OTKA

Zárójelentés a 61814 azonosítójú,

A PPAR-gamma, egy lipidek által aktivált transzkripciós faktor aktivitásának szabályozása a makrofágok különböző állapotaiban

című pályázathoz

Összefoglaló

A Peroxisome Proliferator-activated Receptor γ (PPARγ) egy lipidek által aktivált transzkripciós faktor, mely a lipidanyagcsere és a gyulladás szabályozásával olyan folyamatokban vesz részt, mint az érelmeszesedés és diabétesz. A PPARγ aktivátorai több szinten képesek a gyulladás gátlására. Jelen pályázat célja az volt, hogy tanulmányozzuk a gyulladásos folyamatok PPARγ aktivitására kifejtett hatását, megvizsgáljuk, hogy a gyulladásos mediátorok hogyan kommunikálnak a PPARγ-val és megfejtsük ezeknek folyamatoknak a molekuláris mechanizmusát. Azt találtuk, hogy a gyulladásos molekulák befolyásolják a PPARγ működését. Proinflammatórikus molekulák gátolják, míg az interleukin-4 (IL-4) fokozza a receptor működését makrofágokban és dendritikus sejtekben. Az IL-4 szignálútvonal bekapcsolása egy újonnan feltárt mechanizmussal, a Signal Transducer and Activators of Transcription 6 (STAT6) és a PPARγ interakciója révén erősíti a receptor válaszait a célgének promóterén. Ennek eredményeképpen az IL-4 emeli a PPARγ által szabályozott gének számát és fokozza az egyes gének esetében azok transzkripcióját. Ily módon a PPARγ egy pozitív transzkripciós faktorként működhet a makrofágokban is. Eredményeink szerint létezik egy új mechanizmus, ahogy az immunrendszer sejtspecifikusan képes

szabályozni egy magreceptor működését. Mindez felhívja a figyelmet a sejtek gyulladásos állapota és a lipidanyagcsere kapcsolatára olyan folyamatokban, mint pl. az érelmeszesedés.

Részletes rész

Mivel a pályázat legfőbb témájául szolgáló eredményeinket most rendezzük kézirattá ezért a legjobb összegzés ennek a kéziratnak a bemutatásával lehetséges. Ezt az alábbiakban tesszük meg a szöveg és az ábraanyag bemutatásával.

Itt pedig a lefőbb kísérleti eredményeinket mutatjuk be:

(1) A PPARγ expressziója nem mindig korrelál annak ligand általi aktiválhatóságával.

(2) Gyulladásban szerepet játszó molekulák specifikusan és reverzibilisen befolyásolják mind a receptor kifejeződését, mind válaszkészségét a makrofágokban és a dendritikus sejtekben.

(3) Proinflammatórikus molekulák gátolják,

(4) míg a gyulladásgátló interleukin-4 (IL-4) fokozza a PPARγ expresszióját és aktivitását emberi és egér sejteken.

(5) Az IL-4 kezelt alternatívan aktivált makrofágok és az éretlen dendritikus sejtek a két fő myeloid sejttípus, ahol a PPARγ funkcionális.

(6) Globális génexpresszós analízissel meghatároztuk PPARγ által szabályozott géneket emberi és egér makrofágokban és kb. azonos számú gén indukálódik, mint amennyi represszálódik.

(7) A PPARγ agonisták nem befolyásolják a makrofágok alternatív aktiválásra jellemző általános markereinek a szintjét és maga a receptor nem feltétlenül szükséges az alternatív aktiválás létrejöttéhez.

(8) A receptor viszont képes olyan géneket indukálni, melyek egyébként az IL-4-nek is célpontjai és így a PPARγ képes befolyásolni az alternatív aktiválás lefolyását.

(9) Az IL-4 hatásmechanizmusát tanulmányozva megállapítottuk, hogy az IL-4 a Jak3-STAT6 útvonalon keresztül fejti ki hatását a PPARγ-ra. A STAT6 szükségességét knockout egerekkel bizonyítottuk.

(10) Más mechanizmusokat kizárva megmutattuk, hogy a STAT6 a PPARγ célgének promóterén hat, és

(11) interakcióba lép a PPARγ-val, így engedélyezve a PPARγ célgének átíródását.

(12) Bioinformatikai módszerekkel magasabb ggyakoriságot mutat a STAT kötőhely a PPARγ válaszadó elemek környékén, ami a microarray adatokkal együtt arra utal, hogy a STAT6 a myeloid sejtekben egy általános “licensing” faktor a PPARγ számára.

Molecular mechanism of a crosstalk between PPARγ and IL-4 signaling in macrophages

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Abstract (max. 200 words)

Peroxisome Proliferator-activated Receptor γ (PPARγ) is a lipid-activated transcription factor that regulates lipid metabolism and inflammation, key processes in atherosclerosis and diabetes. PPARγ agonists are known to regulate inflammation on multiple levels. However, it has been poorly studied how the inflammatory milieu regulates the activity of PPARγ and most of the known PPARγ target genes derived from the adipose tissue. We demonstrate here that inflammatory molecules interfere with PPARγ signaling. Proinflammatory molecules inhibit, while interleukin-4 (IL-4) stimulate PPARγ in macrophages and dendritic cells (DCs). Activation of IL-4 signaling augments PPARγ activity through a novel interaction between PPARγ and Signal Transducer and Activators of Transcription 6 on the promoter of PPARγ target genes, like FABP4. As a consequence, IL-4 strongly enhances PPARγ response and acts as a licensing factor by increasing the number of genes regulated and also the magnitude of the responses. Through target gene induction PPARγ can be designated a positive regulator of macrophage gene expression. These findings introduce a new mechanism how inflammatory molecules modulate the activity of a nuclear receptor via cell-type specific factors and highlight the importance of the inflammatory status of cells in lipid metabolism and atherosclerosis.

Introduction (max. 63000 characters)

PPARγ is a member of the nuclear receptor superfamily, a group of ligand-activated transcription factors that regulate expression of their target genes upon ligand binding. For PPARγ oxidized fatty acids serve as endogenous activators {Kliewer, 1997 #82}{Krey, 1997 #83}. PPARγ has been shown to regulate many aspects of lipid metabolism and inflammation. Major biological roles for the receptor include the differentiation of adipocytes {Tontonoz, 1994 #80} and the regulation of lipid/cholesterol metabolism in macrophages {Nagy, 1998 #5}{Ricote, 1998 #90}{Tontonoz, 1998 #81}. Macrophages are not a homogenous population. They originate from bone marrow progenitors committed to the monocytic lineage. The newly formed monocytes are released into the circulation then enter the tissues to differentiate into mature resident macrophages. Alternatively, under inflammatory conditions monocytes themselves are recruited to sites of inflammation. Initially, activated macrophages were defined as cytokine producing inflammatory cells that are able to kill pathogens. However, the immunophenotype and function of these cells depend on the environment and presence of various activator molecules {Gordon, 2003 #50}. Macrophages not only mediate pathogen clearance but act as also key regulators of the resolution after inflammation. Both activities are driven by cytokines and microbial products and manifested in forms of distinct activation states {Ma, 2003 #93}.

Proinflammatory molecules like interferon γ (IFNγ) and tumor necrosis factor (TNF) or activators of pattern recognition molecules (e.g. Toll-like receptors (TLRs)) result in classical activation of macrophages. Consequently, macrophages migrate to the sites of inflammation and degrade pathogens due to increased production of nitrogen radicals and

secretion of proinflammatory molecules such as TNF, IL-1 and IL-6. The classical pathway of IFNγ-dependent activation of macrophages by Th1-type responses is a well-established arm of the cellular innate immunity directed against intracellular pathogens like *Listeria monocytogenes* or *Mycobacterium tuberculosis* {Goerdt, 1999 #37}{Mosser, 2003 #35}.

As part of their studies on the regulation of mannose receptor Gordon and colleagues identified a new class of macrophages, the so-called alternatively activated macrophages that in contrast to the classically activated ones express high level of mannose receptor upon IL-4 stimulus {Stein, 1992 #31}. These cells exert an almost opposite phenotype as their classically activated counterparts. They cannot produce nitrogen-monoxide, able to inhibit T cell proliferation {Schebesch, 1997 #34} and can provoke tolerance or Th2 immune responses {Cua, 1997 #38}. These cells also produce transforming growth factor β (TGFβ), IL-10, IL-1 receptor antagonist {Goerdt, 1999 #37}{Schebesch, 1997 #34}{Fenton, 1992 #42} and inhibit secretion of proinflammatory molecules, like IL-1, TNF, IL-6, IL-12 and macrophage inhibitory protein (MIP)-1α {Cheung, 1990 #43}{Standiford, 1993 #44}{Bonder, 1998 #45}. Alternatively activated macrophages are characterized by the expression of mannose receptor {Stein, 1992 #31}, CD23 {Becker, 1990 #68}, alternative macrophage activation-associated chemokine 1 (AMAC-1 or CCL18) {Kodelja, 1998 #32}, arginase-1 {Munder, 1999 #69}, FIZZ1 and YM1 {Raes, 2002 #33}. The alternative activation of macrophages by Th2 cytokines IL-4 and IL-13 accounts for a distinct macrophage phenotype playing role in humoral immunity and various processes during resolution: wound healing, angiogenesis, tissue repair and extracellular matrix deposition {Goerdt, 1993 #46}{Kodelja, 1997 #53}{Kodelja, 1998

#32}{Gratchev, 2001 #36}{Gordon, 2003 #50}{Mosser, 2003 #35}. Under normal conditions characteristic *in vivo* examples for alternatively activated macrophages are lung alveolar macrophages, perivascular and placenta macrophages {Mues, 1989 #39}{Chang, 1993 #47}{Kodelja, 1998 #32}{Linehan, 1999 #54}{Fabriek, 2005 #62}. Several lines of evidence indicate the existence of a crosstalk between lipid metabolism and inflammation. Obesity is now considered to be an inflammatory disorder accompanied by the accumulation of macrophages {Weisberg, 2003 #104}{Xu, 2003 #105}{Kanda, 2006 #106}{Kamei, 2006 #107}. Two nuclear receptors PPARγ and Liver X Receptor (LXR) have been shown to play pivotal roles in the communication between lipid metabolism and the immune system. Such communication channel could be bidirectional, however in case of PPARγ only one way (from the lipid metabolism to the immune system) has been tested mechanistically so far. Activators of PPARγ like the prostanoid, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) {Forman, 1995 #84}{Kliewer, 1995 #85} or the synthetic anti-diabetic agent thiazolidinedions (TZDs) {Lehmann, 1995 #89} have been shown to inhibit inflammation under certain conditions. Most of our knowledge about the role of PPARγ in inflammation is based on a special inflammatory cell type with largely unknown characteristics; murine thioglycolate-elicited peritoneal macrophages treated with various synthetic agonists and/or proinflammatory molecules. Several reports described PPARγ as a negative regulator of macrophage activation, based on data showing that PPARγ agonists inhibited the expression of proinflammatory molecules, like inducible nitric oxide synthase, gelatinase B, TNF, IL-1β, IL-6 and IL-12 {Jiang, 1998 #18}{Ricote, 1998 #17}{Alleva, 2002 #26}. Importantly, the negative regulatory role assigned to PPARγ was not a direct transcriptional effect of the receptor

but the consequence of the failed induction of inflammatory genes by other transcription factors activated upon proinflammatory molecules e.g. lipopolysaccharide (LPS) {Li, 2000 #91}{Pascual, 2006 #92}. The molecular mechanism, how PPARγ activators exert their anti-inflammatory effects is still controversial. Some of these were reported to be nonspecific, PPARγ-independent effects of the agonists, due to the facts that some of the anti-inflammatory reactions exist in the absence of the receptor and/or these function at higher ligand concentrations than necessary for receptor specific transcriptional activation {Chawla, 2001 #22}. One possible molecular mechanism for the trans-repression of other transcription factors by PPARγ was suggested by Pascual et al., which involves ligand-dependent sumoylation of the receptor targeting it to corepressor complexes. This process prevents recruitment of proteosome machinery that normally removes the corepressor complexes required for gene activation {Pascual, 2005 #28}. Surprisingly, only a few positively regulated genes of PARγ have been identified in the murine macrophages {Welch, 2003 #27}, while PPARγ seems to induce and repress euqal number of genes in human dendritic cells (DCs), a macrophage-related cell type {Szatmari, 2007 #71}. According to these PPARγ responses are considered to be cell-type specific: in murine macrophages it can inhibit proinflammatory gene expression while in adipocytes and human DCs it can induce transcription of lipid metabolism-related genes and no certainties are known about human macrophages. The mechanism of these cell-type specific responses is largely unknown.

Recently, LXR, another member of the nuclear receptor superfamily and target for PPARγ {Chawla, 2001 #6} was also shown to play important role in the communication between lipid signals and the immune system. Activation of LXR results in the inhibition

of inflammatory gene expression in macrophages {Joseph, 2003 #64} and also impacts anti-microbial responses {Joseph, 2004 #100}{Valledor, 2004 #101}. Proinflammatory molecules, bacteria and viral compounds *via* interferon regulatory factor 3 could inhibit LXR-dependent transcriptional activity {Castrillo, 2003 #63}. PPARγ and LXR share many common features {Torocsik, 2009 #103}. The two receptors work together in the regulation of cholesterol metabolism in macrophages {Nagy, 1998 #5}{Tontonoz, 1998 #81}{Chawla, 2001 #6}. A mechanism similar to PPARγ was suggested for the inhibition of proinflammatory target gene expression by LXR {Ghisletti, 2007 #102}. However, the other direction of the hypothesized bidirectional crosstalk between lipid metabolism and inflammation, namely the influence of immune modulators on PPARγ has not yet been analyzed systematically.

There are hints in the literature that such influence exists. In adipose tissue PPARγ could be inhibited by phosphorylation *via* mitogen-activated protein (MAP) kinase {Hu, 1996 #76}{Adams, 1997 #77}. In mouse adipocytes IFNγ induces rapid proteasomal degradation of the receptor {Waite, 2001 #23}. Furthermore, IL-1 and TNF inhibit adipogenesis through nuclear factor κB (NF-κB)-dependent inhibition of PPARγ DNA binding capacity {Suzawa, 2003 #25}. It was also shown previously that IL-4 induced PPARγ and ligand production for the receptor in murine macrophages {Huang, 1999 #19}. However, there is inconclusiveness around PPARγ requirement for alternative macrophage activation. PPARγ was first reported to be required for maturation of alternatively activated macrophages and disruption of the gene impaired alternative macrophage activation in mouse {Odegaard, 2007 #70} while later others in another

mouse strain claimed the dispensability of PPARγ for alternative activation {Marathe, 2009 #111}. Nevertheless, no mechanism of such involvement has been suggested. Although PPARγ has been studied in many reports, it has been analyzed regardless of the inflammatory status of the cells. Besides the trans-repression no effort has been made to find the mechanism of the crosstalk how inflammatory mediators influence ligand-induced transcriptional activity of PPARγ in inflammatory cells like macrophages and DCs. However, that could reveal the cell-type specific differences in receptor-evoked responses.

