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Anti-folate receptor-α IgE but not IgG recruits macrophages to attack tumors via TNF-α/MCP-1 signaling

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Abstract

IgE antibodies are key mediators of anti-parasitic immune responses, but their potential for cancer treatment via antibody-directed cell-mediated cytotoxicity (ADCC) has been little studied. Recently, tumor antigen-specific IgEs were reported to restrict cancer cell growth by engaging high-affinity Fc receptors on monocytes and macrophages, however, the underlying therapeutic mechanisms were undefined and in vivo proof-of-concept was limited. Here, an immunocompetent rat model was designed to recapitulate the human IgE-Fcε receptor system for cancer studies. We also generated rat IgE and IgG monoclonal antibodies specific for the folate receptor (FRα), which is expressed widely on human ovarian tumors, along with a syngeneic rat tumor model expressing human FRα. Compared with IgG, anti-FRα IgE reduced lung metastases. This effect was associated with increased intra-tumoral infiltration by TNFα+ and CD80+ macrophages plus elevated TNFα and the macrophage chemoattractant MCP-1 in lung bronchoalveolar lavage fluid. Increased levels of TNFα and MCP-1 correlated with IgE-mediated tumor cytotoxicity by human monocytes and and with longer patient survival in clinical specimens of ovarian cancer. Monocytes responded to IgE but not IgG exposure by upregulating TNFα, which in turn induced MCP-1 production by monocytes and tumor cells to promote a monocyte chemotactic response. Conversely, blocking TNFα receptor signaling abrogated induction of MCP-1, implicating it in the antitumor effects of IgE. Overall, these findings show how anti-tumor IgE reprograms monocytes and macrophages in the tumor microenvironment, encouraging the clinical use of IgE antibody technology to attack cancer beyond the present exclusive reliance on IgG.

Introduction

Engagement of tumor-specific monoclonal antibodies via their Fc receptors contributes significantly to the anti-tumor effects of the immune system (1). Focusing effector cells such as monocytes/macrophages and natural killer (NK) cells against cancer-associated components may contribute to the functions of therapeutic antibodies such as trastuzumab, cetuximab and the checkpoint inhibitor ipilimumab (2, 3). Antibody engineering strategies to optimize antibody-effector cell interactions and to direct these cells against tumors, may therefore improve therapeutic efficacy (4, 5).

One strategy to influence these interactions is the exploration of changes to the structure of antibody Fc regions. The IgE immunoglobulin class is characterized by high affinity for cognate interaction with Fcε receptors (100-10,000 times higher than that of IgG for FcγR) on distinct, often tumor-resident, effector cells such as monocytes/macrophages (6, 7). Although IgE antibodies play pathogenic roles in allergic inflammation by triggering mast cell degranulation and promoting eosinophil inflammation, they also contribute to the host immune defense against parasitic infections. The potential of IgE to induce inflammatory responses at tumor sites may be harnessed through IgE receptor-expressing effector cells such as monocytes and macrophages in tumors. Strategies to implement this approach include recombinant tumor-associated antigen (TAA)-specific IgEs, and active immunotherapy triggering adaptive IgE responses against cancer (8–12).

Folate receptor alpha (FRα) is overexpressed by several solid tumors, most significantly by epithelial ovarian carcinomas (13), and is a desirable target for TAA-specific IgE due to overexpression in tumors, and no/low expression and restricted distribution in normal tissues. Additionally, evidence of negative associations between allergies and reduced risk of gynaecological malignancies is reported (14), while little is known about IgE immunity against ovarian carcinoma antigens in patients (15). The chimeric (mouse V/human C) IgE antibody hMOv18 IgE, specific for FRα (16, 17), effected superior tumor cell cytotoxicity and improved survival compared with IgG1 of equivalent specificity (18–21). Potential roles of monocytes/macrophages were suggested by loss of IgE-conferred survival advantage following monocyte depletion of human peripheral blood mononuclear cells (PBMCs) introduced with hMOv18 IgE (20). Monocyte-mediated tumor killing was demonstrated through both known IgE receptors: antibody-dependent cell-mediated cytotoxicity (ADCC) via the high affinity FcεRI, and phagocytosis (ADCP) via the low affinity FcεRII (CD23). Since inflammatory infiltrates of many tumors contain macrophages, re-polarizing these against cancer may constitute an important rationale for developing IgE cancer immunotherapy (22). To-date however, the capacity of IgE to recruit macrophages against cancer in an immunocompetent tumor-bearing setting has not been demonstrated and the mechanisms by which IgE may activate these cells against cancer remain unclear. IgE can rapidly mediate parasite neutralization by FcεR-expressing cells including human macrophages (23, 24). Although TNFα, IL-10 and nitric oxide (NO) have been individually reported in these processes (23–25), the mechanisms engendered through cross-talk between immune cells, IgE antibodies and target cell antigens, including parasite or tumor antigens, have not been elucidated.

Lack of cross-reactivity of human IgE with murine FcεRs and absence of trimeric FcεRI on murine monocytes/macrophages, eosinophils and other subsets have provided challenges for the design of immunologically-relevant models with which to study IgE class antibody functions. Previous immunodeficient mouse models, some reconstituted with human immune cells to provide IgE effector cells, were limited by short lifespans of human effector cells and incomplete representation of human immunity. Additionally, certain human effector cell-secreted cytokines may not interact with the murine immune system.

We investigated whether MOv18 IgE can inhibit tumor progression by recruiting and polarizing macrophages. We constructed a syngeneic rat model of FRα-expressing

adenocarcinoma designed to better recapitulate the human IgE-Fcε receptor system and the patient setting. In this model, immune cells are found in their natural anatomical locations, immune cell FcεRI expression and distribution in rats mirrors that of humans, and rat effector cells (*e.g.* monocytes/macrophages) express trimeric FcεRI (αγ2) (26). We generated anti-FRα IgE and IgG with rat Fc sequences (rMOv18 IgE/IgG2b) to examine alongside antibodies with human Fc (hMOv18 IgE/IgG1). We assessed antibody efficacy and IgE-mediated tissue macrophage migration and activation *in vivo*, and TNFα and MCP-1 in tumor environments. We evaluated relevance in the human system and dissected the conditions that promote TNFα and MCP-1, by IgE-mediated human monocyte activation. Our findings support the superior therapeutic efficacy of IgE over IgG, and identify a previously-unappreciated tumor antigen-specific IgE-potentiated axis that promotes effector cell polarization and recruitment towards tumor cells.

Materials and Methods

Human Samples and Ethics

Blood and tumor specimens were collected from 6 ovarian carcinoma patients (Supplementary Table S1) and blood drawn from 13 healthy volunteers (Supplementary Table S2), with informed written consent, in accordance with the Helsinki Declaration. Study design was approved by the Guy's Research Ethics Committee, Guy's and St. Thomas' NHS Foundation Trust.

Cell Lines

The CC531tFR cell line, originally derived from a 1,2 dimethyhydrazine (DMH)-induced colon adenocarcinoma of a WAG-Rij rat (27) (Cell Lines Service), was transfected to express human FRα as previously-described, and selected on the basis of Geneticin resistance (S. Canevari, M. Colnaghi, Instituto Nazionale Tumori, Milan) (28, 29). IGROV1 human ovarian carcinoma cells naturally over-express human FRα (18, 30). A375 (human metastatic melanoma; CRL-11147), SKOV3 (human epithelial ovarian carcinoma, HTB-77), TOV21G (human ovarian clear cell carcinoma, CRL-11730), SKBR3 (human breast carcinoma HTB-30), U937 (human monocytic CRL-1593.2), and THP-1 (human monocytic; TIB 202) cells were from ATCC. FreeStyleTM 293-F cells (R790-07) were from Invitrogen (Supplementary Materials and Methods). Cell lines from ATCC were authenticated by short tandem repeat profiling. Routine *Mycoplasma* testing was performed by PCR regularly on all cell lines.

Production of anti-FRα antibodies with human and rat Fc regions

Chimeric mouse/human antibodies MOv18 IgE and IgG1 recognizing human FRα were engineered as before (18). Chimeric mouse/rat MOv18 IgE and IgG2b antibodies were designed with rat constant and mouse variable domains specific for human FRα (Supplementary Materials and Methods).

Tumor cell cytotoxicity and phagocytosis (ADCC/ADCP) assays

Antibody-dependent cell-mediated killing of FRα-expressing tumor cells was quantified by adapting a previously-described flow cytometric method (Supplementary Materials and Methods).

