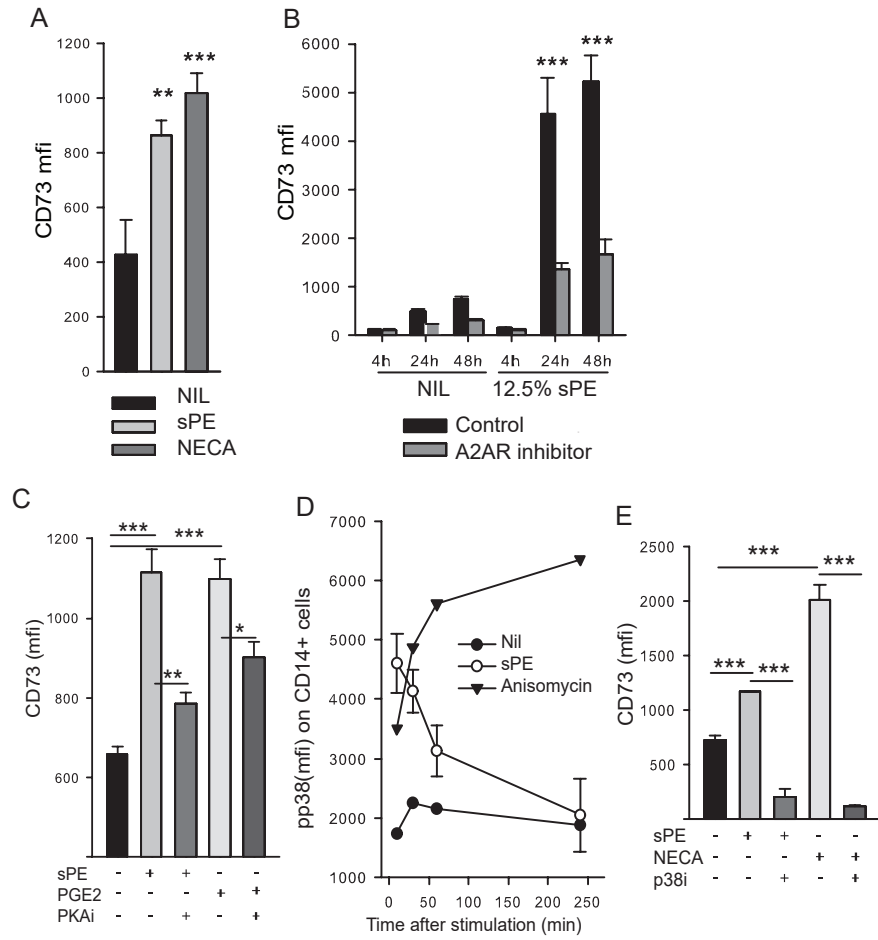
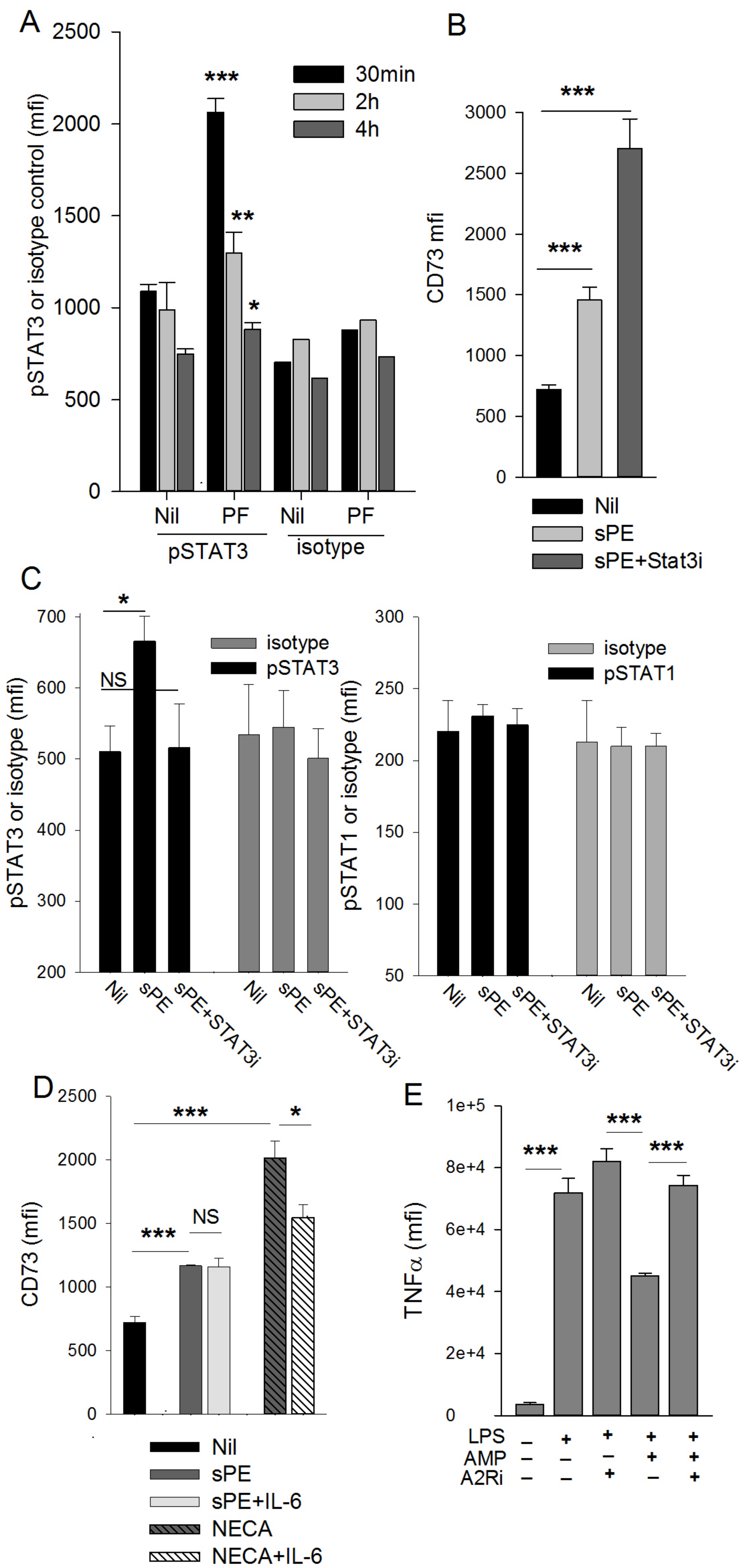