It has not been clarified so far if proinflammatory mediators influence PPARγ expression and/or responses in the macrophages like they do in adipocytes or in case of LXR. It has not been defined in which subpopulation of the macrophages PPARγ is predominantly expressed and functional. So we sought to define such cell-types and determine the molecular mechanism, which makes one cell different from another concerning PPARγ expression and ligand-induced transcriptional activation and wanted to characterize the conditions and permissive factors that besides the receptor level and ligand availability determine PPARγ responses.

In the work presented here we found that (1) expression of the receptor do not always correlate with its ligand-induced transcriptional activity, (2) many inflammatory molecules interfere with PPARγ signaling and the activation type of the macrophages/DCs definitely but reversibly determines PPARγ activity, (3) proinflammatory molecules inhibit, while (4) IL-4 stimulates PPARγ expression and ligand-induced transcriptional activity in human as well as in murine macrophages and DCs, (5) alternatively activated macrophages and immature DCs are the particular cell

types where PPARγ could be functional, while under inflammatory conditions in classically activated macrophages PPARγ response is inhibited. (6) By transcriptional profiling PPARγ was found to induce and repress approximately equal number of genes both in human and mouse alternatively activated macrophages. (7) By an unbiased approach general PPARγ activity was concluded to depend on the presence of IL-4 in myeloid cells. (8) PPARγ agonists do not alter the expression of known markers for alternative macrophage activation and PPARγ is dispensable for this process. (9) The receptor through its target genes acts as a modifier of the alternative activation program since many PPARγ-induced genes are also targets for the IL-4 *per se*. We analyzed the mechanism that makes these two cell types permissive for PPARγ activation and provide a novel molecular mechanism. (10) IL-4 activated Signal Transducer and Activators of Transcription 6 (STAT6) is a determinant factor for PPARγ responses in macrophages and DCs. (11) STAT6 interacts with PPARγ on the promoter of target genes and consequently activates PPARγ and license ligand-induced transcription. (12) Frequency of STAT binding sites in the proximity of PPARγ response elements is increased. With our findings we introduce a new mechanism how inflammatory molecules can determine the activity of a nuclear receptor and highlight the importance of signaling crosstalk and composite gene expression regulation.

Results

Expression and ligand-induced transcriptional activity of PPARγ depends on the activation state of macrophages and DCs

In order to highlight the differences in the expression and activity of PPARγ in myeloid cells we sought to systematically characterize how various inflammatory stimuli influence PPARγ expression and activity in macrophages and DCs. First, we purified human CD14 positive monocytes and cultured them *in vitro* to obtain monocyte-derived macrophages and immature DCs. As a model for macrophage activation we used IL-4 to alternatively activate macrophages and proinflammatory molecules (IFNγ, TNF and LPS) to induce classical activation of macrophages or to induce the maturation of DCs. Under these conditions CD206, CD209, CD23 and AMAC-1 were induced upon alternative activation while CD80, CD83, CD86 and HLA-DR were upregulated by classical activation and CD1a, CD209 were used as markers of DC development (data not shown). First, we analyzed the expression and ligand-induced transcriptional activity of PPARγ in this *in vitro* model of macrophage and DC development. As shown in Figure 1A PPARγ is expressed in the monocytes at a low level, which is induced in the macrophages. Interestingly, IL-4 further increased PPARγ expression, while IFNγ resulted in a decreased expression. In human macrophages we found that LPS and granulocyte-monocyte colony stimulating factor (GM-CSF) also induced the receptor level. In immature DCs differentiated with GM-CSF and IL-4 we measured high PPARγ expression, which was slightly induced upon maturation. Given the expression pattern of PPARγ under the tested conditions we next compared the activity of the receptor by

adding PPARγ specific synthetic agonist, Rosiglitazone (RSG). To follow PPARγ activity we chose the most specific known PPARγ target gene, fatty acid binding protein 4 (FABP4 or aP2) {Tontonoz, 1994 #80}. When measuring FABP4 mRNA levels we obtained dramatic differences (Figure 1B). Under two conditions FABP4 was strikingly induced: in the IL-treated alternatively activated macrophages and in the GM-CSF+IL-4 treated immature DCs. Only slight induction could be observed in the non-activated macrophages and in the IL-4-treated macrophages in the absence of RSG, which resulted in a comparable level as RSG-treated non-activated macrophages. Additionally, IFNγ, IFNγ+TNF and LPS inhibited target gene induction in macrophages and LPS also inhibited FABP4 expression in the mature DCs. We used *Listeria monocytogenes* as a biologically relevant pathogen and found that the bacteria did not change the expression of PPARγ but did inhibit its activity similarly as LPS (data not shown). As a conclusion we got marked differences in the expression and activity of PPARγ. IFNγ reduced the expression of the receptor and also inhibited target gene expression. While LPS slightly increased receptor level it inhibited the activity. IL-4 induced expression of the receptor and it also enhanced ligand-induced transcriptional activity of the receptor. Since the expression of the receptor did not correlate with activity at multiple levels, i.e. LPS or GM-CSF did not results in elevated target gene expression and the degree of target gene induction in the IL-4-activated cells exceeded that could be explained by the PPARγ levels we hypothesized that not only the level of PPARγ but also its activity might be under control by the IL-4. We named this phenomenon IL-4-induced augmented PPARγ response and tested if such IL-4-induced mechanism could be responsible for the cell-type specific differences in PPARγ responses.

We compared the expression of other PPARs in the monocytes, non-activated and activated macrophages. We found that both PPARα and PPARδ are induced during macrophage development but with weaker dynamic (Supplementary Figure 1A and C) than PPARγ (Supplementary Figure 1D). Although PPARα and δ were induced no differences in their levels upon macrophage activation were found (Supplementary Figure 1A and C), while PPARγ showed two fast effects (Supplementary Figure 1B): first it was rapidly induced by the monocyte-macrophage transition and simultaneously IL-4 could further increase its level. IFNγ+TNF treatment caused a delayed repression of the transcript level. When comparing the expression of the three PPARs in the non-activated macrophages all PPARs are present, while in the alternatively activated macrophages PPARγ is the dominant isoform and it is missing in the classically activated cells (Supplementary Figure 1E). Since PPARγ and LXR have been involved in many commonly regulated processes we compared the expression of LXRα to PPARγ and surprisingly we could detect an inverse regulation (Supplementary Figure 1D). LXRα is induced upon macrophage development but much slower than PPARs and IFNγ+TNF could further increase its transcription.

Next, we performed immunohistochemistry on primary macrophages to analyze the protein levels of PPARγ. As shown in Figure 1C consistently with the mRNA results PPARγ protein is expressed at highest level in the IL-4-treated cells (brown nuclear staining), less positive nuclei could be seen in the non-activated cells and IFNγ+TNF treatment resulted in the lowest PPARγ expression (Figure 1C). Figure 1D indicates the expression of FABP4 protein in macrophages under the different activation states.

FABP4 induction was detected only in the alternatively activated macrophages upon RSG treatment correlating with the mRNA levels in Figure 1B.

In order to assess the *in vivo* expression pattern and distribution of PPARγ positive macrophages we carried out a systematic survey using immunohistochemistry. We analyzed human lymphoid tissues such as Peyer's patches, lamina propria of the intestinal vili, reactive lymph nodes, tonsils and known areas of alternatively activated macrophages (Supplementary Figure 2). We used CD68 as a general macrophage and DC-SIGN as a specific and selective marker for alternatively activated macrophages and/or DCs {Geijtenbeek, 2000 #65}, {Relloso, 2002 #66}. The conclusion of our analysis indicated that (1) PPARγ was not expressed in every CD68 positive macrophage, (2) PPARγ positive macrophages were not necessarily DC-SIGN positive, but (3) almost every DC-SIGN positive macrophage expressed PPARγ. (4) PPARγ expressing macrophages were localized in preferential compartments of the lymphoid organs, mainly around the germinal centers in the perifollicular T-cell areas. PPARγ co-localized with DC-SIGN very prominently in perivascular and alveolar macrophages.

We examined if PPARγ responses differ under various activation stimuli when natural sources of ligands such as oxLDL is used. oxLDL had similar effects to RSG on FABP4 gene expression and IL-4 increased the effects of oxLDL similarly to RSG (Figure 1E).

We wanted to see if the explored pathways were restricted to only human monocyte-derived cells. First, we isolated human CD34 positive stem cells and differentiated them to macrophages *in vitro*. After 9 days of maturation we induced activation by IL-4 or IFNγ+TNF. When treating with RSR we obtained similar results as in monocyte-derived macrophages: a moderate induction in the non-activated cells, a large induction in the IL-

4-treated cells while no response was detected in the classically activated macrophages (Figure 1F).

PPARγ responsiveness is reversibly and dynamically changing

Based on our data we hypothesized that the activation state-modulated PPARγ activity of macrophages is not an irreversible end-stage but the result of the changing cytokine environment. To evaluate this scenario we performed sequential treatments: pretreated the macrophages with one cytokine for 6 hours and then added the second cytokine. After 6 hours the cells were treated with vehicle or RSG for an additional 6 hours (Figure 1G and H). Expression of PPARγ changed reversibly upon macrophage activation (Figure 1G). On one hand PPARγ could be repressed by IFNγ+TNF after IL-4 pretreatment, on the other hand PPARγ could be induced by IL-4 in the IFNγ+TNF pretreated cells (Figure 1G arrows). Looking at PPARγ responsiveness, IFNγ+TNF repressed PPARγ activity in the IL-4 pretreated macrophages while IL-4 induced PPARγ activity in the IFNγ+TNF pretreated cells (Figure 1H arrows). These data suggest that PPARγ expression and activity is reversibly and could be dynamically regulated by the cytokine milieu.

We analyzed the specificity of the inflammation-induced changes in PPARγ responses by examining other targets for the receptor and other nuclear receptors. PPARα signaling did not prove to be very active in macrophages, which could be explained probably by the low expression of the receptor and/or tissues specific factors (data not shown). Both PPARγ and δ agonists induced adipose differentiation-related protein (ADRP), however IL-4 could enhance only the effect of the PPARγ agonist but had no effect on PPARδ (Supplementary Figure 1F). PPARδ response was inhibited only by IFNγ, to a lesser

extent by LPS (TLR4), Lipid A (TLR4) or *L. monocytogenes* but not by LTA (TLR2), PolyI:C (TLR3) or CpG (TLR9) treatment (Supplementary Figure 1F). LXR activity did not seem to be fundamentally affected by the activation state of the macrophages as analyzed by the induction of its target genes ABCA1, ABCG1 and LXRα (data not shown). We found only a moderate inhibition with TLR3 and TLR4 activators, similarly as reported previously on murine macrophages {Castrillo, 2003 #63}. Based on these results we concluded that IL-4-induced augmented response is true for PPARγ induced genes and also for an RAR target gene, but not for PPARδ or LXR. Inducers of classical activation eliminated PPARγ activity but had no or moderate effects on PPARδ, while inhibited LXR target gene expression accordingly to the literature.

IL-4 induces augmented PPARγ response in mouse macrophages

We analyzed if our findings are specific for human cells or similar regulation exists in murine macrophages as well. First, we studied C57Black/6 wild type mice. We isolated monocytes from the bone marrow and cultured them in the presence of IL-4 or IFNγ+TNF. Consistently to our results in the human cells the expression of PPARγ was increased by IL-4 and repressed by IFNγ+TNF (Figure 2A). By adding RSG PPARγ could activate gene expression of FABP4 (Figure 2B) and ADRP (Figure 2C) in the non-activated and in the alternatively activated cells but not in the classically activated ones. IL-4 *per se* could induce the expression of both target genes and similarly to the human macrophages increased ligand-induced PPARγ response. Next, we used thioglycolate-elicited peritoneal and bone marrow-derived macrophages and activated them with IL-4, IFNγ+TNF or LPS, respectively. We analyzed the expression of known murine markers

of macrophage activation (Supplementary Figure 3A-L). IL-4 induced the expression of FIZZ1, mannose receptor (MR), YM1, arginase 1 (Arg1) and PPARγ while IFNγ+TNF and LPS increased the level of inducible nitrogen-oxide synthetase (iNOS) in both peritoneal and bone marrow-derived macrophages.

Then, we treated bone marrow-derived macrophages and DCs with RSG to assess PPARγ activity. In Figure 2D RSG was added throughout the differentiation of DCs (Day1) or later as indicated. Interestingly, the mRNA level of the PPARγ target gene changed upon the time when the ligand was added. It seemed that PPARγ activator should be present from the beginning of the differentiation process to obtain maximal induction. However, a smaller response was still present when added on the 8th day. When comparing the levels of FABP4 in the immature and mature DCs there was a remarked repression of gene expression, which required only one day to almost completely eliminate the induction of the target gene (compare immature-mature Day1 values).

In case of the bone marrow-differentiated macrophages we observed a clear difference to the human cells (Figure 2E). The basal expression level of the FABP4 was higher than in the human macrophages. Probably due to this fact the dynamic range for the induction is narrower. Nevertheless, RSG could induce FABP4 more efficiently when added earlier. IL-4 could induce augmented PPARγ response at every time point examined (Figure 2E). IFNγ+TNF repressed PPARγ activity independently of the time, while LPS was more active when added later (Figure 2E).

To further examine the potential of PPARγ to activate target gene expression in murine cells we compared the inducibility of three PPARγ-regulated genes in bone marrow-derived DCs (Figure 2F, H and J) and macrophages (Figure 2G, I and K): PPARγ

Angiopoietin-related Protein (PGAR) (Figure 2F and G) FABP4 (Figure 2H and I) and ADRP (Figure 2J and K). All of them were induced in immature DCs and also in alternatively activated macrophages upon ligand treatment. We also compared PPARα, γ and δ agonists and found that the effects of RSG were impaired by IFNγ, TNF and LPS, while PPARδ agonist still induced ADRP expression in the TNF or LPS-treated cells (data not shown). With these findings we demonstrated that PPARγ can be activated in murine cells (macrophages and DCs), which in turn could induce transcription of target genes. We recognized the importance of the optimal conditions: time and length of ligand treatment, presence of IL-4 and absence of proinflammatory cytokines, bacterial compounds. The major difference in our experiments compared to others' where only a few genes were induced by the receptor {Welch, 2003 #27} was that we added the cytokines and the ligands during the whole differentiation process. We assume that during the differentiation a slightly different cell type is formed in the presence of IL-4, which is capable of responding to PPARγ activators.