Assessments of antibodies *in vivo*

Immunocompetent syngeneic WAG rat model of FRα-expressing lung metastases—Female Wistar Albino Glaxo (WAG/RijCrl) rats (Charles River) were maintained and handled in accordance with the Institutional Committees on Animal Welfare of the UK Home Office (The Home Office Animals Scientific Procedures Act, 1986). Rats were injected *i.v.* with $4x10^6$ CC531tFR tumor cells and subsequently treated with MOv18 antibodies (days 1 and 14 or 1, 7, 14 and 21). Lung tumor burden was determined 26 days following CC531tFR inoculation by: mean number of surface-visible metastases/cm^2; and % tumor occupancy [total white surface area (mm^2) / total lung (black + white) surface area (mm^2)].

Assessment of hMOv18 IgE in immune deficient mouse models—Patient-derived intraperitoneal (*i.p.*) human ovarian carcinoma xenografts in female nu/nu mice were previously described (19). Subcutaneous IGROV1 tumors were established in C.B-17 scid/scid (SCID) mice as before (18) (Supplementary Materials and Methods).

Isolation of rat effector cells from peripheral blood and lungs

Rat primary monocytes were prepared from rat peripheral blood leukocytes (PBL) by flow cytometry cell sorting using a PE-conjugated antibody against CD172 (BD Biosciences) (Supplementary Materials and Methods).

Flow cytometric evaluations of freshly-isolated tumor-infiltrating macrophages

Phenotypic analysis of tumor-infiltrating macrophages from single cell suspensions of tumor-bearing rat lungs was performed with directly-labeled monoclonal antibodies (Supplementary Materials and Methods).

Criteria for evaluating immune cell infiltration of tumors

H&E-stained sections were used to determine the tumor immune cellular infiltrate as a proportion of total tumor areas (Supplementary Materials and Methods). The percentage of tumor occupied by immune cells in each section was derived as follows: % immune cell occupancy = total area occupied by immune cells/total tumor area (Supplementary Fig. S1A).

Evaluations of macrophage infiltration into tumors

Sections double-stained for FRα (AF488, green) and CD68 (AF555, red) were used to determine ratios of within-tumor:peripheral $CD68^+$ cells/mm^2. $CD68^+$ cells and the area covered by tumor (defined by tissue morphology, density of DAPI staining and FRα staining; Supplementary Fig. S1B) and periphery was calculated. (Supplementary Materials and Methods).

Quantitative real-time-PCR analysis of TNFα and MCP-1 expression by tumor cells and monocytes

Cells harvested from ADCC and *ex vivo* stimulation assays were studied for MCP-1 and TNFα relative gene expression. Cells from ADCC assays were CD89-PE- and FRα-FITC-labeled and sorted using a FACSAria II Cell Sorter (BD Biosciences). Sorted cells and cells from *ex vivo* stimulation experiments (Supplementary Materials and Methods) were re-suspended in RLT buffer for RNA isolation by RNeasy Kit (Qiagen).

Chemotaxis assay

To analyze the chemotactic properties of MCP-1 on THP-1 cells and human primary monocytes, a chemotaxis assay was performed using Transwell® plates with a polycarbonate membrane insert and 5μm pore size (Costar®) (Supplementary Materials and Methods).

Statistical methods and analyses of publically-available databases

All statistical analyses (Supplementary Materials and Methods) were performed using GraphPad™ Prism software (version 5.03, GraphPad™). *P* values are represented as follows: *=$P<0.05$, **=$P<0.01$, ***=$P<0.001$, ****=$P<0.0001$. Error bars represent SD and SEM in *in vitro* figures, and in *in vivo* figures and histological analyses, respectively. Clinical associations of tumor gene expression were assessed using publicly-available data (31), in Kaplan-Meier Plotter (http:/kmplot.com/analysis/) (Supplementary Materials and Methods).

Results

Rat and human IgE and IgG display comparable *in vitro* properties

We generated surrogate rMOv18 IgE and IgG2b antibodies (equivalent to mouse IgG2a/b and human IgG1, based on their complement-fixing and ADCC functions) recognizing human FRα in order to construct a syngeneic rat model system. Human FRα-expressing WAG rat syngeneic adenocarcinoma CC531tFR cells served as targets. Rat peripheral blood monocytes (CD172+) expressed FcεRI and bound rMOv18 IgE (Figs. 1A-B). rMOv18 IgE bound to rat FcεRI (αβγ2)-expressing rat RBL-2H3 mast cells. rMOv18 IgG2b bound primary rat monocytes (expressing FcγRI), and RBL-2H3 cells (expressing low levels of rFcγRII/FcγRIII). Rat IgE and IgG2b bound to CC531tFR but not to FRα-negative A375 melanoma cells (Fig. 1B).

Rat primary monocytes activated *ex vivo* with rMOv18 IgE, and rMOv18 IgG2b induced significant CC531tFR cell death compared with isotype control antibodies and equivalent to levels of IGROV1 ovarian tumor cell death triggered by human antibodies (Fig. 1C). Furthermore, monocytic cells stimulated with IL-4 to express the low affinity IgE receptor CD23/FcεRII, and primed with rMOv18 IgE, triggered significant CC531tFR death by ADCP over control antibody, equivalent to levels of ADCP triggered by hMOv18 IgE (Fig. 1D).

This suggests that rMOv18 antibodies are functionally analogous to their human counterparts, at least with regard to binding FcεR+ and FRα+ cells, and their potentiation of particular effector functions.

Anti-tumor activities of rat MOv18 IgE and IgG2b *in vivo*

We next examined the *in vivo* functions of rMOv18 IgE and IgG2b in an immunocompetent rat model of FRα+ CC531tFR lung metastases, designed to better recapitulate the spectrum and functions of human IgE effector cells and the patient setting (Supplementary Fig. S2).

Immunofluorescent staining confirmed *in situ* FRα+ CC531tFR metastases (Fig. 2A). Dose-dependent inhibition of tumors between 5mg/kg, 10mg/kg and 50mg/kg doses (% tumor occupancy of lungs) was observed with rMOv18 IgE (biweekly) (Fig. 2B). Lung metastases and tumor occupancy were significantly lower with rMOv18 IgE compared with rMOv18 IgG2b ($P<0.0001$) or PBS ($P<0.0001$) (Figs. 2C-E) at 10mg/kg doses. IgE and IgG2b significantly reduced lung metastases at 5mg/kg compared with PBS ($P<0.0001$). Given the longer serum half-life of IgG (14-25 days) compared with IgE (1-2 days) (32), we investigated whether dosing frequency could influence efficacy. rMOv18 IgE dosed weekly induced significantly-reduced tumor occupancy at doses as low as 1mg/kg compared with PBS ($P<0.0001$), with a dose-dependent response between 3mg/kg, 10mg/kg ($P=0.0002$) and 50mg/kg ($P<0.001$) (Fig. 2F). Weekly 3mg/kg rMOv18 IgE afforded significantly ($P=0.04$) superior tumor growth restriction compared with IgG2b (Fig. 2G).

Therefore, rMOv18 IgE and IgG2b were functionally active *in vivo*. Rat MOv18 IgE at 3mg/kg weekly and 10mg/kg biweekly doses effected superior tumor growth restriction compared with IgG2b.

MOv18 IgE treatment is associated with rat macrophage infiltration into tumors

We next investigated spontaneous, endogenous macrophage infiltration into tumors in immunocompetent rats. Histological evaluation of rat lungs showed reduced tumor islet density and increased glandular organization in rMOv18 IgE- compared with rMOv18 IgG2b-treated cohorts (Fig. 3A). Tumor areas occupied by immune and stromal cell infiltration were significantly greater with rMOv18 IgE compared to PBS ($P<0.0001$) (Fig. 3B). We observed superior rat macrophage infiltration into tumors of rMOv18 IgE-treated rats. CD68+ macrophage clusters (red) surrounded sparse FRα+ (green) tumor islets. Macrophage density within tumor islets was higher in rMOv18 IgE- and rMOv18 IgG2b-treated rats compared with PBS ($P=0.007$) (Fig. 3C). CD68+ cell ratios within tumor islets:tumor periphery were significantly higher with rMOv18 IgE (rMOv18 IgG2b, $P=0.03$; PBS, $P=0.003$) (Fig. 3C, Supplementary Fig. S1). Additionally the intensity of macrophage infiltration correlated inversely with tumor occupancy in animals treated with rMOv18 IgE, and rMOv18 IgG2b but not PBS (Fig. 3D). Analyses of patient-derived ovarian carcinoma xenografts, where hMOv18 IgE introduced with human PBMCs prolonged survival of tumor-challenged mice compared to controls, showed that human CD68+ macrophage infiltration into tumors correlated with prolonged survival of hMOv18 IgE-treated mice ($r=0.67$, $P=0.009$) (Fig. 3E) (19). Furthermore, in a subcutaneous IGROV1 human ovarian cancer xenograft in immunodeficient mice, significant tumor growth was observed in mice

given PBS (P=0.0054), hMOv18 IgE alone (P=0.049) or human monocyte-enriched PBMCs (P=0.0014), but tumor growth was restricted in mice given hMOv18 IgE plus monocyte-enriched cells (Fig. 3F). These findings suggest that the anti-tumor effects of MOv18 IgE *in vivo,* at least partly reflect immune effector functions of the antibody and enhanced macrophage influx into the tumor mass.

