Online Data Supplement

Host-derived interleukin-5 promotes adenocarcinoma-induced malignant pleural effusion

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Supplementary Table 1. Gene expression of Lewis lung cancer cells after 24 hours of incubation with saline or 22 pM rmIL-5. No significant changes in gene expression were induced by IL-5. Adjusted level of significance: $P < .05$ (*ad hoc*) / 28853 (number of comparisons) = 1.733×10^{-6}. *ΔGE*, fold change in gene expression induced by 22 pM rmIL-5.

Affymetrix Probeset ID	*ΔGE*	*P*
10341210	-2.07983	0.000155407
10341353	-1.90669	0.0463393
10343628	-1.85239	0.00885216
10339479	-1.79559	0.00390373
10341359	-1.78524	0.0192706
10338720	-1.78323	0.0305543
10340103	-1.7799	0.026738
10342705	-1.71943	0.00444225
10342970	-1.70722	0.00190568
10341398	-1.70252	0.0186169
10339511	-1.67343	0.00803461
10343462	-1.66321	0.0345591
10342803	-1.65726	0.00863342
10340623	-1.6511	0.000812849
10343872	-1.64801	0.0289081
10342031	-1.64425	0.0122719
10343549	-1.61672	0.013231
10341940	-1.55966	0.0433129
10341467	-1.55898	0.00304853
10340616	-1.54663	0.0489859
10340317	-1.54252	0.0290594
10344096	-1.54127	0.0331862
10342487	-1.51282	0.0264627
10340679	-1.5083	0.021617
10341454	1.50309	0.0357003
10339543	1.50428	0.0418064
10339116	1.50839	0.0428513
10341800	1.50974	0.029728
10340158	1.51132	0.0322329
10344068	1.51428	0.0469952
10342873	1.51535	0.0224996
10340496	1.51696	0.0317885
10342285	1.51767	0.0017915
10341815	1.5194	0.0292556
10339564	1.52208	0.0257002
10338161	1.52345	0.0224139
10338886	1.52485	0.0265639
10340284	1.52722	0.0250351
10341093	1.53288	0.0230356
10338997	1.53691	0.00734256

10338258	1.5403	0.042514
10339446	1.54746	0.0296148
10341602	1.54908	0.0377937
10341653	1.55021	0.000743042
10341721	1.55168	0.0230421
10343116	1.55262	0.0161803
10339646	1.55712	0.0351343
10343593	1.56328	0.0348556
10342876	1.56663	0.0436713
10344095	1.56778	0.00609033
10344117	1.56835	0.0352524
10342526	1.56959	0.027258
10340989	1.57296	0.0458452
10343209	1.57315	0.0119869
10341293	1.5744	0.0495925
10342535	1.5749	0.00796239
10342418	1.57753	0.0478459
10343782	1.58284	0.0493901
10339429	1.58358	0.0441074
10338238	1.58467	0.0159061
10340526	1.58961	0.0418818
10343793	1.59239	0.00940803
10340577	1.59274	0.0425718
10339998	1.59505	0.0164694
10342144	1.6053	0.0022805
10339269	1.61857	0.00338332
10339677	1.63056	0.0408487
10344085	1.63112	0.0370942
10338895	1.63163	0.0428518
10341012	1.63462	0.0355319
10338250	1.63789	0.0417547
10341357	1.6411	0.00946866
10341410	1.64775	0.0151134
10339628	1.65379	0.0216482
10341919	1.66757	0.00789505
10344264	1.66899	0.0469876
10343148	1.67663	0.00586254
10343235	1.67704	0.00720929
10340583	1.67841	0.0356176
10338432	1.68166	0.0312474
10339234	1.68792	0.0441162
10340098	1.69088	0.0346975
10338187	1.69242	0.0158717
10340746	1.69579	0.0271573
10338794	1.71747	0.0398851
10343210	1.72244	0.0130012

