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Supplementary Materials for

HBEGF$^+$ macrophages in rheumatoid arthritis induce fibroblast invasiveness

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

(available at stm.sciencemag.org/cgi/content/full/11/491/eaau8587/DC1)

Data file S1 (.Microsoft Excel format). Individual subject-level data.

MATERIALS AND METHODS

Real-time PCR

RNA obtained using RNAeasy Mini kit (Qiagen) with DNAse treatment was reverse transcribed into cDNA (Fermentas) and analyzed by real-time quantitative PCR (Fast SYBR Green;Applied Biosystems) on a 7500 Fast Real-Time PCR System (Applied Biosystems). Expression levels were normalized to GAPDH or TBP. Gene-specific primer sequences were as previously described (*32*) or listed here:

AREG (F:GCTGCGAAGGACCAATGAGA, R:CCCCAGAAAATGGTTCACGC);

BTC (F:GCTTGGCATTCCTTAAGCCC, R:GGTCCCTACCTGGTCTCTCC);

CSF3 (F:CCAGGAGAAGCTGGTGAGTG, R:GAAAAGGCCGCTATGGAGTT);

EGF (F:GGATGTGCTTGATAAGCGGC, R:ACGGTCACCAAAAAGGGACA);

EPGN (F:CATCAACGGTGCTTGTGCAT, R:ACAAAGGCCTCACAGTGGTC);

EREG (F:ATCACAGTCGTCGGTTCCAC, R:AGGCACACTGTTATCCCTGC);

GAPDH (F:ATCAAGAAGGTGGTGAAGCA, R:GTCGCTGTTGAAGTCAGAGGA);

*HBEGF (*F:AGGAGCACGGGAAAAGAAAG, R:CTCAGCCCATGACACCTCTC);

IL1A (F:AGTAGCAACCAACGGGAAGG, R:AAGGTGCTGACCTAGGCTTG);

IL33 (F:TGAATCAGGTGACGGTGTTGAT, TGAAGGACAAAGAAGGCCTGG);

IP10 (F:ATTTGCTGCCTTATCTTTCTG, R:TCTCACCCTTCTTTTTCATTGTAG);

STAT4 (F:GAGACCAGCTCATTGCCTGT, R:CAATGTGGCAGGTGGAGGAT);

TGFA (CTCCTGAAGGGAAGAACCGC, R:CAGGCCAAGTAGGAAGGTCTG);

PLAUR (F:GCTGCAACACCACCAAATGC, R:TTTTCGGTTCGTGAGTGCCG);

PLAUR-FAIRE (F:TCACTCTGTCACCCAGGCTA, GTGCCCCTGTAATCCCAGTT).

Western blots on human blood-derived macrophages

Western blot analyses using a STAT4 (Santa Cruz) and HSP90 (Cell Signaling Technology) antibody were performed using standard procedures with the additional step of adding Pefabloc (Sigma-Aldrich) to macrophage cultures before cell lysis to prevent STAT protein degradation.

Flow cytometry on human blood-derived macrophages

Blood-derived macrophages were resuspended in 100uL FACS buffer (PBS with 1% FBS) and stained with FITC-conjugated CD87/PLAUR (VIM5-Miltenyi Biotec), acquired by FACSCanto (BD Biosciences) and analyzed using FlowJo (Tree Star, Inc.) software.

Assay for Transposase-Accessible Chromatin (ATAC)-Seq

ATAC-seq was performed as previously described (*75*). Human blood-derived macrophages were treated for 3 hours with 20 ng/ml TNF (Peprotech) and/or 280 nM PGE_2 (Sigma-Aldrich). 50,000 cells were washed in PBS, lysed (lysis buffer: 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1% IGEPAL CA-630) and centrifuged immediately at 500g for 10 min to obtain nuclei. The pellet was resuspended in the transposase reaction mix [25 µl 2× TD buffer, 2.5 µl transposase (Illumina) and 22.5µl nuclease-free water] and incubated at 37°C for 30 min. DNA was purified using a Qiagen MinElute kit and library fragments amplified for 13 cycles using 1X NEB next PCR master mix and custom Nextera PCR primers (*75*). The libraries were purified using Agencourt AMPure XP PCR Purification kit (Beckman Coulter) and single-end sequenced on a HiSeq 2500 (Illumina).