Identification of PPARγ-regulated transcripts in murine macrophages and DCs

In order to further investigate PPARγ as a positive regulator of gene expression in murine cells we decided to perform microarray experiments and determine PPARγ-regulated genes in the two permissive cell types, alternatively activated macrophages and DCs. To discover PPARγ-specific targets in murine macrophages and DCs we used conditional knockout mice. To induce macrophage-specific recombination we chose the Lysozyme Cre-PPARγ lox system. Lysozyme Cre positive PPARγ$^{+/+}$ mice were used as controls. The degree of recombination was assessed by detecting the wild type and truncated

mRNAs in Supplementary Figure 4A. We analyzed the expression of PPARγ and its target genes in peritoneal macrophages and bone marrow-derived macrophages or DCs (Supplementary Figure 4B and C). PPARγ was induced by IL-4 in both the alternatively activated macrophages and during DC development, while IFNγ, TNF or LPS repressed it. FABP4 mRNA level was slightly increased in the wild type non-activated macrophages upon RSG treatment, which was remarkably increased by IL-4 and repressed by IFNγ. When comparing Lysozyme Cre positive PPARγ$^{+/+}$ and PPARγ$^{fl/fl}$ mice we could still detect the induction of PPARγ target gene at comparable levels as in the PPARγ$^{+/-}$ animals. RSG-induced target gene expression was completely absent in both alternatively activated macrophages and DCs only in the PPARγ$^{fl/-}$ mice suggesting that the recombination was more effective in mice with only one PPARγ floxed allele. Similar results were obtained in peritoneal macrophages (Supplementary Figure 4C).

Next, we performed a microarray experiment and compared the expression of RSG-regulated genes in bone marrow-derived macrophages and DCs from Lysozyme Cre positive PPARγ$^{+/-}$ (control group) and PPARγ$^{fl/-}$ animals (conditional knockout group). Agreeably to our observations we could identify 764 gene probes in DCs and 683 genes probes in alternatively activated macrophages being significantly ($p<0.05$, Benjamini multiple testing correction) regulated at least two-fold by RSG in the +/- but not in fl/- animals based on 4 biological replicates (Supplementary Table 1). From these 416 gene probes were upregulated and 348 were repressed in DCs and 330 gene probes were induced while 353 were downregulated in macrophages. These numbers indicate that under these conditions PPARγ can regulate approximately similar number of genes in both cell types and in both directions and more importantly suggest that under these

circumstances the receptor can function as a transcription factor observed in human DCs previously {Szatmari, 2007 #71} or in human macrophages (see below).

PPARγ is dispensable for alternative macrophage activation

There is a clear difference in the polarization of immune responses in the various mouse strains. C57Black/6 mice are prone to T_H1 while BALB/c mice are prone to T_H2 immune responses. Therefore, we compared if PPARγ could be induced by IL-4 in both mouse strains and found that like other markers of alternative macrophage activation like YM1, Arg1 or FIZZ1 PPARγ could be induced by IL-4 independently to the strain (Figure 4C-J). We also treated these bone marrow-derived macrophages with vehicle or RSG, respectively and importantly we could not detect any consistent effects of PPARγ activation on the expression levels of alternative macrophage markers (Figure 4C-E and G-H). To further prove that PPARγ is dispensable for alternative macrophage activation we used macrophage-specific PPARγ conditional knockout animals on C57Black/6 background and analyzed the induction of markers in peritoneal and bone marrow-derived macrophages (Figure 4K and L). As it is clearly indicated in the figures both Arg1 and YM1 were induced by IL-4 in both macrophages independently of the presence of PPARγ. We should note that the expression of these molecules largely depend on IL-4. Without IL-4 their expression is almost completely shut down and upon IL-4 they are induced rapidly to very high levels. These data indicate that unlike a previous report {Odegaard, 2007 #70} PPARγ is dispensable for alternative macrophage activation. Furthermore, we found that IL-4 is required for the induction of the receptor and more importantly to attain maximum receptor activity.

IL-4 is a general intensifier of PPARγ

After systematically characterizing the activity of PPARγ in human and murine macrophages and DCs we sought to identify the mechanism, how IL-4 activates PPARγ. First, we determined how important this phenomenon is in the general activation of the receptor. To study this we chose an unbiased approach and performed microarray experiments in human differently activated macrophages. We used non-activated, IL-4-treated and IFNγ+TNF-treated macrophages and analyzed PPARγ-regulated global gene expression changes after RSG administration (Figure 5). The results of the microarray analysis explicitly showed that IL-4 influenced PPARγ-regulated gene expression on two levels: it made PPARγ regulate a larger set of genes (Figure 5A) and simultaneously intensified the changes in the individual transcript levels (Figure 5B and Supplementary Table 2). Strikingly, without macrophage activation 120 genes were regulated, which was increased to 624 in the presence of IL-4 and decreased to 63 during classical activation. Interestingly, we identified an interesting correlation between PPARγ-regulated genes and macrophage activation-regulated genes (Figure 5C-F). Most of the PPARγ-induced genes could be also upregulated by IL-4 or downregulated by classical activation (Figure 5C 379 vs. 45 and Figure 5E 128 vs. 54). Generally, PPARγ-repressed genes could be also downregulated by IL-4 (Figure 5D and F). Here, we have to note that a larger set of PPARγ-repressed genes was downregulated than induced by classical activation (Figure 5D and E). A more detailed comparison is available in Supplementary Figure 8. With such correlation between PPARγ and activation-regulated genes it became clear that not only IL-4 activates and enhances PPARγ signaling but also PPARγ can consequently

regulate genes involved in the activation processes, which subsequently leads to a more characteristic alternative activation pattern. This is a novel crosstalk between PPARγ and macrophage activation and also suggests a new mechanism for the anti-inflammatory effects of PPARγ: the receptor induces directly its target genes, which are also targets for IL-4, which is anti-inflammatory. And similar regulation might be true for the PPARγ-repressed genes.

IL-4 acts through STAT6 to activate PPARγ

Classically IL-4 binds to its high-affinity receptor IL-4 receptor α chain {Cabrillat, 1987 #94}{Park, 1987 #95}, which in turn forms a heterodimer with IL-2 receptor common γ chain in hematopoietic cells. IL-4 induces tyrosine phosphorylation of the receptor through recruitment of a Janus tyrosine kinase (JAK). Most commonly tyrosine phosphorylation of JAK3 occurs in response to IL-4 in myeloid cells {Witthuhn, 1994 #98}. That could subsequently activate further proteins like STAT6 and insulin receptor substrate-2 (IRS-2) {Welham, 1995 #96}. Phosphorylated STAT6 forms homodimer, enters the nucleus and binds to DNA acting as a transcription factor. IRS-2 can activate src homology 2 (SH2) domain of proteins, most importantly in this case phosphoinsitol-2 kinase (PI3 kinase) {Izuhara, 1996 #97}.

According to these we wanted to address if IL-4 acts through the classical cytokine signaling via STAT6 to activate PPARγ or signals through SH2 domain binding proteins. To define the signaling pathway we used mice deficient in STAT6 and differentiated macrophages from bone marrow. In Figure 6A-C we show that IL-4 dependent induction of alternative activation markers FIZZ1 and YM1 and also that of PPARγ required the

presence of STAT6 because no induction could be detected in STAT6 knockout animals. The IL-4 induced activation of PPARγ was tested by adding RSG to the cells and PPARγ target genes' expression levels were measured (Figure 6D-F). While RSG could slightly induce target gene expression in non-activated macrophages independently to the presence of STAT6, IL-4 induced PPARγ activation after RSG treatment was almost completely missing from STAT6 knockout mice referring to the requirement of STAT6 to this phenomenon.

We also used JAK inhibitors and PI3 kinase inhibitor in human macrophages. WHI-P131 is a JAK3 inhibitor, while TYRPhostin (or AG490) is more specific for JAK2. Wortmannin was used to inhibit PI3 kinase. The induction of AMAC1, a known IL-4 regulated gene was inhibited by JAK3 inhibitor, while JAK2 and PI3 kinase inhibitors did not inhibit its transcription by IL-4 (Figure 6G). Expression of PPARγ was induced by IL-4 and this induction was inhibited with WHI-P131 but not with Wortmannin (Figure 6H). JAK2 inhibitor increased PPARγ mRNA levels probably via an unknown mechanism. Next, FABP4 was induced by RSG and this induction was further increased in the presence of IL-4 as detailed above (Figure 6I). This IL-4 induced increase was absolutely abolished by the JAK3 inhibitor, WHI-P131, while neither the JAK2 not the PI3 kinase inhibitor affected this. These results are in agreement with the literature where JAK3 was claimed as a dominant JAK kinase in myeloid cells {Witthuhn, 1994 #98}. With data from the STAT6 knockout mice and the pharmacological characterization we can conclude that IL-4 acts via JAK3 and STAT6 to increase PPARγ activity and responses.

STAT6 activates PPARγ on the promoter of PPARγ target genes

After concluding that IL-4 acts through STAT6 to activate PPARγ we took several possible mechanisms into consideration and experimentally tested them. In Supplementary Figure 8 we tested if STAT6 induced the production of a PPARγ agonist, which somehow overwrites the effects of RSG. IL-4 was shown to increase the production of a PPARγ activator, 15d-PGJ_2 via inducing 12/15-lipoxygenase {Huang, 1999 #19}. A possible mechanism could be the recently reported covalent binding of such or similar ligands {Itoh, 2008 #99}. By showing that IL-4 augmented PPARγ response well before the induction of 15-lipoxygenase in human macrophages we excluded that possibility (Supplementary Figure 9 A and B). We also excluded that STAT6 would generate an activator for the Retinoid X Receptor, the permissive dimerization partner for the PPARγ by using RXR antagonist (Supplementary Figure 9C). Next, we tested if STAT6 induces histone acetylation and consequently opening of the chromatin making it easier accessible for PPARγ. We used trichostatin A, an inhibitor of histone deacetylases but no difference could be observed in the non-activated cells suggesting that the histone tails are not deacetylated and chromatin is not closed in the absence of IL-4 (Supplementary Figure 9D and E). Further possible mechanism could be that STAT6 induces the degradation of a repressor for PPARγ. We addressed this by using proteasome inhibitor, MG132 (Figure 6 G-I) but IL-4 still improved PPARγ response in the presence of the proteasome inhibitor. We addressed the opposite scenario as well: induction of an activator and or PPARγ itself. We showed that IL-4 could increase PPARγ mRNA level (Figure 1A). Although, we illustrated that the activity of PPARγ concerning target gene induction did not correlate with the expression level of the

receptor (Figure 1) we tested this scenario by inhibiting new protein synthesis by cycloheximide (CXM) (Supplementary Figure 9F-H). The expression of a STAT6-regulated gene, AMAC1 and that of PPARγ were induced by IL-4, indicating the direct transcriptional event. Obviously, RSG could activate PPARγ independently of new protein synthesis. And IL-4 could still promote the effects of the PPARγ activator, indicating that the STAT-6-augmented PPARγ response did not require new protein synthesis. However, IL-4 could ameliorate PPARγ response, a slight decrease could be observed generally in the mRNA levels of FABP4, which could be due to the impaired production of PPARγ protein after IL-4 treatment. With this experiment we could dissociate the double effect of IL-4 on PPARγ: induction of the receptor and improvement of its responses. The first by definition depends on the protein synthesis but it is dispensable for the second.

To add further evidence that STAT-6 improves PPARγ responses independently of the receptor level we used transiently transfected cells overexpressing PPARγ by a constitutively active cytomegalovirus promoter. In these cells PPARγ target gene expression could be enhanced by STAT6 when cotransfected and activated by IL-4 (Supplementary Figure 9I).

STAT6 interferes with PPARγ signaling on the transcription level

From these data we could conclude that STAT6 presumably acts on the promoter of PPARγ target genes and interferes with PPARγ signaling at the transcription level. We tested the interaction of PPARγ with corepressors and coactivators in mammalian two-hybrid experiments but IL-4 or STAT6 did not influence that interaction (data not

shown). To study PPARγ response on the promoter of its target genes we chose the promoter of the previously analyzed PPARγ target gene, FABP4. We isolated a 5-kilobase (kb) fragment of the FABP4 promoter and studied in a reporter assay. The 5kb fragment responded to PPARγ activators and also to IL-4 (Figure 7A and B). Moreover, IL-4 could augment the effect of RSG consistently to the endogenous response of the target gene (Figure 7A and B). As part of the promoter analysis we generated deletion fragments (Figure 7E and data not shown) and identified the response element for the PPARγ:RXR (Figure 7F). We characterized the enhancer in reporter assays and also in electromobility shift assays (EMSA). The identified response element (Figure 7G), in consistence with the induction of the host gene, showed preferential response (Figure 7F) and binding to PPARγ (Figure 7H and I), which was due to its specific nucleotide sequence (Figure 7G). When we mutated the PPARγ binding site to the consensus AGGTCA we could detect the binding of PPARα, γ and δ as RXR heterodimers and also RXR homodimer (Supplementary Figure 10D). Interestingly, in the promoter of the mouse FABP4 another enhancer was described earlier {Tontonoz, 1994 #80}. In the human gene the homologue of this mouse enhancer was also responsive to PPAR activators very similarly to the newly described element (aP2 A refers to the newly identified element and aP2 PT refers to the homologue of the mouse enhancer) (Supplementary Figure 10A-C).

Unexpectedly, we could detect the presence of a STAT6 binding site downstream to the newly identified enhancer (Figure 7G), which was not present around the other element (data not shown). This STAT6 response element was functional and as efficient as a known enhancer from the promoter of the eotaxin gene when tested in a reporter assay

(Figure 7C and D). A short promoter fragment that contained the composite element (both the newly described DR1 and the downstream STAT6 element) (Figure 7J) behaved similarly as the originally tested 5kb fragment (Figure 7A and B) indicating that this short DNA fragment contained all the required elements for the STAT6-augmented PPARγ response. Mutation of the STAT6 binding site in the composite element resulted in the annihilation of its responsiveness to IL-4 (Figure 7L) without effecting its induction by RSG. Mutation of the DR1 inhibited RSG-induced activation and interestingly almost completely eliminated the effects of IL-4 (Figure 7M). The newly described element clearly differed from the mouse homologue in its responsiveness to IL-4: it did not show any response upon IL-4 (Figure 7K).