Macrophage polarization and elevated TNFα, MCP-1 and IL-10 in lungs of IgE-treated rats

Since antibody treatments were associated with enhanced macrophage infiltration into tumors, we investigated whether IgE was associated with polarization or maturation of tissue-resident macrophages. Consistent with immunohistochemical evaluations (Fig. 3), the mean percentages of freshly-isolated lung CD68+ macrophages within the total CD45+ leukocyte populations (Supplementary Fig. S3) were elevated in rats treated with rMOv18 IgE compared with IgG2b and PBS (Fig. 4A). Macrophages from rMOv18 IgE-treated rats demonstrated enhanced expression of the co-stimulatory molecule CD80, compared with those from rMOv18 IgG2b- or PBS-treated animals (P=0.01). No differences in the alternatively activated (M2) scavenger receptor CD163 (33) expression were found between treatment groups (Figs. 4B-C).

A higher proportion of CD68+ macrophages from rMOv18 IgE-treated rats expressed intracellular TNFα, compared with rMOv18 IgG2b- and PBS-treated cohorts (P=0.04) (Figs. 4B-C). Additionally, a higher proportion of these cells also expressed intracellular IL-10 with rMOv18 IgE treatment, compared with rMOv18 IgG2b- (P=0.04) and PBS-treated cohorts (P=0.04). A proportion of macrophages from rMOv18 IgE-treated rats simultaneously expressed TNFα and IL-10 (Fig. 4D). In conjunction (Figs. 4E-F), we found significantly elevated TNFα, MCP-1 and IL-10 secreted in bronchoalveolar lavage (BAL) fluids of IgE-treated rats compared with IgG (IL-10, P=0.044; TNFα, P=0.017; MCP-1, P<0.001). Levels of IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, and IL-12 did not differ significantly between treatment groups (Fig. 4E).

These data demonstrate TNFα-expressing mature macrophage subsets in rMOv18 IgE-treated rat lungs, along with a significantly-elevated secreted TNFα/MCP-1/IL-10 IgE-associated BAL profile.

Cross-linking of cell surface-bound IgE triggers elevated TNFα by monocytes, and TNFα stimulates MCP-1 by monocytes and tumor cells

We sought to determine whether rMOv18 IgE treatment-associated macrophage activation is relevant in a human setting, and gain insights into how tumor antigen-specific IgE may trigger effector cell activation. Cross-linking of IgE antibodies of different antigen specificities (anti-FRα MOv18, anti-hapten NIP IgE, anti-HER2 against the breast cancer tumor antigen HER2/*neu* and CSPG4 IgE, recognizing the melanoma tumor associated antigen Chondroitin Sulfate Proteoglycan 4 (34, 35)) on monocytes with polyclonal anti-IgE antibody (to mimic engagement by TAA-expressing tumor cells) significantly increased production of mRNA encoding TNFα by human monocytes, compared to IgE alone. Crosslinking of equivalent IgG1s did not trigger TNFα, nor did cross-linking of IgEs on human ovarian IGROV1 tumor cells (Fig. 5A).

We then investigated whether IgE Fc-mediated TNFα upregulation can promote MCP-1 secretion and therefore, putatively, monocyte mobilization. TNFα stimulation of human monocytic U937 cells and primary human monocytes triggered elevated MCP-1 mRNA expression compared with unstimulated cells (Fig. 5B left; $P<0.0001$), along with elevated MCP-1 protein secretion compared with unstimulated cells (Fig. 5B right; U937 $P=0.002$; primary human monocytes $P=0.0116$).

Stimulation of ovarian IGROV1, SKOV3 and TOV21G, breast SKBR3 and melanoma A375 cancer cells with TNFα induced significantly-elevated MCP-1 mRNA expression and secretion compared with unstimulated cells (Fig. 5C). Furthermore, MCP-1 stimulation could effect human monocyte chemotaxis (human primary monocytes, THP-1 monocytes) in a concentration-dependent manner (Fig. 5D).

Therefore, TNFα upregulation, triggered by cross-linking of receptor-bound IgE, but not IgG, on the surface of monocytes, may induce MCP-1 production by human monocytes and a range of tumor cell types, putatively promoting further monocyte chemotaxis and potential accumulation in tumors. These data suggest that IgE-mediated monocyte activation and TNFα upregulation may be relevant in the human setting.

TNFα and MCP-1 are involved in antigen-specific IgE effector functions and may be associated with better patient survival

We investigated whether TNFα/MCP-1 upregulation could be triggered by specific tumor antigen recognition and tumor cell cytotoxicity by IgE *in vitro*. Human monocytes with hMOv18 IgE induced significantly-elevated IGROV1 cell ADCC compared with non-specific NIP IgE controls (Fig. 6A). hMOv18 IgE treatment was associated with significantly-elevated expression (relative to control IgE) of the same mediators found in lung extracts of IgE-treated rats *in vivo* (MCP-1, $P=0.002$; TNFα, $P=0.019$; IL-10, $P=0.025$), confirming their importance in IgE stimulation (Fig. 6B).

In ADCC assays, MCP-1 secretion with hMOv18 IgE was significantly reduced ($P=0.004$) by TNFα receptor-blocking antibodies (Fig. 6C), and tumor-specific hMOv18 IgE, but not the non-specific anti-NIP IgE, triggered upregulated MCP-1 mRNA expression by IGROV1 tumor cells. This effect was abrogated when TNFα signaling was blocked prior to stimulation with antibody ($P=0.032$; Fig. 6D). Therefore, MCP-1 production in the context of tumor antigen-specific IgE cytotoxicity is TNFα-dependent.

To gain insights into a potential clinical relevance of the TNFα/MCP-1 axis, we interrogated publicly-available ovarian carcinoma gene expression datasets (Fig. 6E). We found significant associations of improved 5-year overall survival with elevated levels of our herein reported IgE-mediated immune signatures (TNFα/MCP-1, $P=0.016$; TNFα/MCP-1/IL-10, $P=0.022$). These signatures were still associated with better overall survival alongside the macrophage marker CD68 ($P=0.04$) and with the high- and low-affinity IgE Fc receptors (FcεRI, $P=0.041$; FcεRII/CD23, $P=0.035$). These findings may suggest that, if enhanced, the TNFα/MCP-1 axis may provide a responsive immune signature with protective potential.

In summary, within tumors, cross-linking of MOv18 IgE on macrophages by FRα-expressing tumor cells induces macrophage TNFα upregulation. TNFα promotes elevated MCP-1 production by both tumor cells and macrophages, putatively acting as a potent chemoattractant, drawing macrophages into tumors. Enhanced tumor cell-macrophage interactions further stimulate production of TNFα and thus MCP-1, forming a self-enhancing circuit of macrophage influx into MOv18 IgE-treated tumors (Fig. 6F). This supports a link between TNFα stimulation and MCP-1 expression in response to effector cell engagement and IgE cytotoxic functions against cancer.

MOv18 IgE triggers anti-tumor ADCC by ovarian cancer patient effector cells

We evaluated the capacity of hMOv18 IgE to trigger anti-tumor ADCC by activating human immune effector cells from patients with ovarian carcinomas. PBMCs from patients with FRα-positive, FRα–negative and FRα–status unknown ovarian carcinomas (by immunohistochemistry, Fig. 7A), together with hMOv18 IgE, induced significantly-elevated ovarian carcinoma IGROV1 ADCC compared with non-specific NIP IgE-treated cells (Fig. 7B), and equivalent to that mediated by healthy volunteer PBMCs (Fig. 7C). Additionally, hMOv18 IgE mediated anti-tumor ADCC against 3 FRα-expressing tumor cell lines (human ovarian carcinoma IGROV1 and SKOV3, and rat colon adenocarcinoma CC531tFR), but minimum ADCC against FRα-dim and FRα-negative cancer cell lines (human ovarian carcinoma TOV21G, and human breast carcinoma SKBR3 respectively, Fig. 7D, Supplementary Fig. S4). Therefore, IgE-engendered anti-tumor functions are target antigen-specific, and have potential application with patient immune effector cells and against different target-expressing tumor cells.