10338857	1.72269	0.0254156
10344580	1.72618	0.00422919
10344061	1.72649	0.00828588
10341813	1.73228	0.0122626
10339303	1.7332	0.0289371
10338414	1.73612	0.00977282
10341521	1.73822	0.0142351
10339668	1.74636	0.0249786
10343957	1.74892	0.0114094
10339033	1.76745	0.0281065
10342062	1.7768	0.0339842
10343877	1.77836	0.0372624
10338700	1.78521	0.0396419
10341211	1.79137	0.0371974
10338417	1.7983	0.0382119
10338303	1.80322	0.0137312
10339490	1.80423	0.0061599
10340280	1.80721	0.02079
10338756	1.80896	0.0102024
10340337	1.81795	0.0169445
10341750	1.82176	0.0023629
10339832	1.82449	0.0187162
10343007	1.82937	0.0180884
10341317	1.84473	0.0282066
10339914	1.84525	0.0249933
10339001	1.84534	0.0105515
10342275	1.84558	0.00959874
10339930	1.84709	0.00909966
10338478	1.85037	0.0186569
10339579	1.86831	0.00709304
10342185	1.87407	0.0267704
10342214	1.88149	0.012501
10339942	1.91501	0.0139258
10340411	1.92446	0.00747026
10340724	1.92482	0.0250155
10338884	1.93348	0.00592814
10339743	1.94737	0.040844
10344538	1.96625	0.00828673
10338639	2.00404	0.00753723
10342279	2.02883	0.00355771
10343834	2.04416	0.0256672
10341978	2.04758	0.00533303
10344513	2.0635	0.000751615
10339618	2.07443	0.0453189
10341184	2.121	0.0279251
10342132	2.12788	0.0340778

10341368	2.17703	0.00443723
10342364	2.17891	0.0137549
10343651	2.18676	0.047059
10341488	2.19042	0.0373971
10343645	2.19253	0.044078
10341037	2.22633	0.0401524
10341338	2.30682	0.0124736

DETAILED MATERIALS AND METHODS

Reagents: rmIL-5 was from R&D Systems (Minneapolis, MN); 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay from Promega (Madison, WI); anti-proliferating cell nuclear antigen (PCNA) antibody from SantaCruz Biotechnology (Santa Cruz, CA); terminal deoxynucleotidyl nick-end labeling (TUNEL) kit from Roche Molecular Biochemicals (Penzberg, Germany); anti-factor VIII-related antigen (fVIIIra) antibody from Invitrogen (San Francisco, CA); Biebrich's scarlet from Sigma Chemical (St Louis, MO); and phycoerythrin-Cy7-conjugated anti-CD11b and allophycocyanin-conjugated anti-Gr1 antibodies from BD Pharmingen (Palo Alto, CA). TRFK5 rat anti-mouse IL-5 antibody was prepared from the supernatants of TRFK5 hybridoma as described previously (E1). Rat IgG_{2a} was from Peprotech EC (London, UK).

Human Pleural Effusions: Pleural fluid and matched serum were obtained during the initial diagnostic thoracenteses performed in 55 consecutive patients with MPE (causative neoplasms: 10 non-small cell lung cancers, 21 adenocarcinomas, 3 small cell lung cancers, 1 large cell lung cancer, 6 mesotheliomas, 7 breast adenocarcinomas, 1 each of gastric adenocarcinoma, thyroid adenocarcinoma, Hodgkin's and non-Hodgkin's lymphoma, chronic myelomonocytic and lymphocytic leukemia, and adenocarcinoma of unknown primary), and in 20 patients with congestive heart failure (CHF) who were treated at the General Hospital Evangelismos, Athens, Greece, from September 2006 through May 2008. The study was approved by the ethics committee of the hospital, and all patients gave written informed consent. Human MPE fluids and matched sera were handled as described

previously (E2,E3). MPE was diagnosed by positive pleural fluid cytology or pleural tissue histology (E4,E5). Pleural effusion due to CHF was diagnosed using a constellation of clinical, radiologic, and laboratory findings, including: 1) transudative effusion, 2) echocardiographic evidence of left ventricular dysfunction, 3) serum pro-brain natriuretic peptide levels above 1500 pg/ml, 4) increased heart size on the chest X-ray, 5) negative pleural fluid bacteriology and cytology, and 6) improvement with diuretic and/or inotropic drugs (E6).