FAIRE qPCR

FAIRE experiments were performed as previously described (*76*). Chromatin was crosslinked by treating cells with 1% formaldehyde for 7 min and the reaction quenched with 0.125 M glycine for 5 min. Cells were washed with cold PBS and scraped, followed by a second wash. Fixed cells were lysed in buffer LB1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100, and protease inhibitors) for 10 min. Pelleted nuclei were resuspended in buffer LB2 (10 mM Tris–HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and protease inhibitors) and incubated on a rotator for 10 min. The nuclei were pelleted and lysed in buffer LB3 (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-deoxycholate, 0.5% N-lauroylsarcosine, and protease inhibitors).

Chromatin was sheared using a Bioruptor Pico device (Diagenode). A total of 10% of sonicated nuclear lysates were saved as input. Phenol-chloroform-purified nuclear lysates and de-crosslinked input DNA were used for qPCR analysis using specific primers for the upstream region. Chromatin accessibility is displayed relative to total input.

Prostaglandin E2 EIA Kit

Culture supernatants were collected from the culture after 24 hours, diluted 1:50 in plain RPMI, and prostaglandin concentrations measured using the Prostaglandin E2 EIA Kit-Monoclonal (Cayman Chemical). ELISA plates were read on Varioskan Flash Multimode Reader (ThermoFisher Scientific).

Neutrophil viability assay

Supernatants were collected from cultures of human macrophages and synovial fibroblasts after 24 hours and frozen at -80 °C. Whole blood from healthy human subjects was collected into heparin coated tubes. Neutrophils were isolated with the EasySep Direct Human Neutrophil Isolation Kit (Stemcell Technologies) with "The Big Easy" EasySep Magnet. In a 12-well plate, 600,000 neutrophils were plated in 800 µL of supernatant for 24 hours. Neutrophil viability was measured using Muse Annexin V and Dead Cell Assay Kit on a Muse Cell Analyzer mini-flow cytometer (EMD Millipore).

Luminex

Supernatants were collected from macrophage-fibroblast cultures or synovial cell cultures at 48 hours. Customized Luminex panels were ordered from R&D Systems. Protein concentrations were read by a MAGPIX from EMD Millipore using xPONENT 4.2.