Discussion

We report the superior anti-tumor efficacy of TAA-specific IgE in an immunocompetent syngeneic rat model of cancer, uniquely suitable for the study of IgE anti-tumor effector functions. We furthermore describe a previously-unappreciated putative contribution of a TNFα/MCP-1 cascade to monocyte and macrophage repolarization and recruitment, delineated in the human IgE and human effector and tumor cell functional context.

The functional significance of this IgE-induced TNFα/MCP-1 axis is supported by: a) enhanced intra-tumor infiltration by macrophages following IgE treatments; b) a TNFα-expressing lung macrophage compartment, and elevated TNFα and MCP-1 concentrations in BAL from IgE- but not IgG-treated rats; c) macrophage recruitment correlating with tumor growth restriction in IgE-treated syngeneic rat and human xenograft mouse models of cancer; d) the ability of human IgE to trigger monocytes to upregulate TNFα in a class-specific manner; e) the induction of MCP-1 production by TNFα in monocytes and tumor cells, in turn shown to promote a monocyte chemotactic response, f) the involvement of TNFα/MCP-1 in antigen-specific human IgE tumor ADCC, abrogated with TNFα receptor-specific blockade on monocyte effector cells and, g) publicly-available ovarian carcinoma gene expression datasets indicating associations between elevated levels of TNFα/MCP-1 with better survival. These findings reveal an immune cascade through which TAA-specific IgE may focus macrophages towards tumor cells.

Rat and human MOv18 IgE showed comparable monocyte-mediated tumor cell killing. In immunocompetent rats, rMOv18 IgE demonstrated potential to compete favorably with IgG in a metastatic setting, and to restrict tumors in highly-vascularized organs like the lung, where IgG (based on its long half-life in the circulation) would have a major advantage. Rat and human MOv18 IgE treatment-associated macrophage infiltration into tumors correlated with tumor growth restriction and better efficacy in the immunocompetent rat and human xenograft mouse models. This supports a function of recruiting macrophages towards tumor cells associated with IgE therapy *in vivo*. Stromal macrophages can express matrix-degrading, matrix–producing and pro-angiogenic factors (36), allowing regulation of stromal remodeling and neo-vascularization to support tumors. Conversely, macrophage density within tumor islets has been positively associated with patient survival (37, 38), and tumor islet-resident macrophages can express IL-1α, IL-1β, IL-6, NOS and TNFα, the latter two thought to be involved in target cell–killing mechanisms of macrophages (39, 40). These mechanisms may fail to be effectively deployed against tumors, perhaps partly due to alternatively-polarized humoral immunity in cancers favoring antibody isotypes that fail to activate key effector cells such as macrophages (41–45). Here we show that tissue-resident macrophage subsets in IgE-treated cohorts are polarized to express the macrophage maturation and co-stimulatory marker CD80, as well as higher levels of pro-inflammatory TNFα. TNFα levels were also enhanced in BAL fluids of IgE-treated but not of IgG-treated rats. TNFα can also be expressed by activated macrophages known to act as effector cells in IgE-mediated parasite control (23–25). Our findings now directly link tumor-immune cell interactions engendered by IgE with potentiating TNFα production, re-education and recruitment of monocytes and macrophages into tumors. These functions may be further explored in future studies that may include effector cell depletion or enrichment with different polarized phenotypes. Based on expression of TNFα, but not IL-4 *in vitro* and *in vivo* in the context of tumor antigen-specific IgE, our findings also suggest that macrophage activatory, but most likely not allergic, mechanisms might be employed by TAA-specific IgE to restrict the growth of tumor metastases. IL-4 has been shown to be a negative factor in IgE/FcεRI cross-presentation by DCs and generation of cytotoxic T lymphocytes (12). It is therefore possible that during Th2-type inflammation, IL-4 may prevent a TNFα/MCP-1 axis-driven macrophage response.

The CC chemokine, M1 macrophage mediator and potent chemoattractant MCP-1 was also elevated in BAL fluid of rMOv18 IgE-treated rats. Cross-linking of macrophage-bound IgE by densely-expressed antigens on the surface of target cells, more likely to occur in tumor lesions, can trigger TNFα, which in turn may stimulate MCP-1. Our findings suggest that cross-linking on the monocyte surface by IgE, but not by IgG, may initiate this TNFα response. Monocytes and cancer cells respond to TNFα signals by producing MCP-1, consistent with increased recruitment of macrophages into tumors following rMOv18 IgE treatment. The high affinity of IgE for FcεRs on tissue-resident monocytes and macrophages may result in sustained antibody retention, thus favoring cross-linking of IgE by multivalent TAAs. This class-specific immune complex formation may potentiate TNFα production in tumor microenvironments and subsequent sustained *in situ* MCP-1 secretion by different cell subsets, providing an advantage for IgE immunotherapy against cancer.

In human IgE, human monocyte and human tumor cell functional studies, TNFα stimulated tumor cells to upregulate MCP-1, which can trigger monocyte chemotaxis. We confirmed the relevance of both TNFα and MCP-1 in ADCC assays, where cross-linked IgE on the surface of human monocytes upregulated TNFα and MCP-1 production. Loss of MCP-1 production by tumor cells, and significant reduction in IgE-dependent tumor cell cytotoxicity when TNFα signaling was blocked, also suggest that MCP-1 upregulation and IgE anti-tumor functions require TNFα. We and others also demonstrate that tumor cells can be stimulated to produce MCP-1 (46–49). Additionally, potential synergistic effects between MCP-1 and IgE-mediated anti-tumor mechanisms were shown with an anti-MUC1 IgE antibody co-administered with MCP-1, to MUC1-expressing tumor-bearing hFcεRIα transgenic mice (50). Together, our findings thus point to the putative involvement of a TNFα/MCP-1 cascade triggered by IgE-mediated cross-talk between effector cells and tumor cells, and implicated in tumor cell growth restriction *in vitro* and *in vivo*. Conversely, we observed elevated levels of IL-10 in rMOv18 IgE-treated rat lungs and in effector cell stimulation assays. *In vitro* infection of human macrophages with *Toxoplasma gondii* also upregulated IL-10, inversely correlating with iNOS and NO generation, and IL-10 was found to attenuate IgE-mediated parasite elimination by macrophages (25). IL-10 could therefore provide an *in vivo* mechanism for restricting IgE-induced effector cell activation, and requires further study.