We identified a composite response element in the promoter of the FABP4 containing a DR1 for PPARγ:RXR and another binding site for the STAT6. IL-4 could activate the transcription through the STAT6 element and could also improve PPARγ response through this element. These results indicate the requirement of DNA binding of the STAT6 to its response element to exert its effect on PPARγ. When isolated consensus DR1s (consensus or the one isolated from the enhancer of the FABP4) were tested STAT6 was not effective (Supplementary Figure 10E and F). Similarly, activity of Gal-fusion PPARγ could not be augmented by STAT6 (Supplementary Figure 10G).

With chromatin immunoprecipitation (ChIP) we could show the *in vivo* binding of PPARγ to the new composite element and also to the mouse homologue element (Figure 8A and B). In consistence with our previous results STAT6 binding could be detected only on the new element (Figure 8C and D).

STAT6 interacts with PPARγ

Since the two binding sites are in close proximity we sought to analyze if the two transcription factors could interact. First, we performed co-immunoprecipitation and we could detect the binding of STAT6 to PPARγ (Figure 8E and F). In order to characterize the interaction more intensively we performed pull-down experiments and STAT6 could be pulled down with resin-bound PPARγ (Figure 8G). We could detect interaction in the absence of IL-4 but IL-4 increased this interaction.

By using various fragments of the STAT6 we could localize the interaction site to the XXX part of the STAT6. This is in agreement with or other results obtained from mammalian two-hybrid experiments (Supplementary Figure 11). We also analyzed the effects of these STAT6 fragments when transfected into 293T cells and analyzed which fragment could augment PPARγ responses (Supplementary Figure 11).

Our results indicate that IL-4 augments PPARγ response via activation of STAT6, which consequently translocates to the nucleus binds to its response elements and interacts with PPARγ in order to improve its activity after ligand binding. With an unbiased method, using global gene expression profiling we showed that this phenomenon could work with most of the PPARγ-regulated genes (Figure 5A and B). However, to find and prove the exact molecular mechanism we characterized FABP4. We found a mechanism, which suggests that it might work in case of other target genes if response elements are present in the promoter of target genes. So, we used a bioinformatics approach to analyze the occurrence of STAT6 elements in the proximity of PPARγ binding sites. We could demonstrate that the incidence of STAT6 and also STAT1 response elements is higher in the neighborhood of PPARγ response elements (Figure 8H).

Discussion

Estrogen receptor β inhibits ligand-induced PPARγ activity in adipocytes {Foryst-Ludwig, 2008 #108}.

Crosstalk: ERβ-PPARγ, LEPR-STATs, adipocyte-IL-4-PPARδ, PPARγ alternative activation Cell Metabolism by B. Steals {Bouhlel, 2007 #109}

Disc.: The above model can be used only for conditions where PPARγ is considered mainly as a negative regulator of gene expression. However, it does not explain how the specificity of the liganded receptor can distinguish positive and negative regulation of transcription.

We also determined the PPARγ-regulated genes and biological processes in the macrophages under the various inflammatory conditions. We performed analysis on multiple levels: 1. We studied PPARγ expression in various types of macrophages 2. We analyzed the activity of PPARγ on the level of the individual target genes. 3. With a systematic, non-biased approach we determined the global changes caused by PPARγ activation regarding the activation status of the macrophages.

Analyzing the expression of PPARγ in various tissues we demonstrated that PPARγ is expressed mainly in the macrophages and its distribution shows an interesting pattern. Not every macrophage expresses PPARγ, but most of the macrophages carrying the alternative activation marker also have PPARγ. These highly PPARγ-positive cells are preferentially localized in the perifollicular areas of the lymphoid organs within the tissues and can be found in force at regions where macrophages first meet external stimuli (alveolar, perivascular, intestinal macrophages).

When we activated macrophages in vitro we found that PPARγ is not only induced upon alternative activation but also its activity is highly enhanced in the alternatively activated macrophages while classical activation decreases the level of the receptor and almost totally abolishes PPARγ-driven responses concerning both the positive and negative transcriptional events.

We also identified the transcriptional targets of the receptor finding more upregulated than downregulated genes. Genes from either group belong dominantly to the alternative activation state. By analyzing PPARγ-regulated genes we determined the basic biological processes being modulated by the receptor and conclude that PPARγ influences two major programs: lipid metabolism and immune responses.

With the data presented here it became important to study the inflammatory environment of macrophages and probably other cells when PPARγ is analyzed. Based on our work these conditions have dramatic effects on PPARγ activity, which should be addressed in future studies.

It became also apparent from our results that PPARγ is a key transcription factor in the biology of alternatively activated macrophages. By inducing and activating the receptor IL-4 turns on PPARγ-regulated processes, which in turn will strengthen immunological changes IL-4 induced and also activate other types of responses e.g. lipid metabolism-related ones that are not part of the IL-4-induced closely defined program.

Our findings also suggest a new mechanism for the anti-inflammatory effects of the PPARγ: as a participant of the alternative activation program many anti-inflammatory effects ascribed to PPARγ may be due to its direct contribution to the transcriptional changes of alternative activation by which it shifts the macrophage from the classical

towards the alternative activation and as a part of this program several inflammatory genes are repressed directly or indirectly. PPARγ serves as a modulator of the alternative activation-induced transcriptional program by 1. further increasing many IL-4-induced genes and 2. inducing many genes repressed by classical activation which further enhances IL-4 dominance.

Our present results indicate that the sequence of the various stimuli is of critical importance since pathogen-stimulated macrophages have no functional PPARγ so its agonists could hardly express receptor-dependent anti-inflammatory reactions while generation of IL-4 dominant environment and subsequent activation of the receptor is capable to inhibit inflammation by inducing its target genes. This hypothesis is supported by the findings of Alleva et al. who showed that treatment of murine macrophages with IFNγ prevented PPARγ activators from suppressing pro-inflammatory cytokines {Alleva, 2002 #26}. We also claim that PPARγ is a predominantly positive regulator of transcription in human cells by inducing many genes being also induced during alternative activation or repressed during classical activation or being independent of activation.

Our results throw new light upon the relation of PPARγ and LXR. Both PPARγ and LXR agonists expose anti-inflammatory effects {Joseph, 2003 #64}, both receptors are influenced by the inflammatory signals of the environment {Castrillo, 2003 #63}. In the human macrophages we found similar results as it was reported by Castrillo et al. {Castrillo, 2003 #63} in mouse macrophages: TLR3 (PolyI:C) and 4 (LPS) agonists but not TLR9 activators (CpG) inhibited LXR-induced target gene transcription and according to our findings all these signals inhibit PPARγ signaling as well. An interesting

observation is the opposite regulation of PPARγ and LXRα mRNAs upon inflammatory cytokines, which influence only PPARγ but not LXRα raising the possibility of a macrophage where PPARγ and LXRα signaling can be separated. The functional consequences of such a cell type are elusive and require further investigation.

Upon these results we assign the spatiotemporal niche, namely the alternatively activated macrophages, for PPARγ in terms of the various inflammatory conditions of the macrophages throughout the body and also define the functional consequences of the active PPARγ by laying down the receptor-regulated biological programs. Our findings also identify PPARγ as a novel marker and functional participant of the alternative activation process in macrophages that regulated the transcription of many activation-related genes.

It was reported previously that IL-4 induced 12/15 lipoxygenase in murine macrophages that activated PPARγ by producing endogenous activators for the receptor {Huang, 1999 #19}. To test this hypothesis in the human system we measured the expression of 15-lipoxygenase and it is clear from our time course experiments that the IL-4 enhanced PPARγ activity is detectable much earlier than the lipoxygenase mRNA could be detected (data not shown). Therefore it is unlikely that the lipoxygenase pathway contributes to this enhanced activity at early time points.

The role of Th2 cytokines in atherosclerosis is more controversial. Both Th1 and Th2 responses are involved in the pathogenesis of atherosclerosis with a predominant role for the pro-inflammatory molecules in the pathogenesis {King, 2002 #73}{Davenport, 2003 #72}. This is also supported by the observation that C57Black/6 mice (prone to Th1 responses) can develop atherosclerosis while BALB/c mice (prone to Th2 immune

responses) are relatively resistant to atherogenesis {Paigen, 1985 #74}{Huber, 2001 #75}. Targeted deletion of the transcription factor STAT6 through which IL-4 regulates gene expression in BALB/c mice makes them susceptible to atherogenesis {Huber, 2001 #75}.

Experimental procedures

Materials

Cells were treated with the following ligands: AM580 (Biomol), LG268, a gift from R. Heyman (Ligand Pharmaceuticals), Wy14643, Rosiglitazone (Rosigl.), T0901317 (Alexis Biochemicals), GW501516 and GW9662 were gifts from T. M. Willson (GlaxoSmithKline), oxidized LDL and diI-oxidized LDL (Intracel). Cytokines were obtained from Peprotech. All other reagents were obtained from Sigma or as indicated.

Flow cytometry

Analysis of cell surface expression of proteins was performed on a Beckton Dickinson FACSCalibur Flow Cytometer. Briefly, cells were washed in PBS (phosphate buffered saline) pH7.4 supplemented with 0.5% (BSA) bovine serum albumin then were incubated with antiCD14-PE, antiCD86-PE, antiCD23-PE, anti-mannose receptor-PE, antiDC-SIGN-FITC, anti HLA-DR-PE, antiCD1a-PE or isotype control (Beckton-Dickinson) antibody, respectively for 1 hour at 4^{o}C, finally cells were washed in PBS-BSA and 10000 cells were counted on the cytometer.

Immunohistochemistry

For immunocytochemistry macrophages ($6x10^{6}$ cells/group) were pelleted and fixed in 4% paraformaldehyde (pH 7.3) for 24 h at 4^{o}C. Cell blocks were then embedded in paraffin followed by serial sectionings (4 μm thick). After deparaffinisation and dehydration, serial sections from each cell group, mounted on the same glass slides, were

used for peroxidase-based indirect immunohistochemistry (IHC). Briefly, sections were treated with 3% H_2O_2 in methanol for 15 min at room temperature to block the endogenous peroxidase. For antigen unmasking, sections were heated in antigen retrieving citrate buffer (pH 6.0, Dako) for 2 min at 120^{o}C using a pressure cooker. Immunostainings of cells for PPARγ were carried out using the biotin-free Catalyzed Signal Amplification IHC detection kit according to the manufacturer's instructions (CSAII, Dako,). After blocking the non-specific binding sites, sections were incubated with the primary antibodies for 1 h at room temperature prior to use the biotinylated secondary antibodies. The peroxidase-mediated color development was set up for 5 min using the VIP substrate (Vector Labs). Finally, the sections were counterstained with methylgreen. Immunofluorescence (IF) stainings were carried out on normal human tissues obtained from the formalin-fixed and paraffin-embedded surgical specimen archives of the Department of Pathology, University of Debrecen. Following the incubations with the primary then the horse-radish peroxidase (HRP) conjugated secondary goat anti-mouse antibodies, IF for PPARγ staining was carried out using the tetramethyl-rhodamine (TMR)-tagged tyramide reagent of the fluorescent amplification kit according to the manufacturer's instructions. All other IF stainings (CD68, DC-SIGN) were made using biotinilated secondary antibodies and streptavidin-FITC. For double IF, sequential immunostainings were used. Briefly, following the red fluorescence TMR development for PPARγ staining, the second primary antibodies were applied and then developed with FITC (green fluorescence) using the fluorescent ABC kit. After rinsing, the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue nuclear fluorescence). Fluorescent microphotographs were made with single exposure

using excitation filter to visualize simultaneously both green (FITC) and red (TMR) together with the blue (DAPI) fluorescence lights.

Isolation and culture of human monocytes and stem cells

Human monocytes were isolated from healthy volunteer's buffy coat obtained from the Regional Blood Bank. Monocyte separation was carried out according to the manufacturer's instructions using CD14 MicroBeads (Miltenyi Biotec). Monocytes were differentiated for the indicated time. Cells were cultured in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, penicillin and streptomycin and treated with vehicle (ethanol:dimethyl-sulfoxide 1:1) or as indicated. For the activation we used IL-4 (100 ng/ml), IFNγ (100 ng/ml) and TNFα (50 ng/ml), IL-13 (100 ng/ml), Dexamethasone (2.5 μM), *E. coli* (O55:B5 serotype) LPS (100 ng/ml), E. coli Lipid A (50 μg/ml), CpG (100 nM), Polyinsinic-polycitydilic acid (PolyI:C) (5 μg/ml), *S. aureus* lipoteichoic acid (LTA) (5 μg/ml) or *L. monocytogenes* (ATCC 43251) at a MOI (multiplicity of infection) of 10.

Human CD34 positive stem cells were isolated with CliniMax (AmCell GmbH, Bergisch Gladbach, Germany) from peripheral blood of granulocyte colony stimulating factor-treated patients according to the protocol. Stem cells were expanded with recombinant human Flt-3L (25 ng/ml), stem cell factor (20 ng/ml), IL-6 (20 ng/ml) and IL-3 (20 ng/ml) for 10 days and then differentiated to macrophages with recombinant human M-CSF (10 ng/ml) for 8 days.

RNA isolation and real-time quantitative RT PCR

Total RNA was isolated from cells using Trizol Reagent (Invitrogen) according to the instructions. RNA was transcribed into cDNA via random hexamer priming using SuperScript II (Invitrogen) reverse transcriptase. Transcript quantification was performed by quantitative real-time RT (reverse transcriptase) PCR (polymerase chain reaction) using Taqman probes (self-made assays) or Taqman Gene Expression Assays (Applied Biosystems). Transcript levels were normalized to the level of cyclophilin D. Sequences of primers and Taqman probes or Taqman Assays IDs used in transcript quantification are listed in Supplemental Table 1.

For the validation of the microarray data we used Taqman Low Density Arrays (TLDA). The Assay IDs are listed in Supplemental Table 2. For these experiments RNA samples were transcribed with High Capacity cDNA Archive Kit (Applied Biosystems).