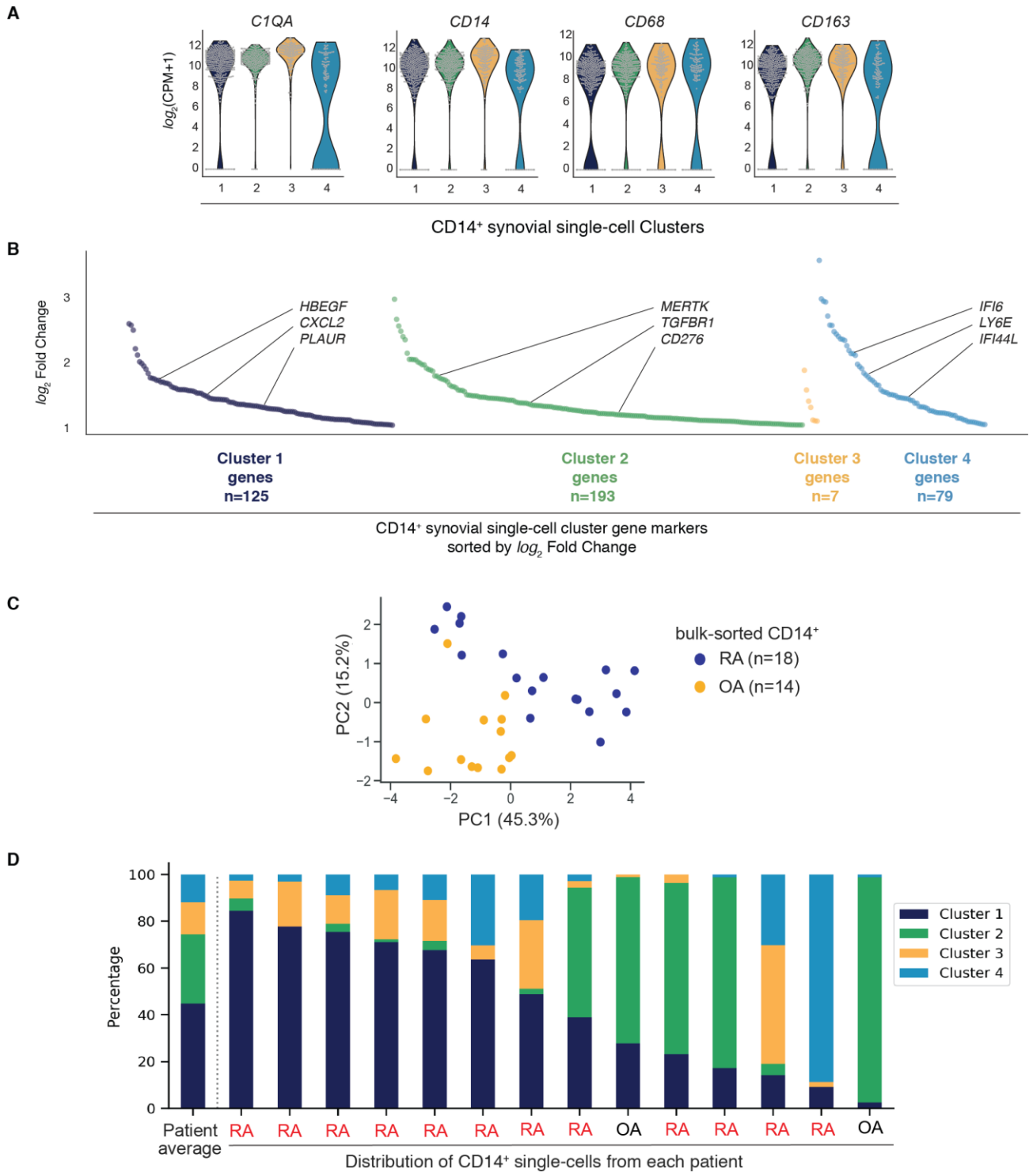

Fig. S1. Genes defining the CD14⁺ single-cell clusters and variance across patient samples. (**A**) Expression of monocyte-macrophage marker genes in each of the four synovial CD14⁺ single-cell clusters. Gene expression represented as the $\log_2$ counts per million + 1 (CPM+1). (**B**) Marker genes for each of the four synovial CD14⁺ single-cell clusters were selected based on a $\log_2$ fold-change greater than 1 relative to all other clusters (y-axis) and are sorted here in descending (x-axis). (**C**) Variance in bulk-sorted CD14⁺ synovial cells from 18 RA and 14 OA patients for the top 100 most variable genes. The majority of the RA samples separate collectively from the OA samples in principal component 1 (PC1), with the remaining RA samples separating from most of the OA in PC2. %, variance explained by each component. (**D**) Percentage of the single-cells from each patient that clustered into the four CD14⁺ subtypes (~100 cells per patient). For 7 out of 12 RA samples, Cluster 1 HBEGF⁺ (SC-M1) is the most abundant subtype found in these tissues.