In summary, MOv18 IgE-potentiated tumor-restricting effects are superior to those of IgG in immunocompetent rats. IgE immune complex formation on monocytes and macrophages, initiated through cross-talk with TAA-expressing cancer cells, promotes effector cell polarization, activation and recruitment. This process is driven by TAA IgE-dependent TNFα and MCP-1 upregulation. Our findings draw parallels with physiological roles of IgE in anti-parasitic immune surveillance but not in allergy. Engineering antibodies with Fc regions that confer unique effector cell-polarizing properties against cancer may open a new avenue for addressing tumor resistance to immune clearance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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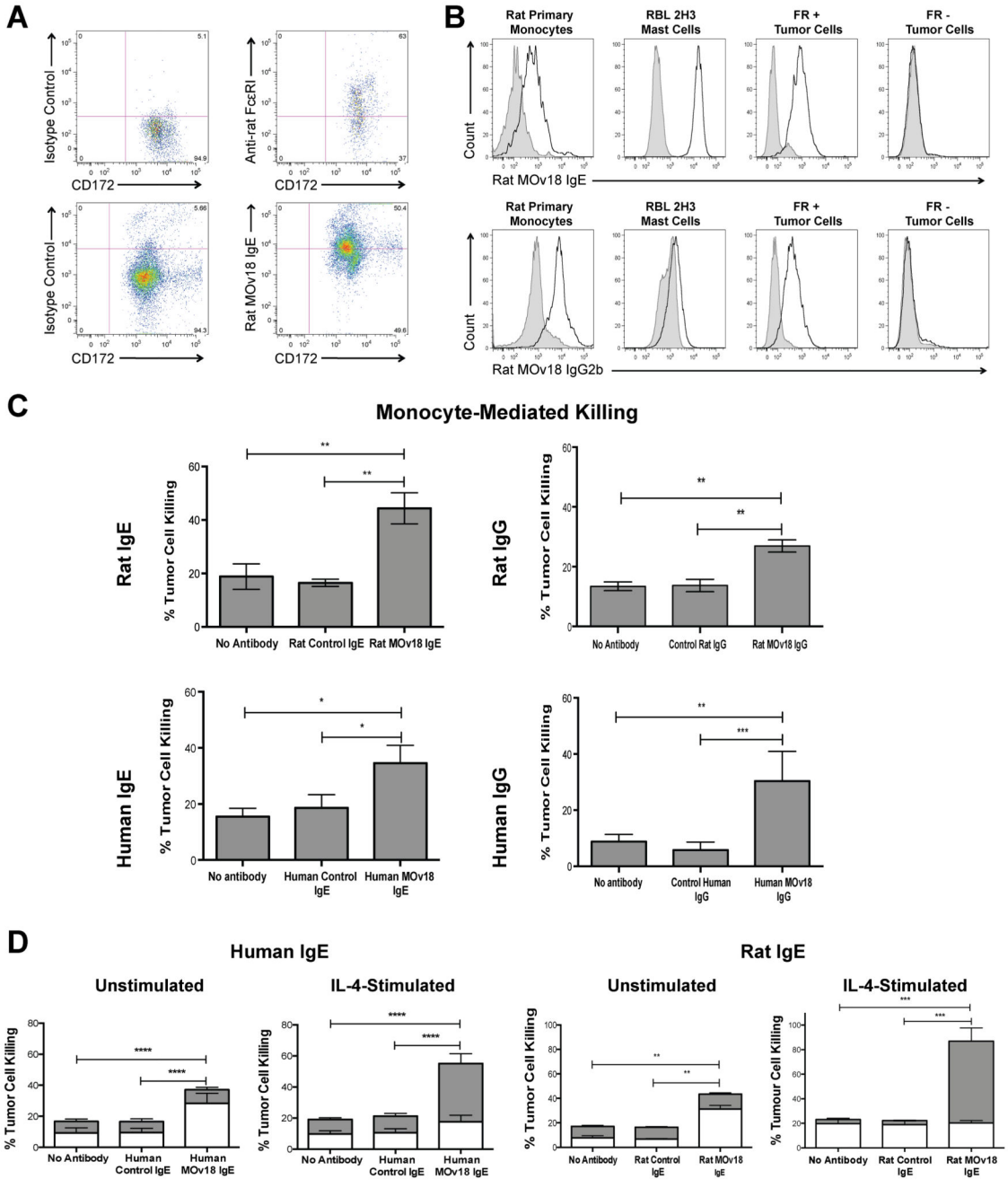

Figure 1. Rat and human MOv18 antibodies display comparable effector functions.

(A) Anti-rat FcεRI antibody or rMOv18 IgE bind to primary (CD172+) rat monocytes. (B) rMOv18 IgE and IgG2b bind to rat primary monocytes, RBL-2H3 rat mast cells, FRα-expressing rat colon adenocarcinoma CC531tFR cells, and non-FRα-expressing human melanoma A375 cells (grey: non-specific isotype control or detection antibody control). (C) Quantification of ADCC against FRα-expressing tumor (rat CC531tFR and human ovarian carcinoma IGROV1) cells by rat or human MOv18 IgE- or IgG-primed primary monocytes. (D) Quantification of ADCC and ADCP against FRα-expressing CC531tFR tumor cells by

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rat or human MOv18 IgE-primed unstimulated (CD23-) or IL-4 stimulated (CD23+) monocytic cells, compared with non-specific antibody treatments or cells alone (n=3).

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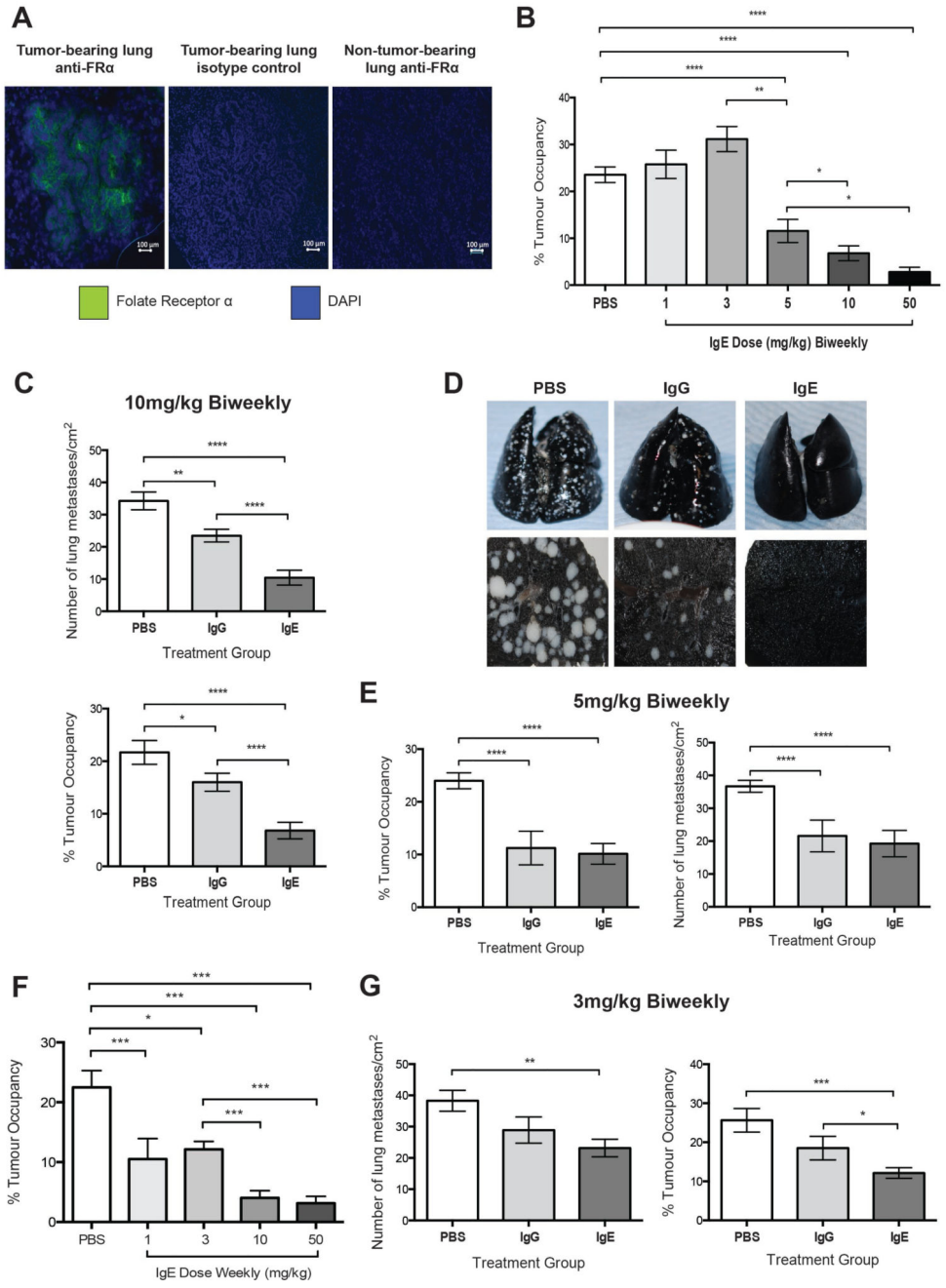

Figure 2. Rat MOv18 IgE demonstrates superior tumor growth restriction.

(A) FRα expression *in vivo* 26 days post-tumor challenge. Representative immunofluorescent microscopy images from lungs of tumor-bearing and healthy rats (n=5). (B) Percentage (%) tumor occupancy quantified following two biweekly (Q2W) doses of PBS (n=20), or 1 (n=3), 3 (n=2), 5 (n=11), 10 (n=11) and 50 (n=2) mg/kg rMOv18 IgE. (C, E) Number of metastases/cm^2 and % tumor occupancy quantified for PBS-treated rats (n=22) and rats treated with rMOv18 IgE (n=11) and IgG2b (n=11) at 10mg/kg and 5mg/kg Q2W. (D) Representative images of Indian ink-stained lungs from rMOv18 IgE-, IgG2b-

(10mg/kg) and PBS-treated rats. (F) Percentage tumor occupancy quantified following weekly doses of rMOv18 IgE (n=10 per dose) or PBS (n=17) (mean of 1-3 independent experiments for B, C, E and F). (G) Number of metastases/cm^2 and % tumor occupancy quantified for rats treated weekly with PBS or with rMOv18 IgE or IgG2b at 3mg/kg.