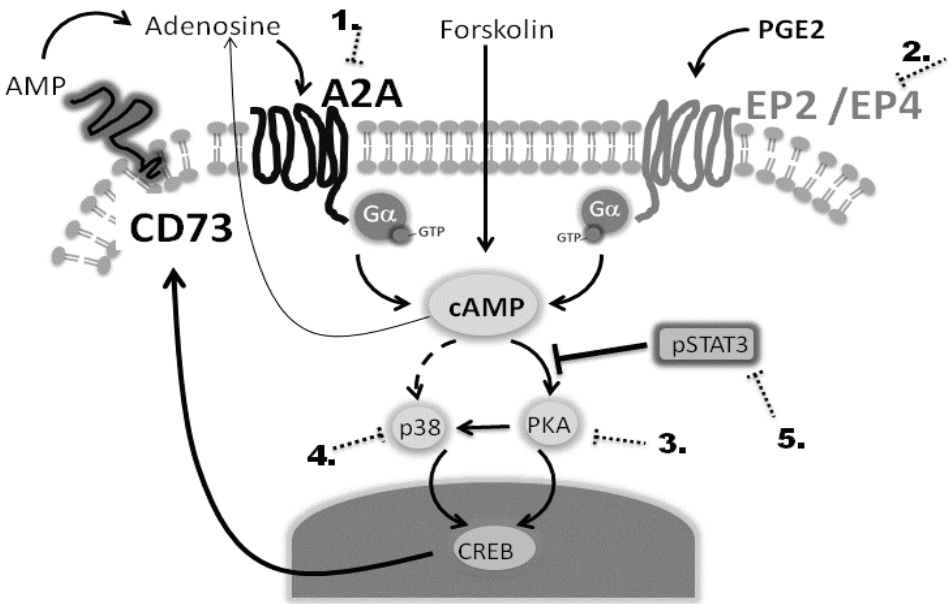