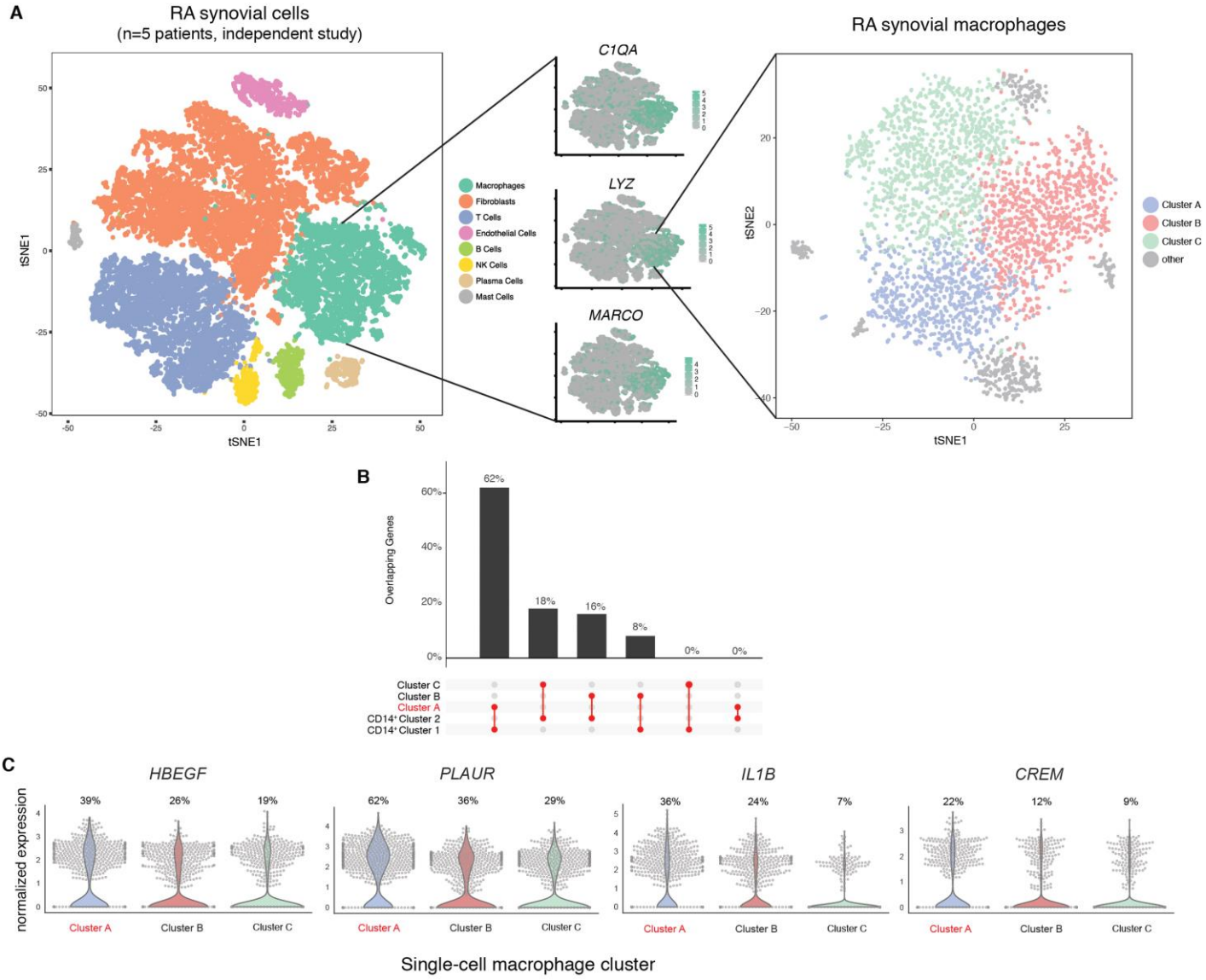

Fig. S2. Identification of synovial HBEGF$^+$ inflammatory macrophages in an independent RA patient study. (**A**) From an independent RA patient synovial tissue study [Stephenson *et al.* (*31*)], tSNE plots of single-cell RNA Drop-seq data clustered by cell type (left, 20,031 cells) and macrophage specific cells re-clustered (right, 4,212 cells). Center panel depicts cells expressing three macrophage lineage markers in light green. n = 5 RA. (**B**) The percentage of overlapping markers between the CD14$^+$ synovial cell Clusters 1 and 2 from Fig. 1 (labeled here as 'CD14$^+$ Cluster 1-2') and macrophage clusters from Stephenson *et al. (31)* (Clusters A-C). The top markers from the CD14$^+$ Clusters 1-2 (n = 50) were compared to the top makers (n = 300) from Clusters A-C. (**C**) Expression of HBEGF$^+$ inflammatory macrophage marker genes found in Clusters A-C. Values represent the percentage of cells in each cluster in which some amount of expression was detected (non-zero cells).