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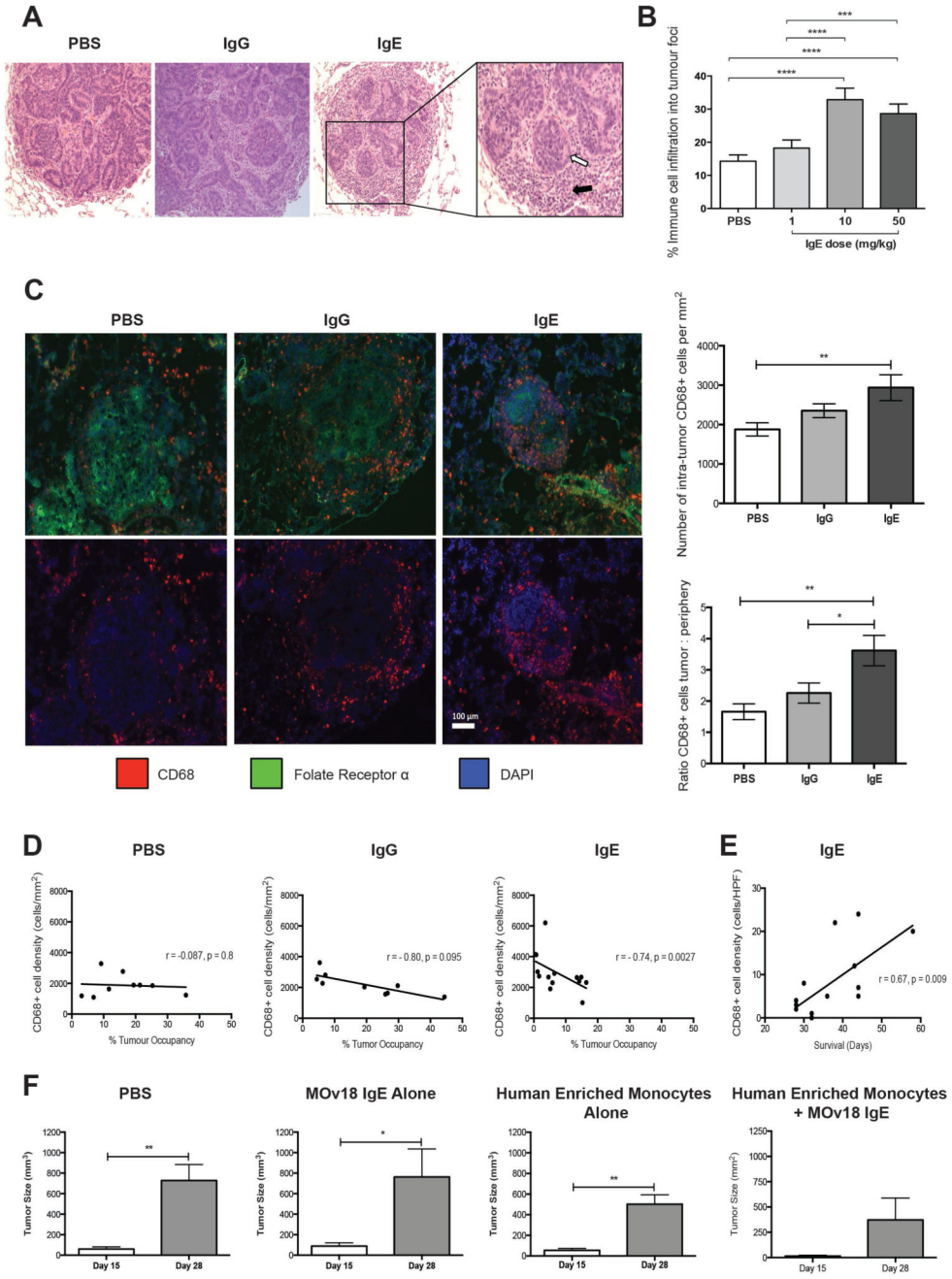

Figure 3. MOv18 IgE treatment induces CD68+ macrophage infiltration into tumors.

(A) Paraffin-embedded lung sections from PBS-, rMOv18 IgG-, and rMOv18 IgE-treated rats stained with H&E, and (B) percentages of immune cell infiltration into tumor foci quantified (demarcation strategy: Supplementary Fig. S1) (5 data points/animal, 5 animals/group). Magnification 200x. White arrow: glandular tumor islet; black arrow: immune and stromal cells. (C) Representative cryosections of lung from PBS-, rMOv18 IgG-, and rMOv18 IgE-treated rats co-stained with anti-human FRα (green) and anti-rat CD68 (red) antibodies. Intratumoral CD68+ cell density/mm^2 (right panel, top) and ratio of

tumour:peripheral CD68+ cells (right panel, bottom) were calculated. IgE (n=14), IgG (n=17), PBS (n=24). (D) Correlation of within-tumor CD68+ macrophage density with % tumor occupancy in rats treated with PBS (n=9), rMOv18 IgE (n=13) and rMOv18 IgG2b (n=9). (E) Correlation of within-tumor CD68+ macrophage density with survival of nude mice with intraperitoneal human ovarian carcinoma xenografts, treated with human PBMC and hMOv18 IgE (n=13). (F) Inhibition of subcutaneous IGROV1 tumor growth in SCID mice treated with PBS (n=4), hMOv18 IgE (n=4), monocyte-enriched effector cells (n=5), or monocyte-enriched effector cells plus hMOv18 IgE (n=5).

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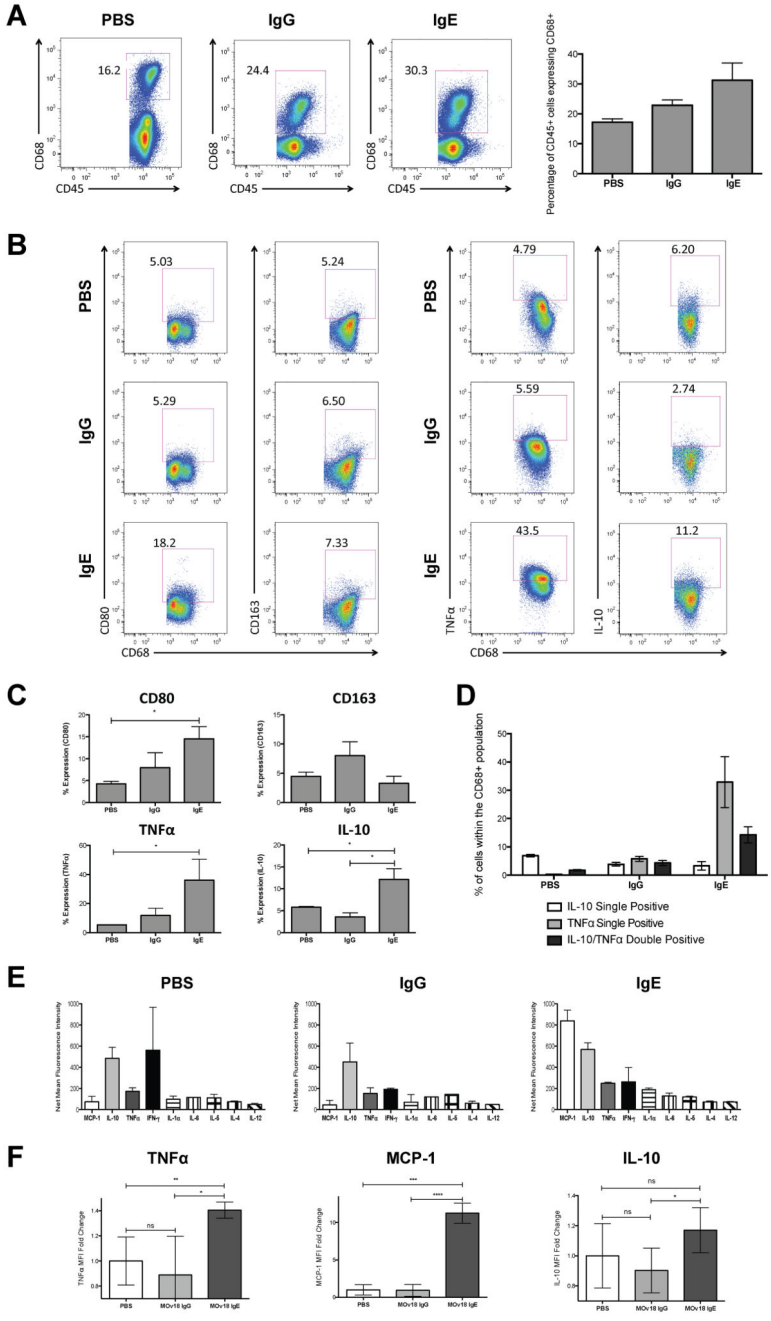

Figure 4. Macrophages infiltrating tumors in IgE-treated rats are differentially-polarized.