Figure 4





ONLINE DATA SUPPLEMENT

Title: Prostaglandin E2-mediated adenosinergic effects on CD14+ cells: self-amplifying immunosuppression in cancer

Authors: Saly Al-Taei, Josephine Salimu, Lisa K Spary, Aled Clayton, Jason F Lester and Zsuzsanna Tabi

Methods for Supplementary Data

Human Cytokine Array

Cytokine Array Kit (ARY005B) from R&D Systems was used to simultaneously detect the relative levels of 36 different cytokines and chemokines in sPE and plasma, according to instructions. Pixel density was determined using Image-J software. Means of duplicate samples were calculated from average pixel density values minus negative control values.

Mesothelioma cell proliferation following NECA treatment

Cells of the primary mesothelioma line 15 (established by us, refs ^{17,18} in main paper) were plated into a 96 well flat bottomed tray at 5×10^3 cells/well in the presence of increasing concentrations of NECA as indicated on the figure. After 24h, the cells were labeled with $0.5 \mu\text{Ci } ^3\text{H}$ -thymidine overnight. The cells were harvested onto a filtermat and counted in a β -counter (Wallace, Perkin-Elmer).

sPE treatment of monocytes and flow cytometry

CD14⁺ cells were isolated from freshly prepared PBMC by positive isolation (StemCell Technologies). CD14⁺ cells were incubated for 48h in the presence or absence of 12.5% sPE. Cell surface labeling was carried out on ice for 30min using antibodies specific for CD3, CD14, CD16, CD86, HLA-DR, PDL-1, CD1c, CD62L, CD163 and CD73 (Affymetrix) and (BD-Biosciences). A FACSVerse cytometer (BD-Biosciences) was used for cell phenotyping, and FACSDiva software for data analysis.

Figure S1

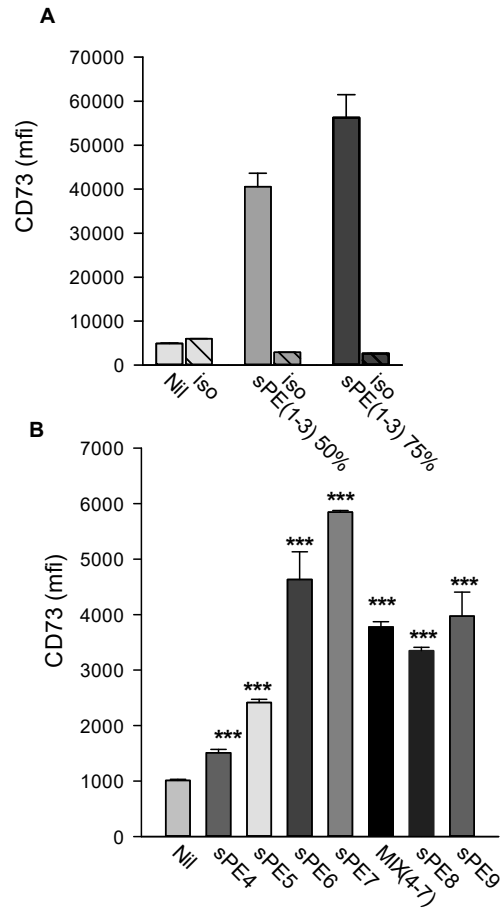


Figure S1. CD73 is upregulated on CD14+ cells by MPM PE. Means+SD of CD73 or isotype mean fluorescence intensity (mfi) from triplicate samples are shown following 48h treatment with (A) Nil, or 50% of 75% sPE pooled from three donors, or (B) with nil and sPE from a further 6 individual donors. The Mix from samples 4-7 represents the pooled sPE from donors as listed in Table 1 of the main paper, and used in all experiments. sPE9 represents a sample from a patient with sarcomatoid subtype of mesothelioma.