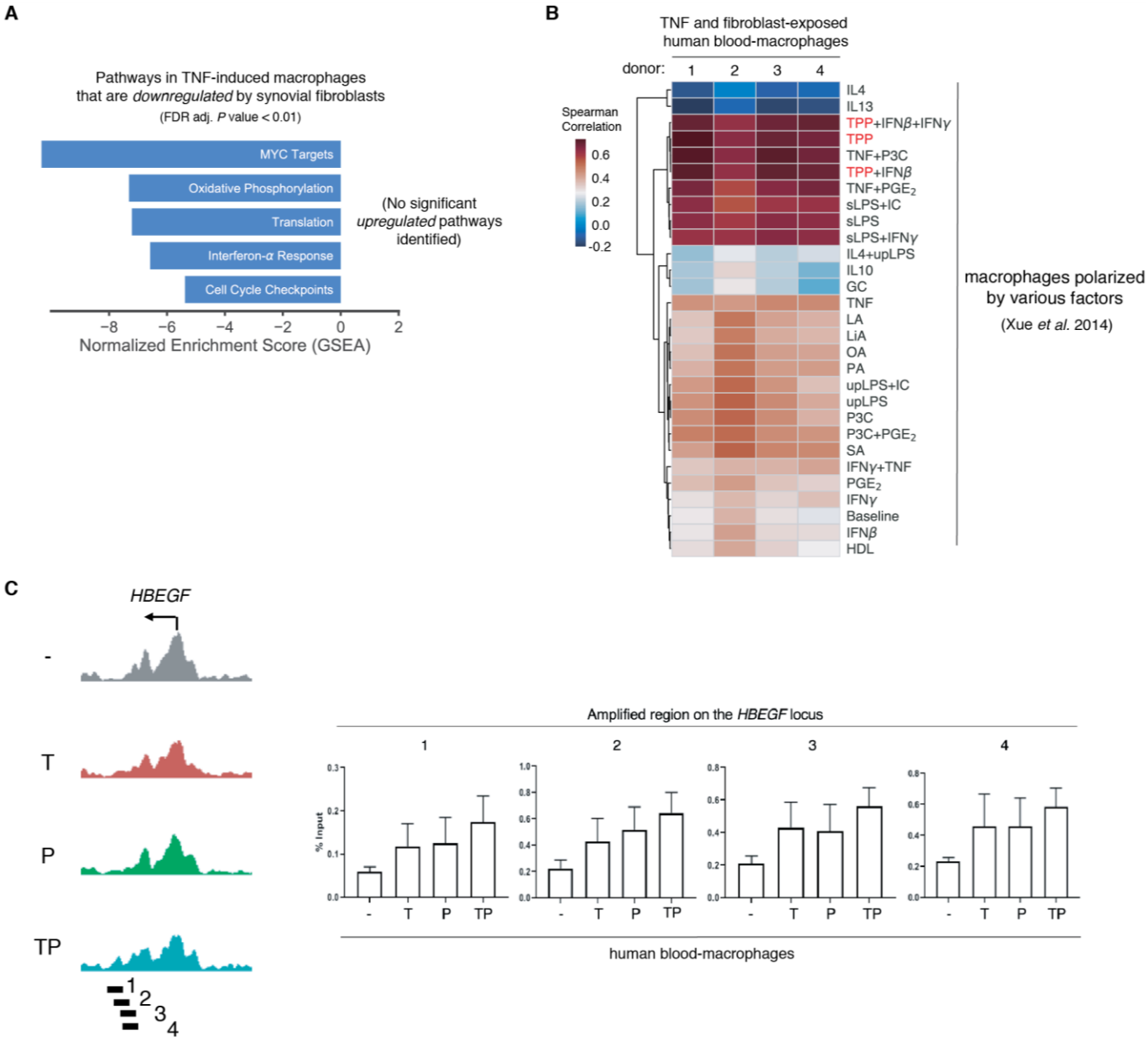

Fig. S3. Synovial fibroblasts down-regulate several pathways in TNF-induced macrophages and impose a transcriptome consistent with TNF and prostaglandin exposure. (**A**) Pathways in TNF-induced macrophages that were downregulated upon synovial fibroblast exposure (pathways with a negative enrichment score). Pathways listed were identified independently in a GSEA and IPA (Qiagen) analysis. There were no pathways identified as being induced upon fibroblast-exposure. (**B**) Transcriptome correlation between four human donor blood-derived macrophages exposed to synovial fibroblasts and TNF (columns; RNA-seq normalized expression data) and macrophages stimulated under a variety of polarization conditions [rows; microarray expression data from Xue *et al*. (*12*)]. Colors represent the spearman rank correlation values using the top 250 most variable and overlapping genes. PGE_2, prostaglandin E2. TPP, TNF+PGE_2+Pam-3Cys. (**C**) ATAC-seq peaks (left) and corresponding regions amplified in FAIRE-qPCR analysis (right, n = 3) at the HBEGF promoter. Blood-derived macrophages treated with TNF (T), PGE_2 (P) or TNF plus PGE_2 (TP), similar to Fig. 2 I-J.