(A) Single cell suspensions, isolated from lungs of rats inoculated with CC531tFR tumor cells 26 days previously, were stained with anti-CD45 and anti-CD68 antibodies. Infiltrating CD68+ macrophages were identified following successive gating on $FSC^{hi}SSC^{low\text{-}hi}$ and CD45+ populations (Supplementary Fig. S3) and the percentage of CD68+ cells in the CD45+ populations calculated (right panel). (B, C) Percentages of lung CD68+ macrophages expressing CD80, CD163, TNFα and IL-10. (3 independent experiments for Figs. A-C). (D) Percentages of single- and double-positive (TNFα+/IL-10+) cells within the

infiltrating CD68+ macrophage populations (n=3). (E) Cytokine/chemokine production measured in BAL fluid from tumor-bearing rats given rMOv18 IgE, rMOv18 IgG2b, or PBS (n=3 per group). (F) For selected analytes, fold change values represent mean fluorescence intensity (MFI) for each replicate within the treatment (IgE or IgG) groups, divided by that of the PBS group. Results represent 2 independent experiments.

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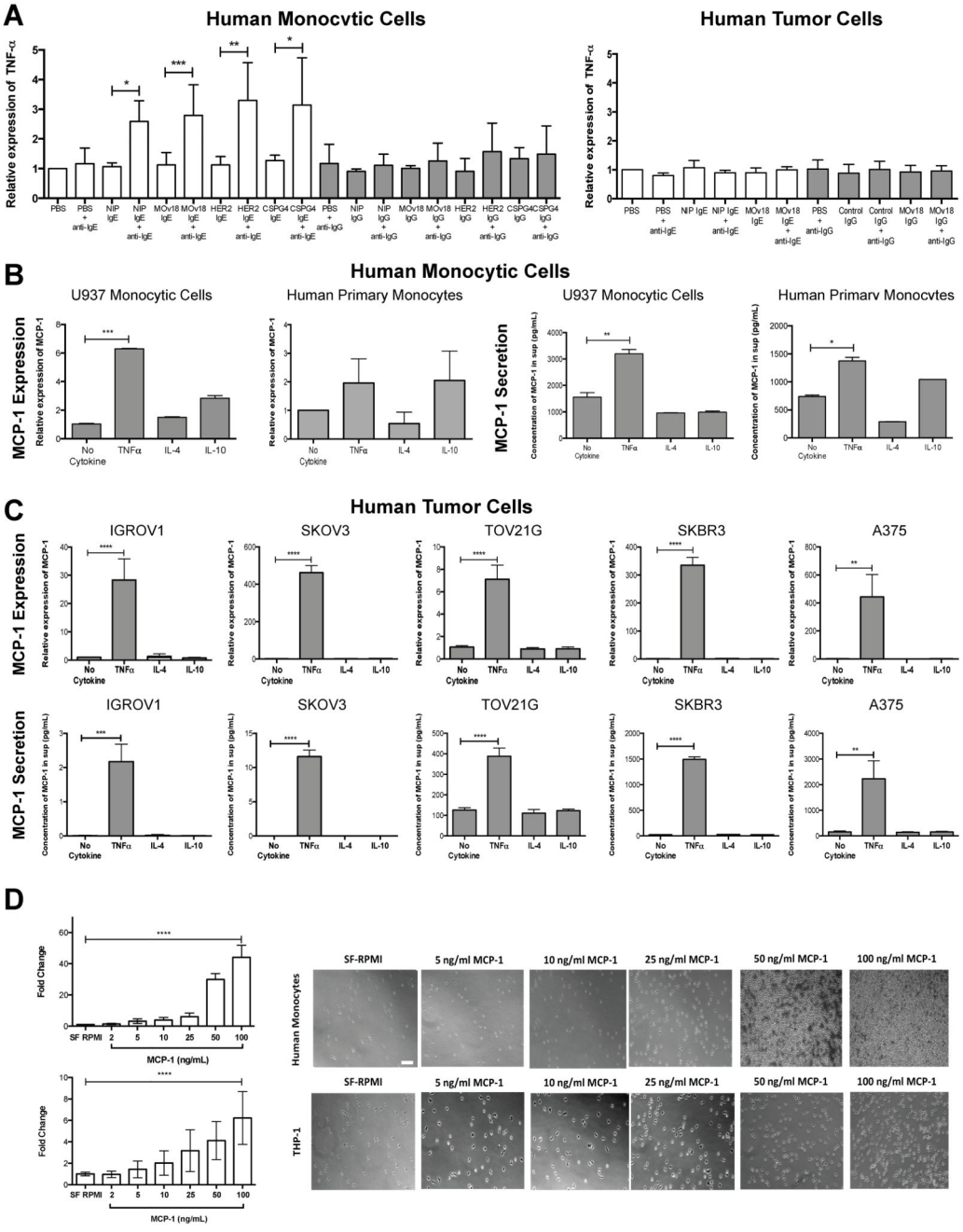

Figure 5. IgE cross-linked on monocytes triggers TNFα, which stimulates MCP-1.

(A) Comparative real-time PCR analysis of TNFα expression by U937 monocytes (left) or FRα+ human ovarian carcinoma IGROV1 tumor cells (right) following cross-linking of human IgEs and IgGs of different antigen specificities by polyclonal antibodies for 1 hour (n=4). (B, C) Effects of TNFα, IL-4 and IL-10 (20ng/mL) stimulation on relative mRNA expression and secretion of MCP-1 by U937 monocytes (B; 10-hour stimulation), human primary monocytes (B; 5-hour stimulation) and human ovarian carcinoma IGROV1, SKOV3 and TOV21G, human breast carcinoma SKBR3, and human melanoma A375 tumor cells (C;

10-hour stimulation). (D) Quantitation of human primary monocytes (top left) or THP-1 human monocytic cells, (bottom left) (representative images, right) migrating through a trans-well membrane in response to increasing MCP-1 concentrations (n=3).

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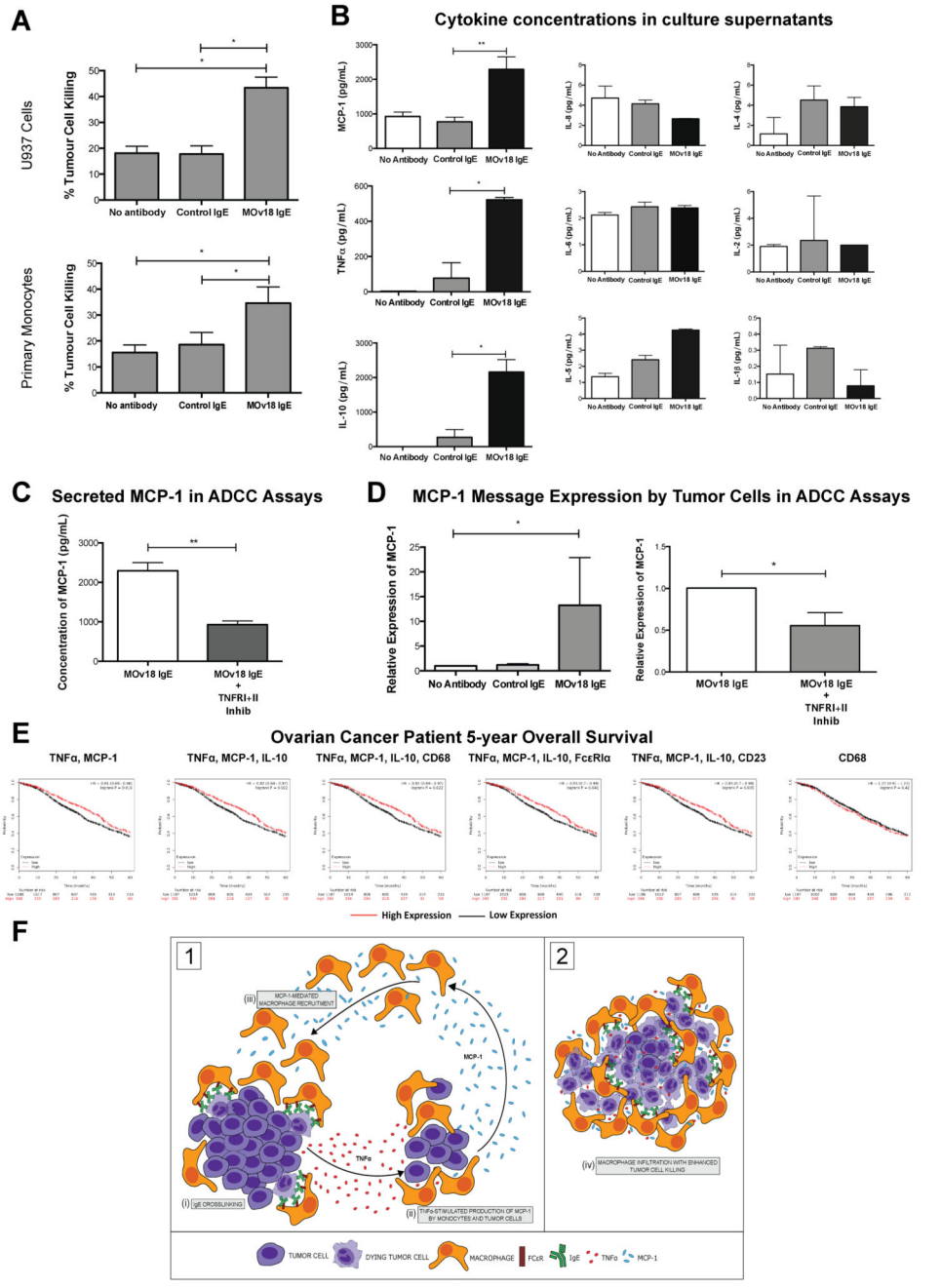