Animal Models: *il5+/+* and *il5-/-* mice generated on a C57BL/6 background (E7) were purchased from the Jackson Laboratory (Bar Harbor, MN) or the Biomedical Sciences Research Center Alexander Fleming (Vari, Greece) and inbred at the Animal Care facilities of Vanderbilt University Medical Center (Nashville, TN) or the General Hospital Evangelismos (Athens, Greece). Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee at Vanderbilt University or the Veterinary Administration Bureau of the Prefecture of Athens, Greece, and were conducted according to International Animal Care and Use Guidelines (http://grants.nih.gov/grants/olaw/GuideBook.pdf). Mice used for experiments were sex-, weight (19-24 g)-, and age (8-10 week)-matched. For MPE generation, intrapleural injections of LLC and MC38 cells (1.5×10^5/50 μl PBS/mouse) (day 0), as well as sacrifice and specimen collection (day 14 after LLC and day 11 after MC38 cells) were performed as described previously (E2,E3,E8). For subcutaneous tumor formation, LLC or MC38 cells (5×10^5/50 μl PBS/mouse) were injected into the dermis of the rear flank. Three vertical tumor dimensions (δ1, δ2, δ3) were measured weekly, and tumor volume (V) was determined using the formula $V = \pi \times (\delta 1 \times \delta 2 \times \delta 3) / 6$.

Bioluminescence imaging: Serial bioluminescence imaging of live mice bearing *pNGL* LLC or MC38-induced MPEs was done using intravenous injection of 1 mg D-luciferin, and of cultured *pNGL* LLC or MC38 cells using addition of 10 mM D-luciferin. Imaging was performed using a Xenogen IVIS cooled charged couple device (Xenogen, Alameda, CA). Data were analyzed using Living Image v.2.50 (Xenogen) and IgorPro (Wavemetrics, Lake Oswego, OR) (E2,E3,E8).

Cell Culture: LLC mouse and A549 human lung adenocarcinoma, and mouse skin melanoma (B16F10) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and MC38 colon adenocarcinoma cells from the Tumor Repository of the National Cancer Institute (Frederick, MD). Cells were cultured at 37^0C in 5% CO_2-95% air using Dulbecco's modified Eagle's medium (DMEM) 10% fetal calf serum (FCS) supplemented with glutamine and 100 mg/l penicillin/streptomycin. *pNGL* MC38 cells stably expressing a NF-κB reporter (NF-κB.GFP.LUC; *pNGL*) were generated similar to *pNGL* LLC cells, as has been described elsewhere (E8).

Cytokine determinations: Mouse and human IL-5 (detection limits: 7.0 and 3.0 pg/ml, respectively), mouse VEGF (detection limit: 31.3 pg/ml), mouse monocyte chemoattractant protein (MCP)-5 (detection limit: 3.0 pg/ml), and mouse macrophage inflammatory protein (MIP)-2 (detection limit: 15.6 pg/ml) were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems and Peprotech EC). Mouse tumor necrosis factor (TNF)-α, interferon (IFN)-γ, (MCP)-1, and IL-6, IL-10, and IL-12p70 were measured using a cytometric bead array (CBA; BD, San Diego, CA) (detection limits: 7.3, 2.5, 52.7, 5.0, 17.5, and 10.7 pg/ml,

respectively) (E2,E3,E8). Cellular expression levels of these proteins were normalized for total protein.

In vivo delivery of exogenous rmIL-5: Forty ng rmIL-5 in 100 µl phosphate-buffered saline (PBS) or PBS alone (control) were delivered intravenously to the retro-orbital veins of mice at days 2, 4, 6, 8, 10, and 12 after LLC cells, a regimen previously shown to effectively reconstitute endogenous levels of the cytokine in *il5*-/- mice (E9).

In vivo IL-5 neutralization: For *in vivo* IL-5 neutralization, mice with LLC-induced MPE received intraperitoneal injections of 1 mg/kg TRFK5 in 100 µl PBS (rat IgG_{2a} served as control), a dose reported to achieve steadily effective levels of circulating antibody (E10). Mice were treated with TRFK5 in two protocols: a "prevention" protocol on days 0 and 8 after LLC cells and a "regression" protocol on day 8 after tumor cells. This latter study design was aimed at unveiling potential therapeutic effects of TRFK5 against already established MPE, since they develop around day 8 in the original mouse model (E8,E11).

Pleural tumor enumeration and processing: Pleural tumors were enumerated by three independent and blinded readers under a dissecting microscope, and the average number was used for analyses.