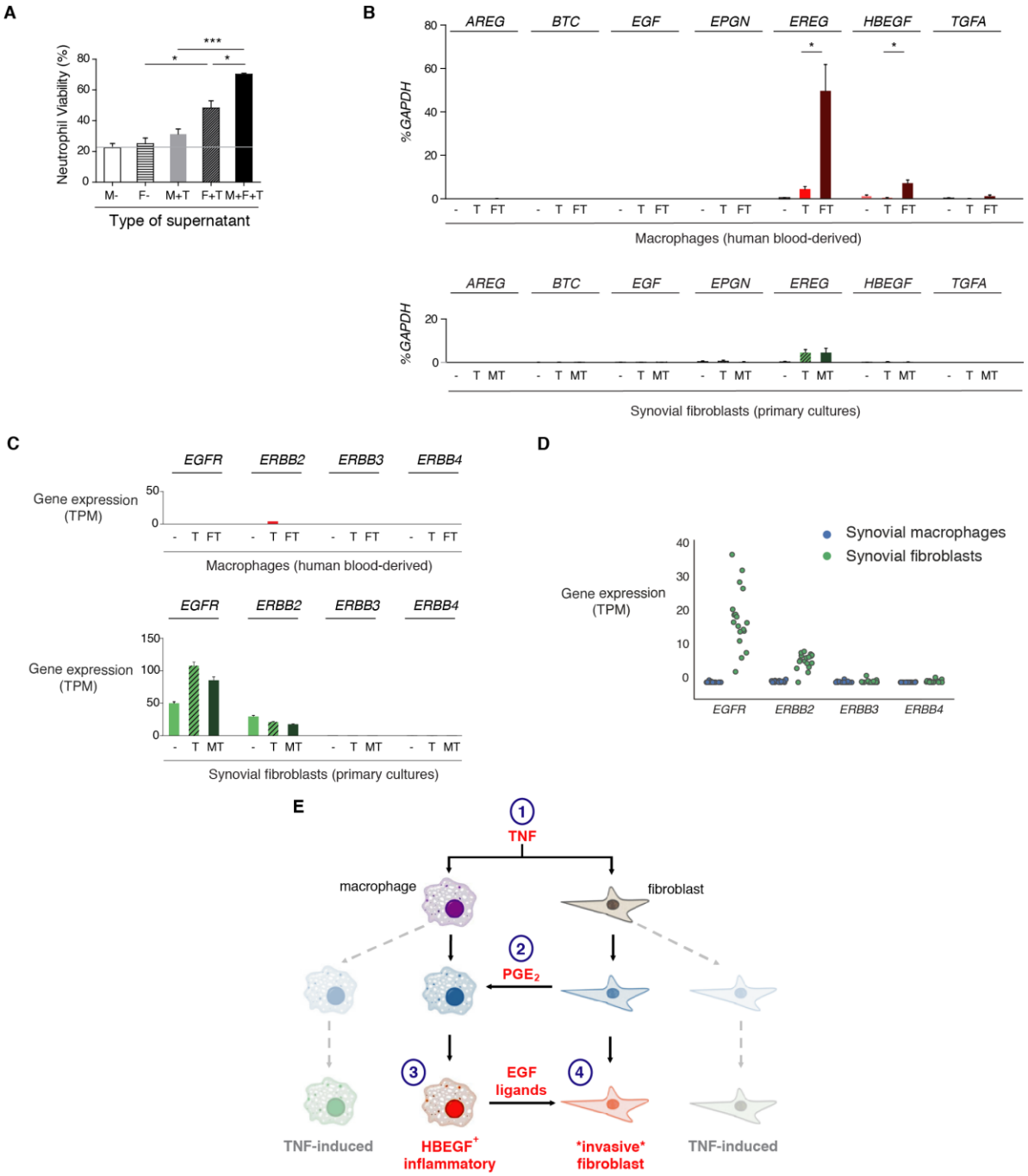

Fig. S4. Synovial fibroblasts express EGF receptors whereas HBEGF$^+$ inflammatory macrophages express two EGF ligands. (**A**) Neutrophil viability as detected by flow cytometry for cell death markers after 24 h incubation with supernatants from macrophage and fibroblast mono- and co-cultures. *n* = 4 unique donors for all cell types, reported as mean with standard error of the mean. *, **, *** represent p-value <0.05, 0.01, 0.001 by paired Student's t-test, respectively. (**B**) qPCR expression analyses of blood-derived macrophages or synovial fibroblast primary cultures for seven EGF ligands after 24 h culturing alone untreated (—), TNF-treated (T), or co-cultured with TNF (FT or MT, respectively). Gene expression plotted as percent (%) of GAPDH; error bars represent standard error, *n* = 4 unique donors for each cell type. *, p<0.05 by paired Student's t-test. (**C**) Synovial fibroblast RNA-seq expression of four EGF receptor subunits after culture conditions similar to panel B. TPM, transcripts per million; error bars represent standard error, *n* = 4 blood-derived macrophage and *n* = 2 synovial fibroblast donors. (**D**) RNA-seq expression of EGF receptor subunits in RA synovial CD14$^+$ macrophages (blue dots, *n* = 20 patients) and PDPN$^+$ fibroblasts (green dots, *n* = 18 patients). (**E**) Nearby synovial macrophages and fibroblasts crossregulate to drive distinct inflammatory responses, wherein [1] TNF induces [2] production of prostaglandin PGE_2 by fibroblasts, which together with TNF drives the [3] HBEGF$^+$ inflammatory phenotype and [4] EGF ligand production that then feeds back to induce fibroblast invasive behaviors.