Western blotting

Cells were treated for two days as indicated and were washed in PBS then lysed in buffer A (Tris-HCl pH7.5, 1mM EDTA, 15mM β-mercaptoethanol, 0.1% Triton X 100, 0.5mM PMSF (phenyl-methyl-sulfonyl fluoride). 25μg total protein was separated on 10% SDS-PAGE (polyacrylamid gel electrophoresis) and transferred to PVDF membrane (Bio-Rad Laboratories). After blocking with 5% dry milk the membrane was probed with anti-FABP4 antibody (Cayman Chemical Company) or anti-GAPDH (glyceraldehyde-3-phosphate-dehydrogenase) antibody (Abcam) and subsequently with peroxidase-conjugated secondary antibody. ECL detection kit (Pierce) was used for signal detection.

Mice

Mice carrying null or floxed alleles of *Pparγ* were created as described previously {Barak, 1999 #4}, {Miles, 2000 #56}, {Hevener, 2003 #57}. These mice were backcrossed to the C57BL/6J strain for eight generations. Mice were bred with Lysozyme-Cre (Lys-Cre) transgene animals to create the following genotypes: $Ppar\gamma^{+/+}$ Lys-Cre, $Ppar\gamma^{fl/fl}$ Lys-Cre, $Ppar\gamma^{+/-}$ Lys-Cre and $Ppar\gamma^{fl/-}$ Lys-Cre. Genotypes were determined by PCR of tail DNA. PCR genotyping was carried out by using the following primers: for the Cre transgene, 5'-GCATTACCGGTCGATGCAACGAGTG-3' and 5'-GAACGCTAGAGCCTGTTTTGCACGTTC-3'; for the upstream loxP site, 5'-CTAGTGAAGTATACTATACTCTGTGCAGCC-3' and 5'-GTGTCATAATAAACATGGGAGCATAGAAGC-3'; and for the null allel, 5'-AGGCCACCATGGAAAGCCACAGTTCCTC-3' and 5'-GCTGGCGAAAGGGGGATGTGCTGCAAG-3'. Genomic DNA was amplified by 35 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 55 s.

RT PCR was performed with SuperScript II (Invitrogen). Sense (5'-GTCACGTTCTGACAGGACTGTGTGAC-3') and antisense (5'-TATCACTGGAGATCTCCGCCAACAGC-3') primers were designed to anneal to regions in exons A1 and 4 of PPAR1, respectively, which distinguish the full-length (700-bp) and recombined (300-bp) transcripts {He, 2003 #58}. PCR was performed by 40 cycles of 94°C for 20 s, 60°C for 30 s, and 72°C for 60 s. Lysozyme-Cre animals were obtained from I. Förster (University of Munich) {Clausen, 1999 #61}. All animal experiments were carried out under ethical guidelines, which were established by the 28th Act in 1998 of the Parliament of the Republic of Hungary. Animals were housed under minimal disease *(MD)* conditions in a laboratory animal facility seeing the requirements

of FELASA recommendations and DIN EN ISO 9001 standards. Animal boxes were ventilated with HEPA filtered air, animals received sterilized pellet diet (Altromin) and tap water (*ad libitum).* The cages contained sterilized bedding material. The room lightning was automatically switched on at 6:00 and off at 18:00. The room temperature was 20± 2 °C, the relative humidity was 50%.

Isolation and culturing of mouse peritoneal and bone marrow cells

Thioglycolate-elicited macrophages were harvested from the peritoneal cavity 4 days after injection of 3 ml 3% thioglycolate solution. Cells were washed in saline and cultured in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, penicillin and streptomycin for two days. Bone marrow cells were isolated from the femur of mice then were washed in saline then cultured in RPMI 1640 supplemented with 10% FBS, 2mM glutamine, penicillin and streptomycin. These cells were differentiated to macrophages by M-CSF (20 ng/ml) or to dendritic cells by GM-CSF (20 ng/ml) and IL-4 (20 ng/ml) for 10 days. For activation of macrophages similar methods were used as in the human macrophages: IL-4 (20 ng/ml), IFNγ (100 ng/ml), TNFα (20ng/ml), *E. coli* (O55:B5 serotype) LPS (100 ng/ml). Fresh medium containing cytokines and ligands were added every third day to complement the old medium.

Microarray analysis

Total RNA was isolated using RNeasy kit (Qiagen). cRNA was generated from 5 μg of total RNA by using the SuperScript Choice Kit (Invitrogen) and the High Yield RNA transcription labeling kit (Enzo Diagnostics). Fragmented cRNA was hybridized to

Affymetrix (Santa Clara, CA) arrays (Human Genome U133 Plus 2.0) according to Affymetrix standard protocols. Analysis was performed using GeneSpring 7.2 (Agilent). The Affymetrix .cel files were loaded into GeneSpring and analyzed by GC-RMA. 3 biological replicates (3 for each condition, non-activated, alternatively activated and classically activated plus/minus Rosigl. treated) of the 12h treated samples were analyzed together as replicates. After a per chip normalization to the 50th percentile of expression values obtained from the whole array we performed per gene normalization to the median expression of the given gene during the various conditions and finally each chip were normalized to its specific vehicle-treated control. After determining the changing genes based on a T-test (parametric, variances assumed to be equal, using Benjamini and Hochberg false discovery rate as multiple testing correction), p-value cutoff 0.05 we selected those genes that showed at least 1.5 fold changes. For the validation on the TLDA we chose changing genes based on two independent analyses. We reanalyzed the original microarray data with MAS 5 algorithm and determined the changing changes in a similar manner as with the GC-RMA and selected genes that showed significant changes upon Rosigl. treatment with both methods. 4 replicates of a 6h long Rosigl. treatment of alternatively activated macrophages were also analyzed similarly and based on these data we completed the list of genes selected for validation. Activation-specific changes were analyzed similarly. TLDA results were analyzed in a similar manner as the microarray data. We loaded the raw Ct values obtained in the real-time Q-PCR runs into GeneSpring and handled as Q-PCR data. For normalization we chose three methods: first we normalized to the housekeeping gene cyclophilin D, second to another housekeeping gene, 36B4 and third we performed a global scaling by normalizing everything to the

median expression of all measured transcripts. We went through the analysis as described above and took those genes that showed significant changes in at least two normalization methods.

Statistical tests

All data are presented as means ±SD. In real-time quantitative PCR the mean and standard deviation were calculated for both the normalized and the normalizer values. To incorporate the random errors of the measurements we used the propagation of errors to determine the standard deviation of the normalized values. For all experiments we made at least four biological replicates and on the fold changes we performed an F test followed by an unpaired (two tail) t test and results were considered significant with $p<0.01$.

Acknowledgement

We thank Ibolya Furtos, Brigitta Menyhart and Anita Farkas for the technical assistance. L.N is an International Scholar of HHMI and holds a Wellcome Trust Senior Research Fellowship in Biomedical Sciences and also supported by a grant from the Hungarian Science Research Fund (OTKA NK72730 to L.N). A.Sz. is supported by the Hungarian Academy of Sciences (Bolyai Scholarship) and by grants from Hungarian Science Research Fund (OTKA/61814) and from the Univ. of Debrecen (Mecenatura).

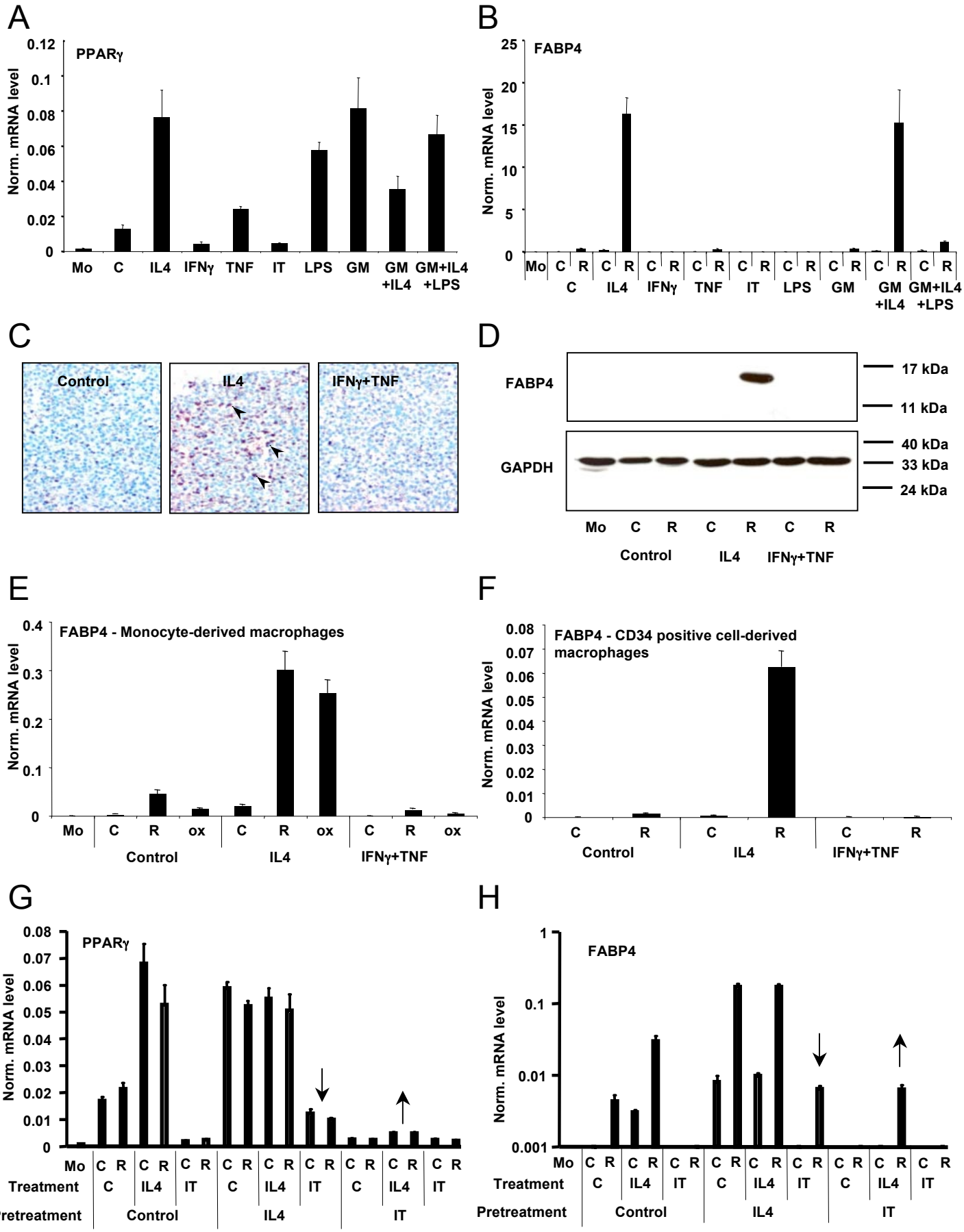

A
PPARγ
Norm. mRNA level
0.12
0.1
0.08
0.06
0.04
0.02
0
Mo
C
IL4
IFNγ
TNF
IT
LPS
GM
GM +IL4
GM+IL4 +LPS
B
FABP4
Norm. mRNA level
25
20
15
10
5
0
Mo C R C R C R C R C R C R C R C R C R C R
C
IL4
IFNγ
TNF
IT
LPS
GM
GM +IL4
GM+IL4 +LPS
C
Control
IL4
IFNγ+TNF
D
FABP4
GAPDH
17 kDa
11 kDa
40 kDa
33 kDa
24 kDa
Mo C R C R C R
Control
IL4
IFNγ+TNF
E
FABP4 - Monocyte-derived macrophages
Norm. mRNA level
0.4
0.3
0.2
0.1
0
Mo C R ox C R ox C R ox
Control
IL4
IFNγ+TNF
F
FABP4 - CD34 positive cell-derived macrophages
Norm. mRNA level
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
C R C R C R
Control
IL4
IFNγ+TNF
G
PPARγ
Norm. mRNA level
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Mo C R C R C R C R C R C R C R C R C R C R
Treatment C IL4 IT C IL4 IT C IL4 IT
Pretreatment Control IL4 IT
H
FABP4
Norm. mRNA level
1
0.1
0.01
0.001
Mo C R C R C R C R C R C R C R C R C R C R
Treatment C IL4 IT C IL4 IT C IL4 IT
Pretreatment Control IL4 IT