Figure 6. TNFα and MCP-1 are involved in IgE-mediated tumor killing and may be associated with better patient survival.

(A) hMOv18 IgE effects significantly-elevated killing of human ovarian carcinoma IGROV1 cells by human U937 monocytes and primary monocytes *in vitro*, compared with non-specific anti-NIP IgE controls (n=3). (B) Concentrations of cytokines/chemokines secreted in cultures of human primary monocytes incubated with IGROV1 cells and hMOv18 IgE, control antibody NIP IgE or no antibody. (C) MCP-1 secreted in cultures of U937 monocytes incubated with IGROV1 cells and hMOv18 IgE was reduced when cells were incubated with blocking antibodies against the human TNFα receptors I and II prior to

incubating with hMOv18 IgE (representative of two independent experiments). (D) MCP-1 mRNA expression by IGROV1 cells was elevated following co-incubation with U937 monocytes and hMOv18 IgE compared to controls (left), and reduced MCP-1 expression detected when IGROV1 cells were incubated with blocking antibodies against the human TNFα receptors I and II prior to treatment with hMOv18 IgE (right). (E) Kaplan-Meier curves showing higher TNFα/MCP-1, TNFα/MCP-1/IL-10, TNFα/MCP-1/IL-10+CD68, FcεRIα or CD23 expression in 1582 ovarian cancers is significantly associated with improved 5-year overall survival. Ovarian tumor CD68 expression alone does not correlate with patient outcome. (F) Model summarizing a proposed mechanism of MOv18 IgE immunotherapy. (1) A TNFα/MCP-1 axis promotes potent recruitment of further macrophages into tumors, (2) resulting in enhanced tumor cell-macrophage interactions and subsequent tumor cell death.

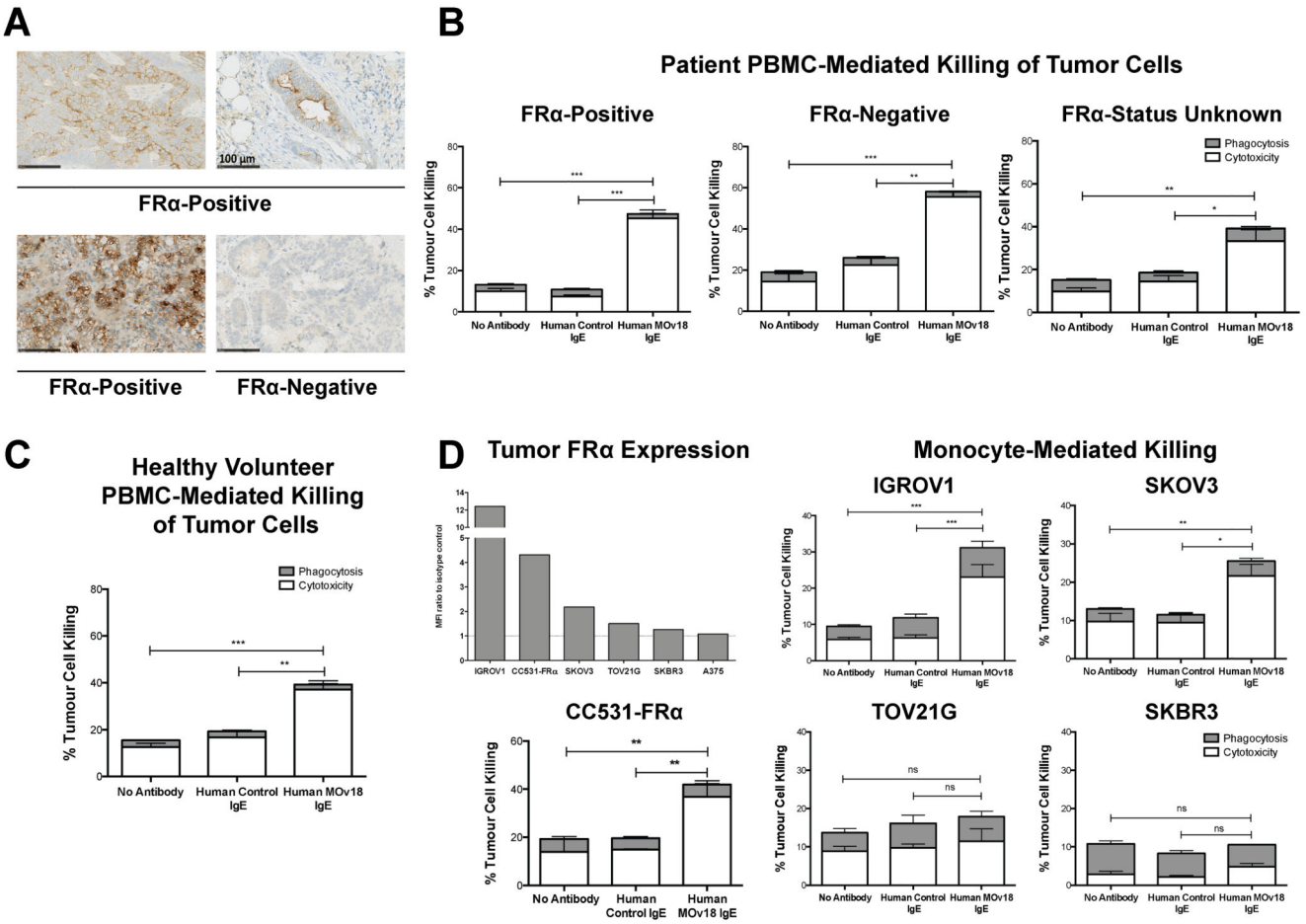

Figure 7. MOv18 IgE triggers ADCC of different FRα-expressing tumor cell lines by human effector cells from patients with ovarian cancer

(A) Paraffin-embedded sections of tumors from patients with ovarian cancer were stained to detect patients with FRα-positive (n=3) and FRα-negative (n=1) tumors. (B, C) ADCC/ADCP of FRα-expressing human ovarian carcinoma IGROV1 cells by hMOv18 IgE-primed PBMCs from patients (B) with FRα-positive (n=3), FRα-negative (n=1) and FRα-status unknown (n=2) tumors and from healthy volunteers (n=2) (C). (D) hMOv18 IgE effects significantly-elevated killing of FRα-bright human ovarian carcinoma IGROV1 (n=3) and SKOV3 (n=3), and rat colon adenocarcinoma CC531tFR (n=2) tumor cells by monocytic cells, compared with non-specific anti-NIP IgE. Minimal killing of FRα-dim human ovarian carcinoma TOV21G (n=3) and human breast carcinoma SKBR3 (n=2) tumor cells by hMOv18 IgE was detected.