Histology: Mouse lungs and attached tumors were fixed in 10% neutrally buffered formalin (24 hours) and 70% ethanol (3 days). Tumors were dissected and embedded in paraffin. 5-µm-thick sections were cut, mounted on glass slides, and stained with hematoxylin and eosin. Alternatively, tissue sections were immunostained using antibodies for PCNA, TUNEL, and fVIIIra, and immune-

labeling was quantified, as described previously (E2,E3,E8,E11), or stained with Biebrich's scarlet for eosinophils using Luna's technique (E12).

Polymerase chain reaction (PCR) and gene expression profiling: Total RNA from PBS or rmIL-5-treated (1 ng/ml = 22 pM = KDa for binding of IL-5 to its receptor IL5R) LLC cells (pooled samples from triplicates from three independent experiments) was isolated by homogenization in ice-cold Trizol reagent (Invitrogen, Carlsbad, CA) followed by a single passage through an RNAeasy column (Qiagen, Hilden, Germany). Isolated total RNA was reverse transcribed with Superscript Reverse Transcriptase II (Invitrogen), and the cDNA was indirectly labeled using the amino-allyl cDNA labeling method. Primers and conditions used for PCR were: sense 5'-GCCCTTTGATCAGCTGTTCAGTCCAC-3'; antisense 3'-CTGGTCCAACAAAGGTGGCCAAGGC-5'; 35 cycles with an annealing temperature of 61 °C, as described previously (E13). For microarray, experimental samples were mixed with isomolar amounts of the baseline sample (which was used as a common reference sample throughout) and hybridized in triplicates to cDNA glass microarray slides (GeneChip Mouse Gene 1.0 ST Arrays, Affymetrix, Santa Clara, CA) interrogating 28853 genes. After image analysis, all microarray data were subjected to preprocessing, Lowess normalization, centering, and/or averaging. To select differentially expressed genes, we employed both a parametric and a nonparametric ANOVA (Kruskal-Wallis), using proprietary algorithms implemented in MATLAB v7.1.14 (MathWorks, Natick, MA). No filtering criteria were applied and gene expression was directly compared between groups. Validation of 20 genes was performed at the RNA and protein levels using RT-PCR, ELISA, Western blot, and/or CBA, as appropriate. Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MEXP-2490.

Cytology: 50,000 pleural fluid cells were used for cytocentrifugal specimen (cytospin) preparation. The slides were air dried, fixed in methanol for 10 s, and stained with May-Gruenwald-Giemsa. Distinct cell types were enumerated as a percentage of 500 cells on the slide.

Biochemical and cellular assays: Protein was determined using the Bio-Rad protein assay (Hercules, CA). A MTS assay was used to assess cell viability (Promega). For cell experiments, cells were plated at equal densities in 12- or 96-well culture dishes. Indicated treatments were applied when cells were 20-30% confluent. All cell experiments were done in triplicate.

Flow cytometry: After red blood cell lysis using 30-minute exposure to 1% NH_4Cl, blood and pleural fluid cells were suspended in PBS 1% bovine serum albumin, stained with phycoerythrin-Cy7-conjugated anti-CD11b and allophycocyanin-conjugated anti-Gr1 antibodies at 0.1 $\mu g/10^6$ cells for 60 minutes, were fixed in 1 % paraformaldehyde, and were analyzed by flow cytometry for CD11b and Gr1 expression using a FACS-SCAN automated flow cytometer (Becton-Dickinson, Palo Alto, CA). Flow cytometric data were analyzed using Windows Multiple Document Interface for Flow Cytometry (WinMDI) v2.8 (J. Trotter, The Scripps Research Institute, La Jolla, CA; freely available at http://facs.scripps.edu/software.html).

Statistical analysis: All values given represent mean ± SEM. Intergroup differences in frequencies, means, and medians were examined by χ^2 test, t-test or one-way ANOVA with LSD post-hoc tests, and Mann-Whitney or Kruskal-Wallis test with Dunn's post-hoc tests, respectively. All *P* values are two-tailed; *P* values < 0.05 were considered significant. All statistical analyses were performed using the Statistical

Package for the Social Sciences v.13.0.0 (SPSS, Chicago, IL).

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