Figure 1

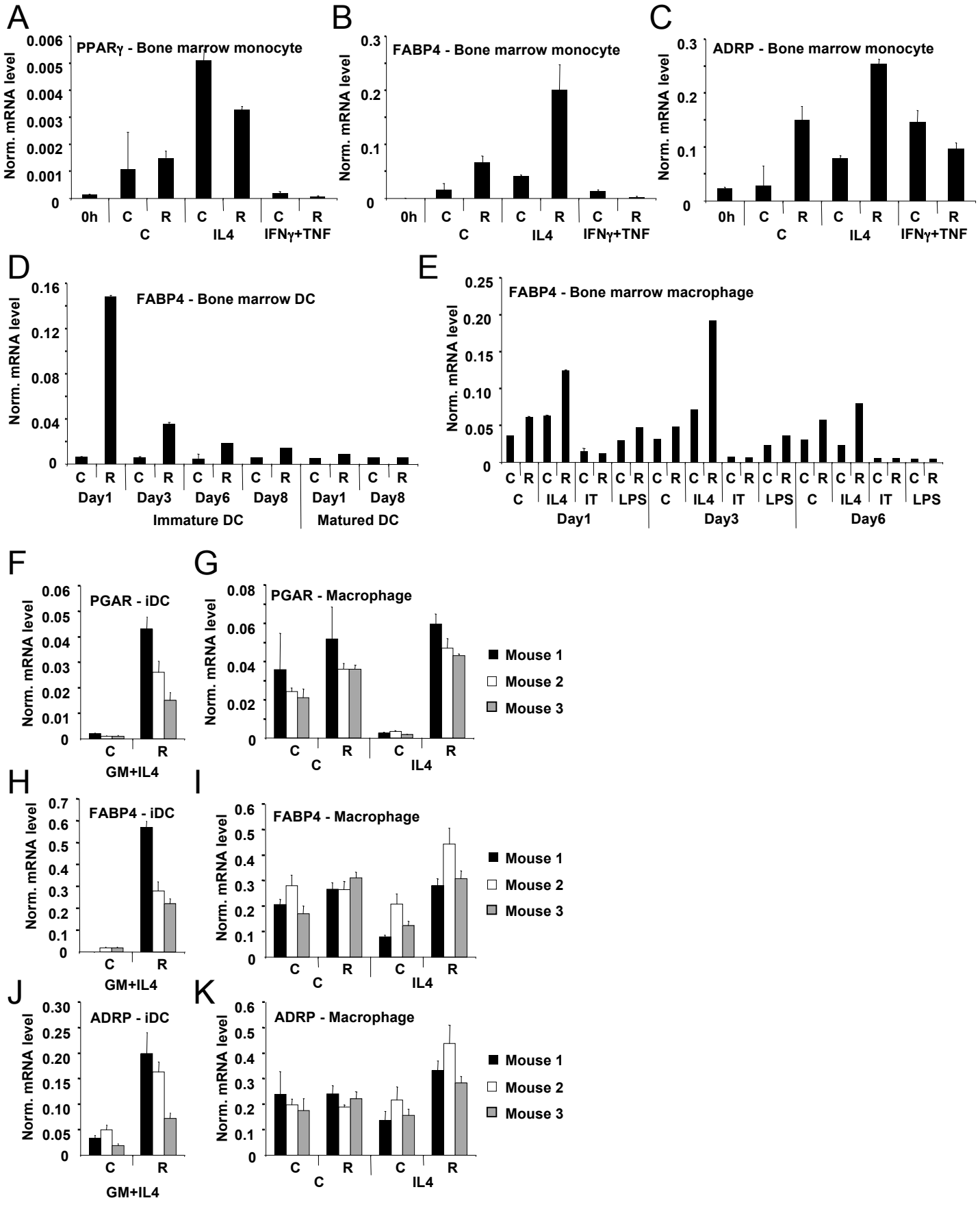

A
PPARγ - Bone marrow monocyte
Norm. mRNA level
0.006
0.005
0.004
0.003
0.002
0.001
0
0h C R C R C R
C IL4 IFNγ+TNF
B
FABP4 - Bone marrow monocyte
Norm. mRNA level
0.3
0.2
0.1
0
0h C R C R C R
C IL4 IFNγ+TNF
C
ADRP - Bone marrow monocyte
Norm. mRNA level
0.3
0.2
0.1
0
0h C R C R C R
C IL4 IFNγ+TNF
D
FABP4 - Bone marrow DC
Norm. mRNA level
0.16
0.12
0.08
0.04
0
C R C R C R C R C R C R
Day1 Day3 Day6 Day8 Day1 Day8
Immature DC
Matured DC
E
FABP4 - Bone marrow macrophage
Norm. mRNA level
0.25
0.20
0.15
0.10
0.05
0
C R C R C R C R C R C R C R C R C R C R C R C R C R C R C R C R C R C R
C IL4 IT LPS C IL4 IT LPS C IL4 IT LPS
Day1
Day3
Day6
F
PGAR - iDC
Norm. mRNA level
0.06
0.05
0.04
0.03
0.02
0.01
0
C R
GM+IL4
G
PGAR - Macrophage
Norm. mRNA level
0.08
0.06
0.04
0.02
0
C R C R
C IL4
Mouse 1
Mouse 2
Mouse 3
H
FABP4 - iDC
Norm. mRNA level
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
C R
GM+IL4
I
FABP4 - Macrophage
Norm. mRNA level
0.6
0.5
0.4
0.3
0.2
0.1
0
C R C R
C IL4
Mouse 1
Mouse 2
Mouse 3
J
ADRP - iDC
Norm. mRNA level
0.30
0.25
0.20
0.15
0.10
0.05
0
C R
GM+IL4
K
ADRP - Macrophage
Norm. mRNA level
0.6
0.5
0.4
0.3
0.2
0.1
0
C R C R
C IL4
Mouse 1
Mouse 2
Mouse 3

Figure 2

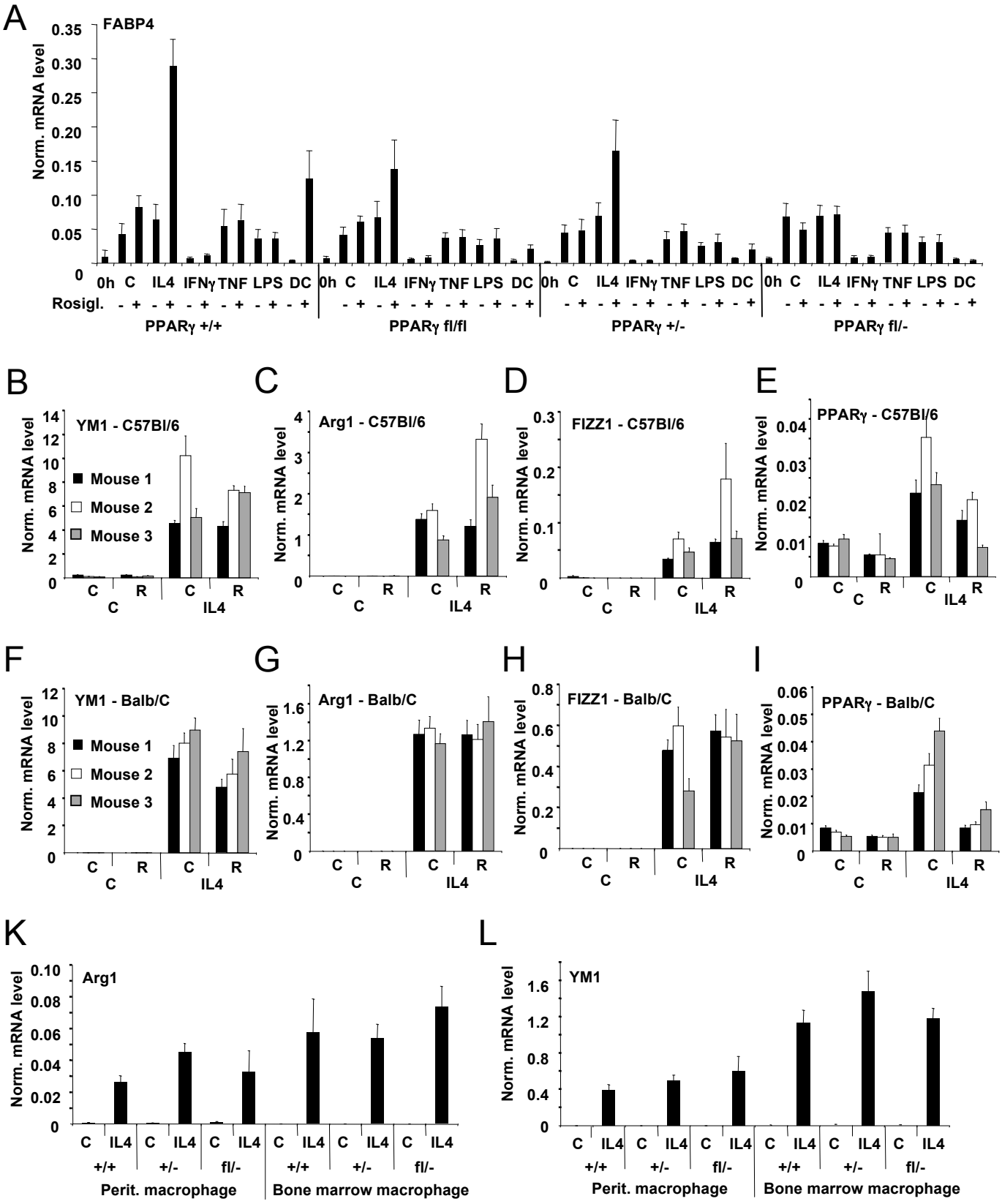

A
FABP4
Norm. mRNA level
0h C IL4 IFNγ TNF LPS DC
Rosigl. - + - + - + - + - + - +
PPARγ +/+
PPARγ fl/fl
PPARγ +/-
PPARγ fl/-
B
YM1 - C57Bl/6
Mouse 1
Mouse 2
Mouse 3
C
Arg1 - C57Bl/6
D
FIZZ1 - C57Bl/6
E
PPARγ - C57Bl/6
F
YM1 - Balb/C
G
Arg1 - Balb/C
H
FIZZ1 - Balb/C
I
PPARγ - Balb/C
C R C R
C IL4
K
Arg1
L
YM1
C IL4 C IL4 C IL4 C IL4 C IL4 C IL4
+/+ +/- fl/- +/+ +/- fl/-
Perit. macrophage
Bone marrow macrophage

Figure 3

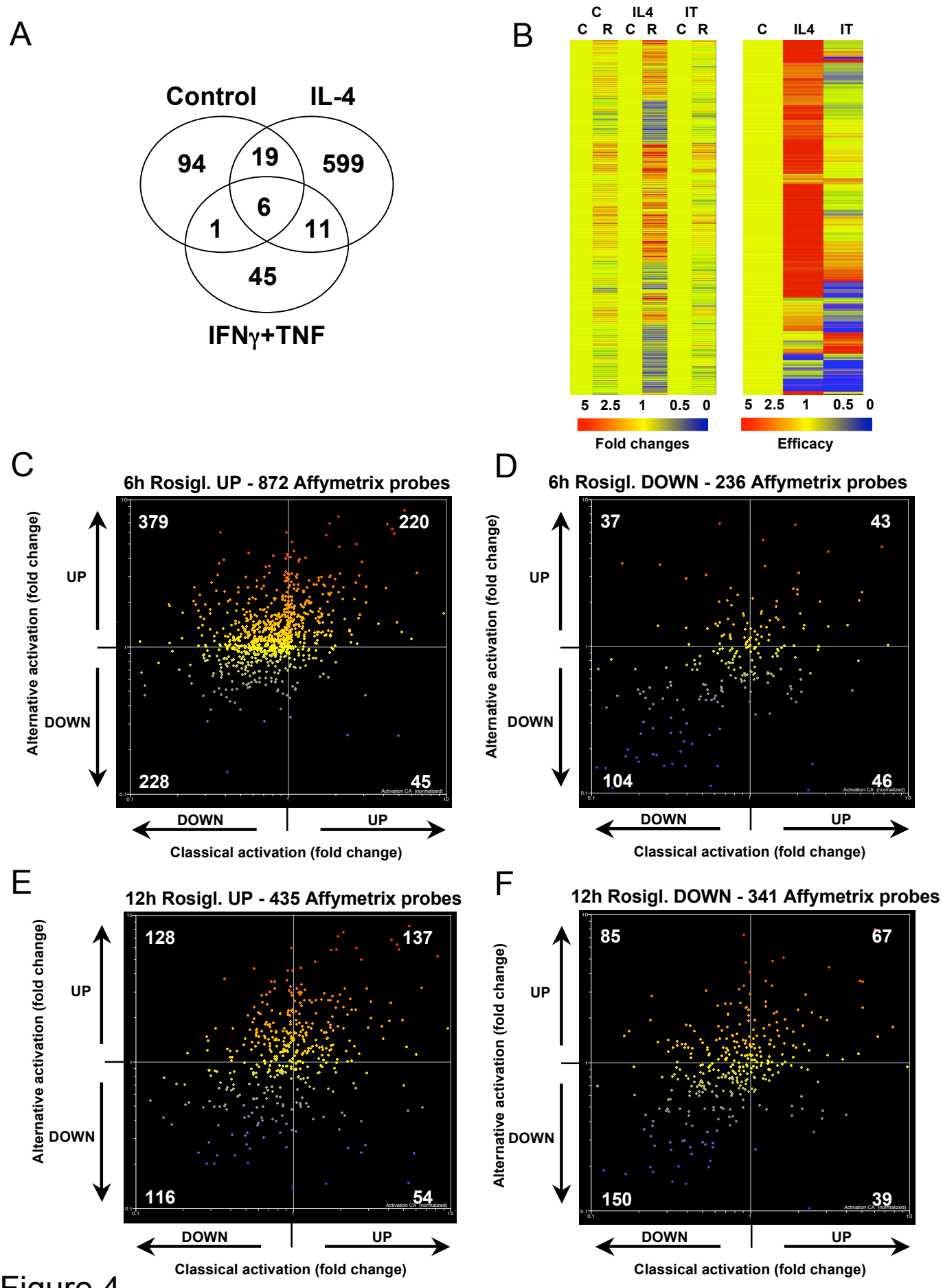

A
Control
IL-4
IFNγ+TNF
94
19
599
6
1
11
45
B
C
IL4
IT
C R C R C R
C
IL4
IT
5 2.5 1 0.5 0
5 2.5 1 0.5 0
Fold changes
Efficacy
C
6h Rosigl. UP - 872 Affymetrix probes
379
220
228
45
UP
DOWN
Alternative activation (fold change)
DOWN
UP
Classical activation (fold change)
D
6h Rosigl. DOWN - 236 Affymetrix probes
37
43
104
46
UP
DOWN
Alternative activation (fold change)
DOWN
UP
Classical activation (fold change)
E
12h Rosigl. UP - 435 Affymetrix probes
128
137
116
54
UP
DOWN
Alternative activation (fold change)
DOWN
UP
Classical activation (fold change)
F
12h Rosigl. DOWN - 341 Affymetrix probes
85
67
150
39
UP
DOWN
Alternative activation (fold change)
DOWN
UP
Classical activation (fold change)