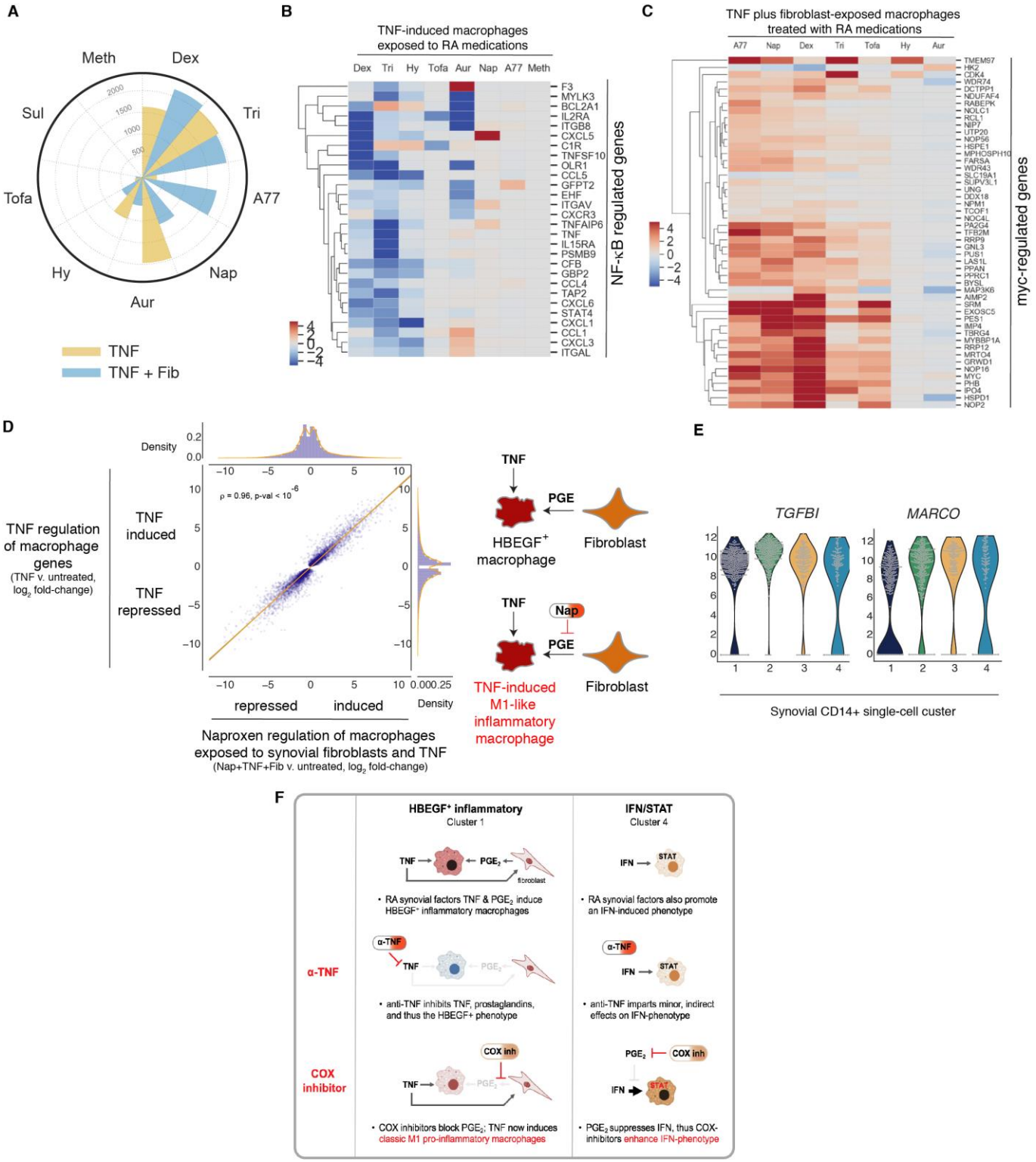

Fig. S5. Synovial fibroblast exposure modifies how RA medications affect TNF-induced macrophages. (**A**) Number of genes modified (FDR adjusted p-value < 0.1) by nine different drug conditions in TNF-induced macrophages (TNF, yellow bars) or in TNF-induced macrophages exposed to synovial fibroblasts (TNF+Fib, blue bars). (**B**) Expression changes for NF-κB regulated genes in TNF-induced macrophages exposed to RA medications. Several of the anti-inflammatory RA medications suppressed induction of the NF-κB targets (blue color indicates suppression by medication). Colors represent z-scores from IPA Upstream Regulator analysis. Color intensity depicts statistical significance, defined as the -$\log_{10}$ FDR adjusted p-value multiplied by the sign of the change in expression. (**C**) Expression changes for myc-regulated genes in macrophages exposed to TNF plus synovial fibroblasts and RA medications. (statistics as in panel B).