Figure S2

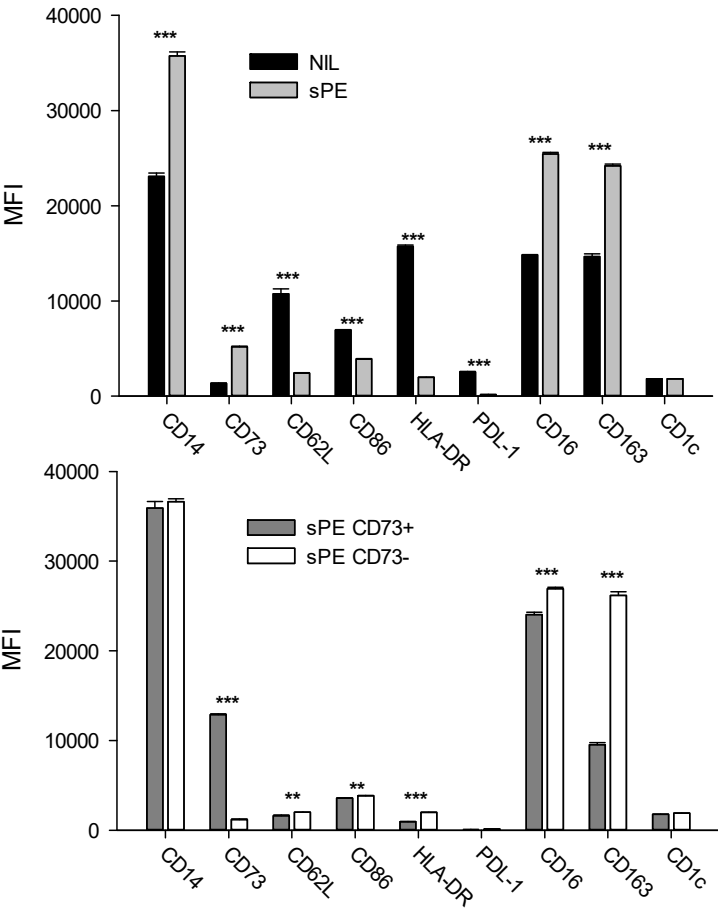


Figure S2. Phenotypic changes due to sPE-treatment on monocytes. (A) Marker expression on sPE - treated or untreated (Nil) CD14+ cells. (B) Marker expression on sPE-treated CD73⁺ vs. CD73⁻ cells. Means+SD of antigen expression (as mean fluorescence intensity, mfi) from triplicate samples are shown. Significant differences are shown as **p<0.01 and ***p<0.001.

Figure S3

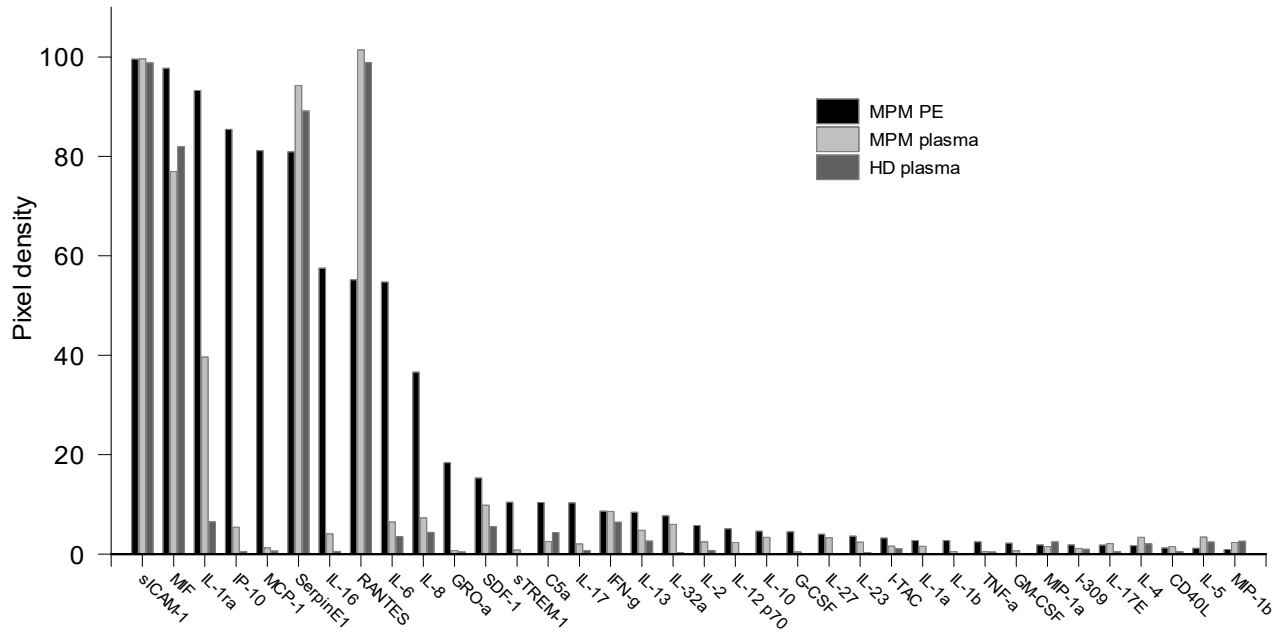


Figure S3. Cytokine array results from two PE, two MPM plasma and two healthy donor plasma samples. Means of duplicate readings of pixel densities for each cytokine shown are expressed as relative pixel densities, after background deduction, determined with Image J software.

Figure S4

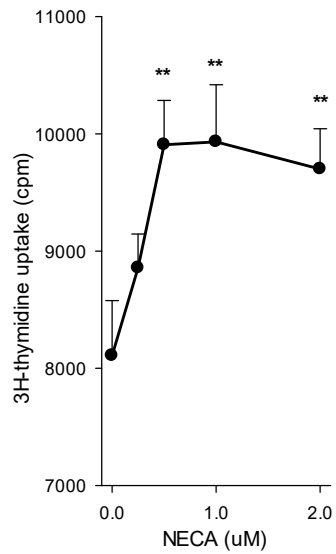


Figure S4. NECA enhances mesothelioma cell proliferation (48h assay). Means+SD of ^3H -thymidine uptake (cpm) by mesothelioma cells from 14 replicates per treatment group are shown. Significant differences are shown as ** $p < 0.01$.