Figure 4

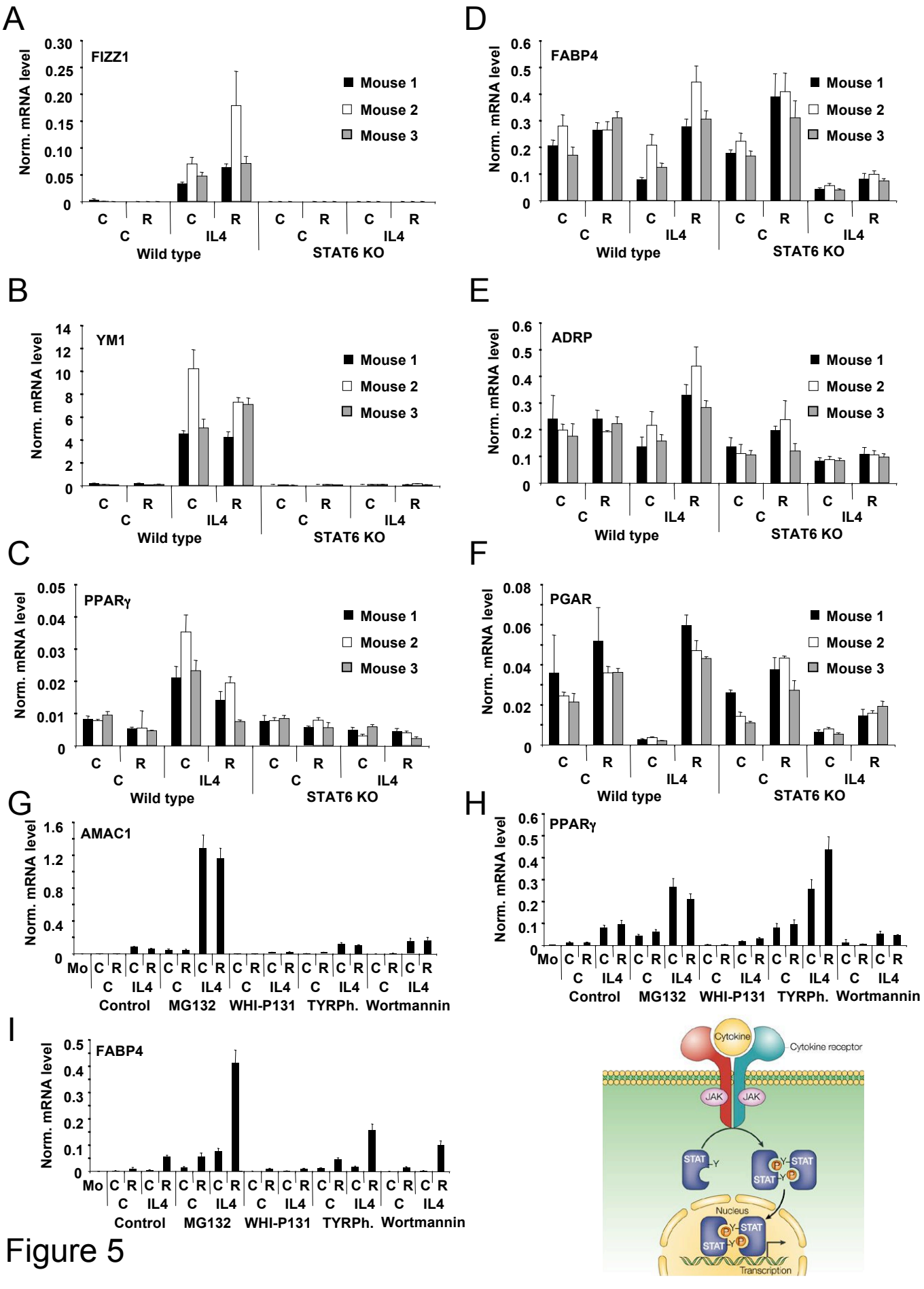

A
FIZZ1
Norm. mRNA level
Mouse 1
Mouse 2
Mouse 3
C R C R C R C R
C IL4 C IL4
Wild type
STAT6 KO
B
YM1
C
PPARγ
D
FABP4
E
ADRP
F
PGAR
G
AMAC1
Mo C R C R C R C R C R C R C R C R C R
Control
MG132
WHI-P131
TYRPh.
Wortmannin
H
PPARγ
I
FABP4
Cytokine
Cytokine receptor
JAK
STAT
Nucleus
Transcription

Figure 5

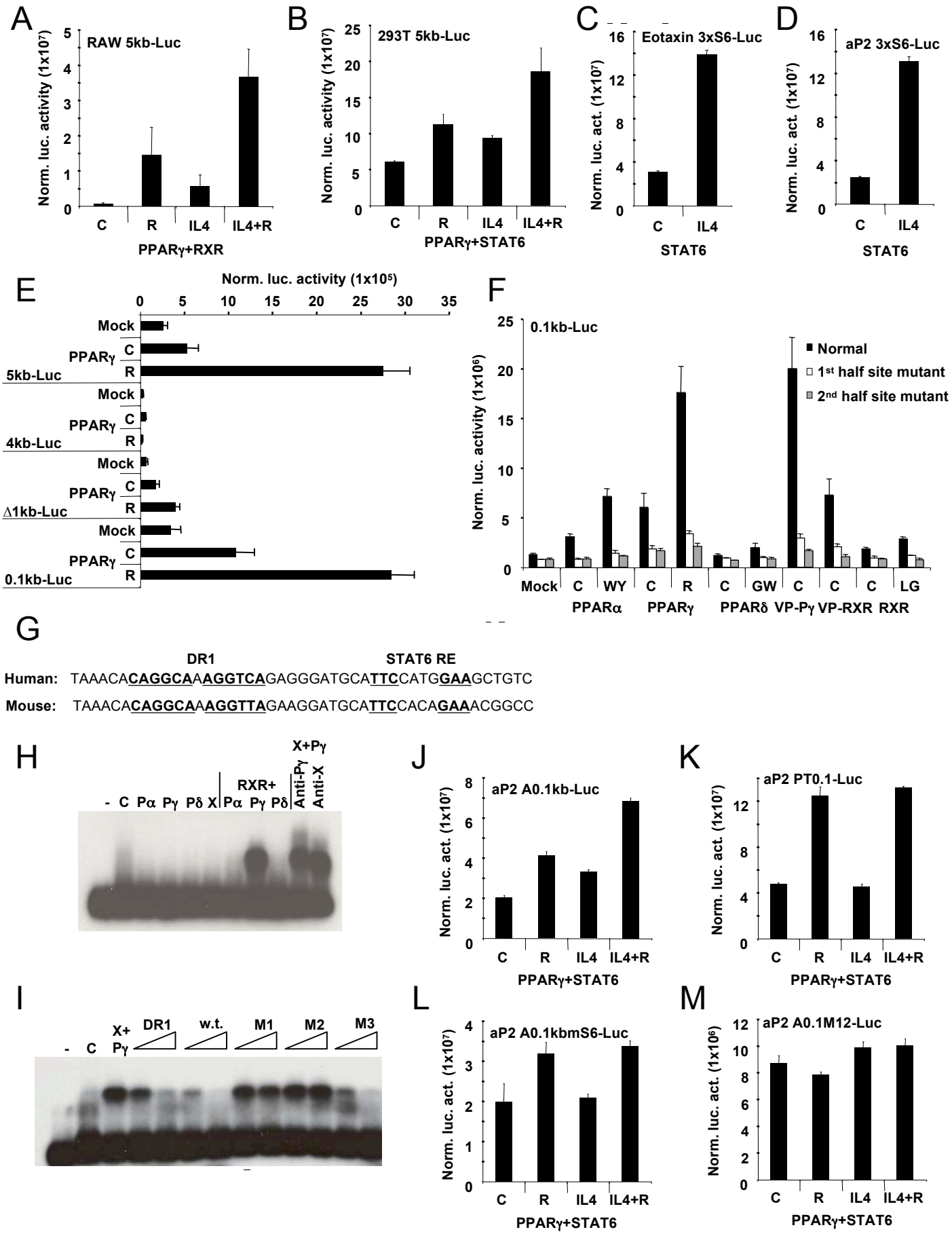
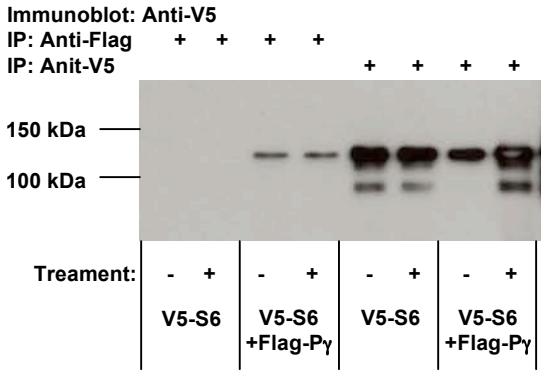

A
RAW 5kb-Luc
Norm. luc. activity (1x10⁷)
C
R
IL4
IL4+R
PPARγ+RXR
B
293T 5kb-Luc
Norm. luc. activity (1x10⁷)
PPARγ+STAT6
C
Eotaxin 3xS6-Luc
Norm. luc. act. (1x10⁷)
STAT6
D
aP2 3xS6-Luc
E
Norm. luc. activity (1x10⁵)
Mock
PPARγ
5kb-Luc
4kb-Luc
Δ1kb-Luc
0.1kb-Luc
F
0.1kb-Luc
Normal
1st half site mutant
2nd half site mutant
Mock
WY
GW
PPARα
PPARγ
PPARδ
VP-Pγ
VP-RXR
RXR
G
DR1
STAT6 RE
Human: TAAACACAGGCAAAGGTCAGAGGGATGCATTCCATGGAAGCTGTC
Mouse: TAAACACAGGCAAAGGTTAGAAGGATGCATTCCACAGAAACGGCC
H
X+Pγ
RXR+
Anti-Pγ
Anti-X
- C Pα Pγ Pδ X Pα Pγ Pδ
I
X+ Pγ
DR1
w.t.
M1
M2
M3
J
aP2 A0.1kb-Luc
K
aP2 PT0.1-Luc
L
aP2 A0.1kbmS6-Luc
M
aP2 A0.1M12-Luc