Whereas synovial fibroblasts suppress myc-regulated genes in TNF-induced macrophages (fig. S3A), several RA medications reversed this suppression (red color indicates reversed suppression). (**D**) Gene expression profile of macrophages exposed to TNF, synovial fibroblasts and naproxen (Nap+TNF+Fib, x-axis) compared to macrophages exposed only to TNF (y-axis). Both cellular profiles were relative to untreated human macrophages (n = 6,613; $\log_2$ fold-change; FDR adjusted p-value < 0.1). The orange line represents the regression between the $\log_2$ fold-change values (Pearson's $\rho = 0.96$, p-value < 10^{-6}). Cartoon on the right depicts how these data suggest that, in the co-culture system, naproxen targets the fibroblast-mediated effects on macrophages but not the TNF-mediated effects. As a Cox-inhibitor, naproxen therefore likely blocks fibroblast prostaglandin production and thereby the polarization of macrophages towards the $HBEGF^+$ inflammatory phenotype, but does still permit TNF polarization towards an M1 inflammatory phenotype. (**E**) Violin plots of selected marker genes from $CD14^+$ single-cell RNA-seq represented by the $\log_2$ counts per million (CPM) (y-axis) across the four identified clusters (x-axis). (**F**) Schematic of the effects of anti-TNFs and the COX inhibitor naproxen on the two RA-enriched synovial macrophage subsets.

Table S1. Patient characteristics for CD14$^+$ synovial single-cell samples. *n* = 12 RA patients; *n* = 2 OA samples also included in CD14$^+$ synovial single-cell dataset to populate putative underrepresented cell subsets in RA tissues. OA age range, 64-81 years. Both male, both arthroplasty tissue.

Characteristic	Average value	Range or percentage
Age (years)	58.3	38 - 79
Female	7	58%
RF positive	8	67%
CCP positive	7	58%
Duration Dx (years)	6.5	<1 - 51
DAS28	4.8	2.2-6.7
On steroids	3	25%
On methotrexate	3	25%
On a biologic, or within 3 months	2	17%
Hospital collection sites	6	n/a
Biopsies (v. arthroplasty)	9	75%

Table S2. Patient characteristics for CD14$^+$ synovial bulk-sorted samples. n/a, not applicable.

	RA patients (*n* = 18)		OA patients (*n* = 13)	
Characteristic	Average value	Range or percentage	Average value	Range or percentage
Age (years)	56.4	38 - 79	70.5	60 - 85
Female	11	61%	9	69%
RF positive	15	83%	n/a	n/a
CCP positive	14	78%	n/a	n/a
Duration Dx (years)	4.9	<1 - 26	n/a	n/a
DAS28	5.4	3.1 - 7.6	n/a	n/a
On steroids	5	28%	n/a	n/a
On methotrexate/hydroxychloroquine	3	18%	n/a	n/a
On a biologic, or within 3 months	3	18%	n/a	n/a
Hospital collection sites	6	n/a	4	n/a
Biopsies (v. arthroplasty)	18	100%	0	0%

Table S3. RA patient characteristics for the ex vivo synovial tissue assay.

Characteristic	Average value	Range or percentage
Age (years)	66.1	61 - 79
Female	5	62.5%
Race: White	5	62.5%
BMI (kg/m^2)	28.1	20.9 - 33.2
Duration Dx (years)	8.6	0.1 - 22.4
Duration Sx (years)	9.5	0.2 - 22.4
ESR	36	20 - 54

Table S4. Individual patient disease diagnosis, serology, and histology for the ex vivo synovial tissue assay. RA diagnosis: both indicates 2010 ACR/EULAR criteria and 1987 ACR criteria; Serology: high positive* signifies >3x ULN; Histology: symbols correspond to pathologist scored gradient from mild (+) to severe (+ + + +); RA medication: DMARD (Disease-modifying antirheumatic drugs)

	RA diagnosis	Serology		Synovial tissue histology		RA medication	
	Criteria	RF result interpretation	anti-CCP interpretation	Lymphocytic infiltration	Lining hyperplasia	DMARD	Biologic
Patient 1	Meets both	Positive	High positive*	+ + +	+ + + +	Yes	Yes
Patient 2	Meets both	Positive	High positive*	+ + + +	+ + + +	Yes	Yes
Patient 3	Meets both	Positive	High positive*	+ + + +	+ + +	No	No
Patient 4	Meets both	Positive	High positive*	+ + + +	+ + +	Yes	Yes
Patient 5	Meets both	Positive	High positive*	+ + +	+ + + +	Yes	No
Patient 6	Meets both	Negative	Negative	+ + + +	+ + + +	Yes	Yes
Patient 7	Meets 2010	Negative	Negative	+ +	+ + + +	No	Yes
Patient 8	Meets 2010	Negative	High positive*	+ + +	+ + + +	Yes	No