Figure 6

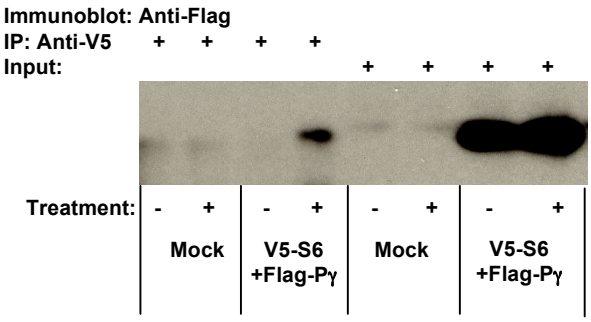
A B C D

ChIP

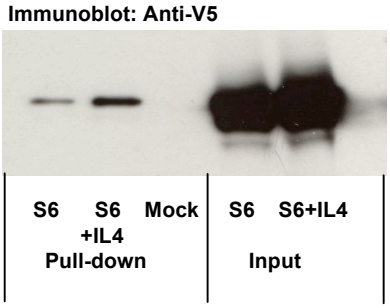
E



F



G

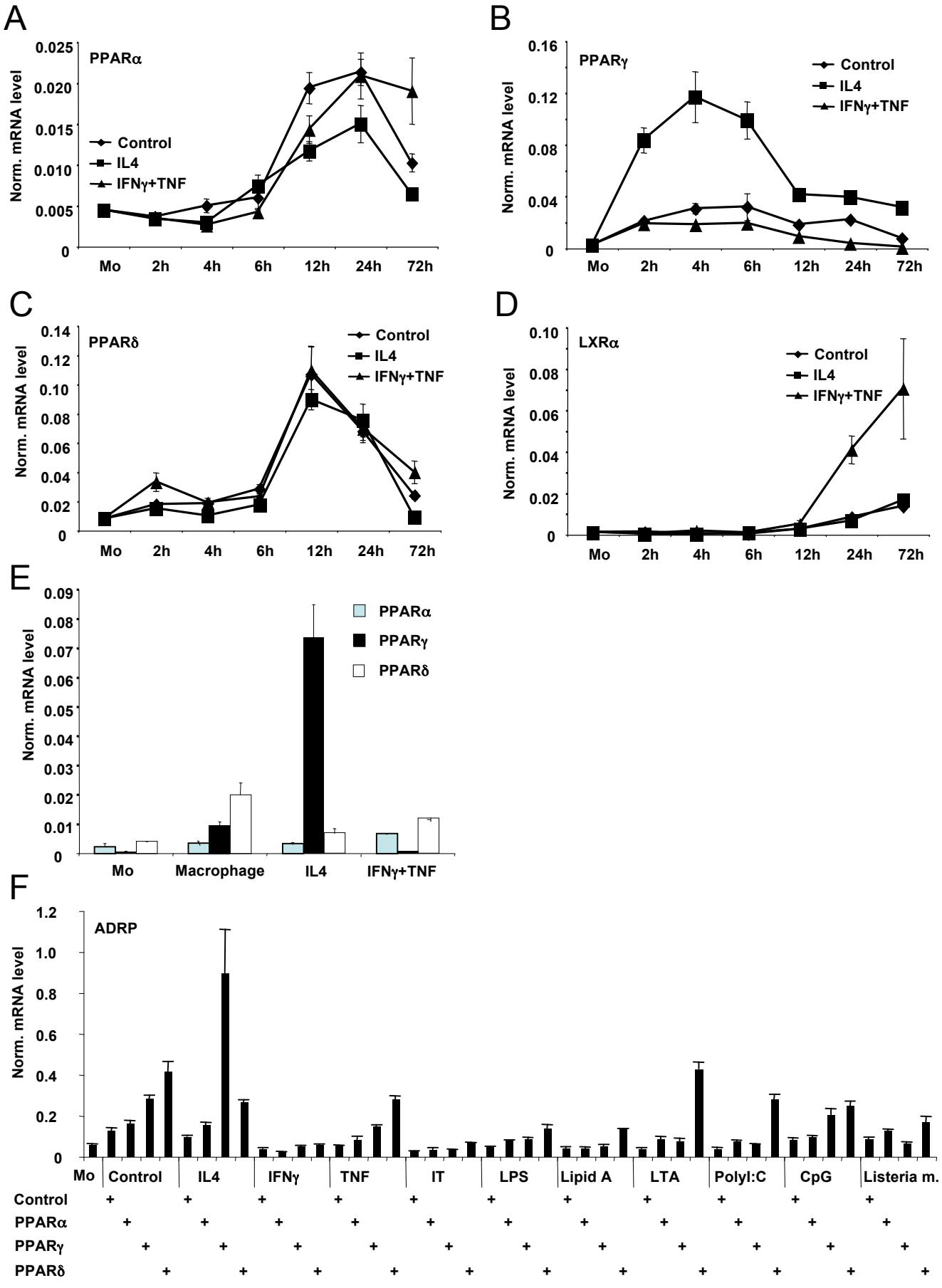


H Response element distribution

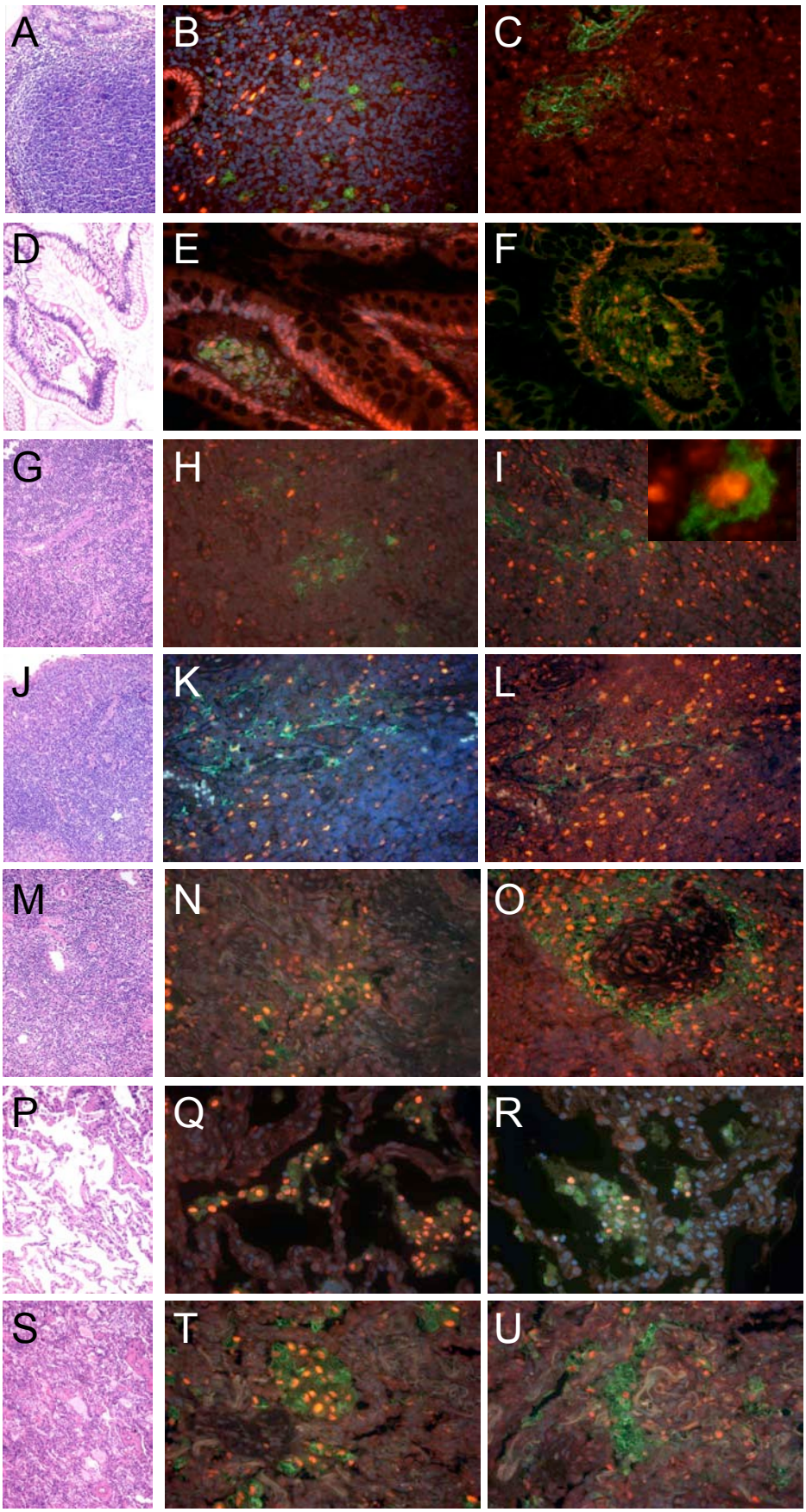
I

Adipocyte-MQ model

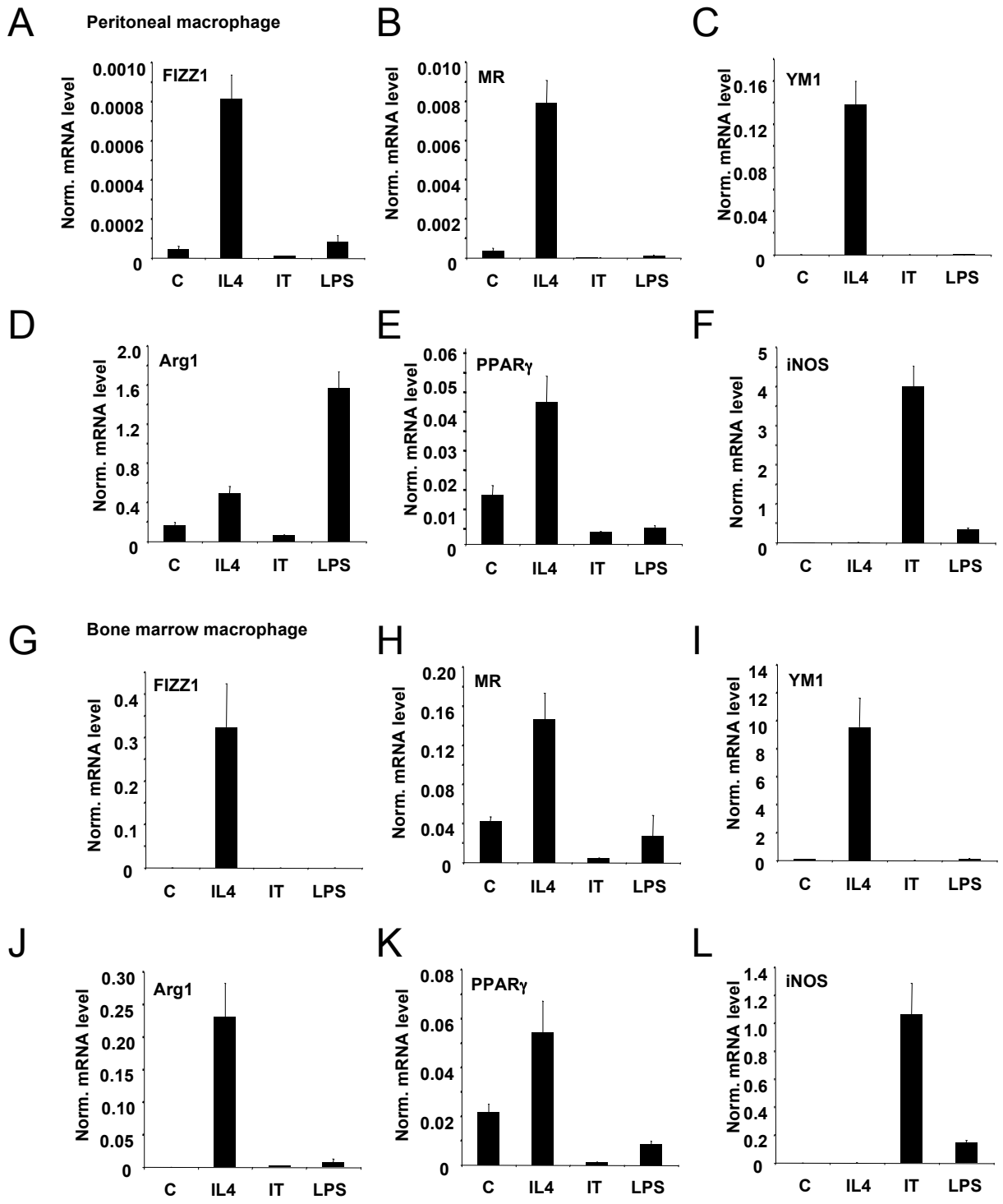
Figure 7



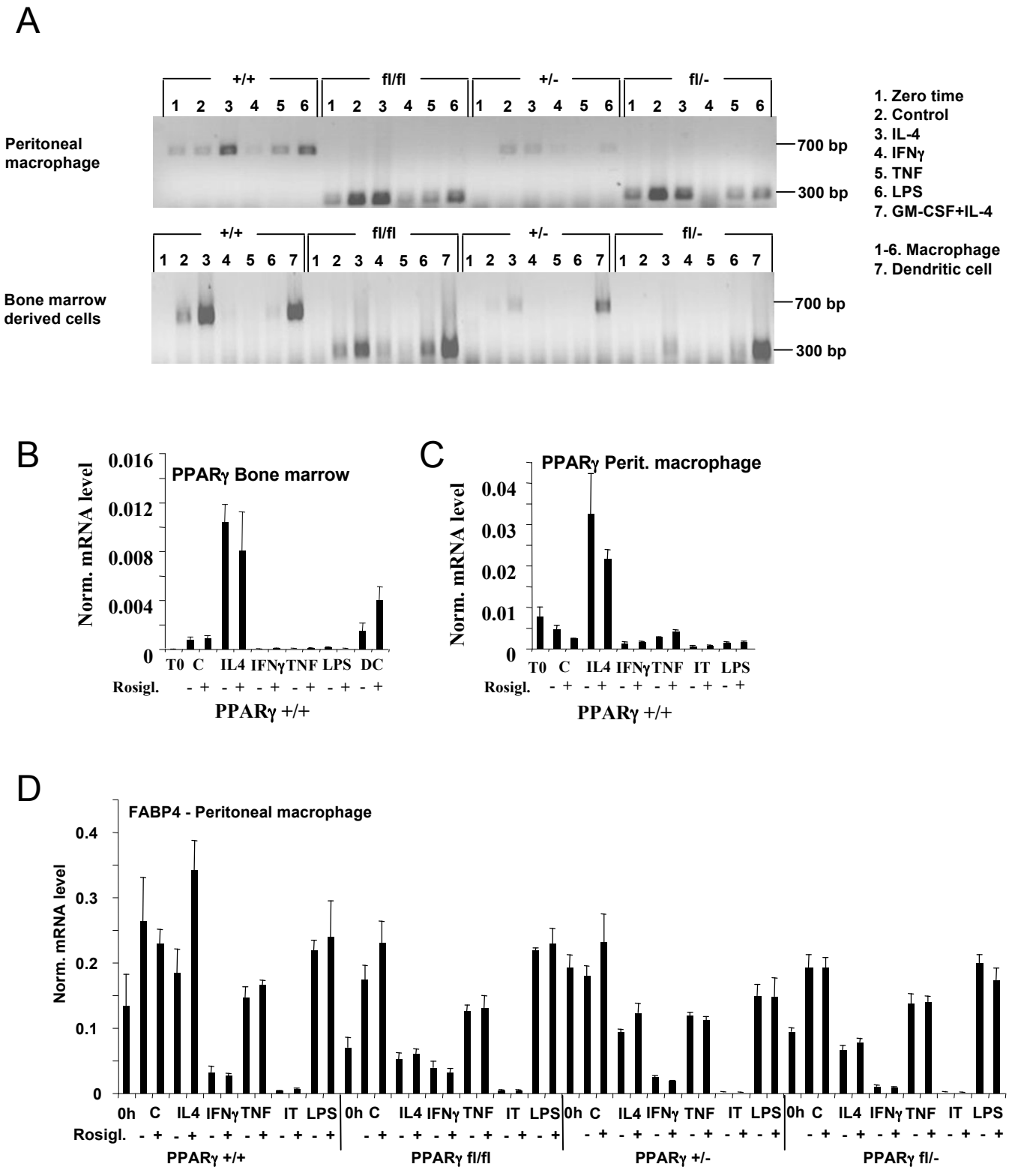
Supplementary Figure 1



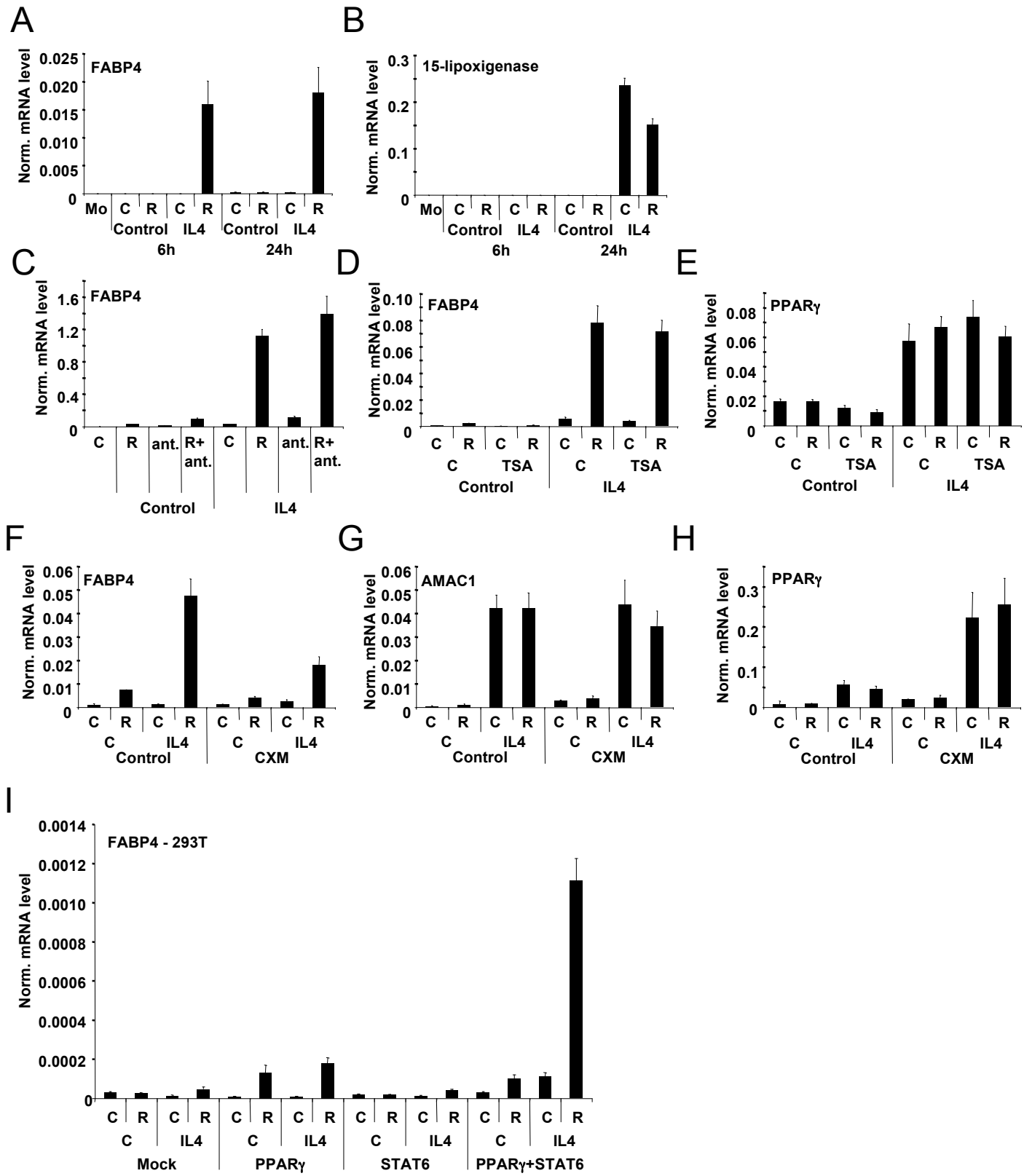
Supplementary Figure 2



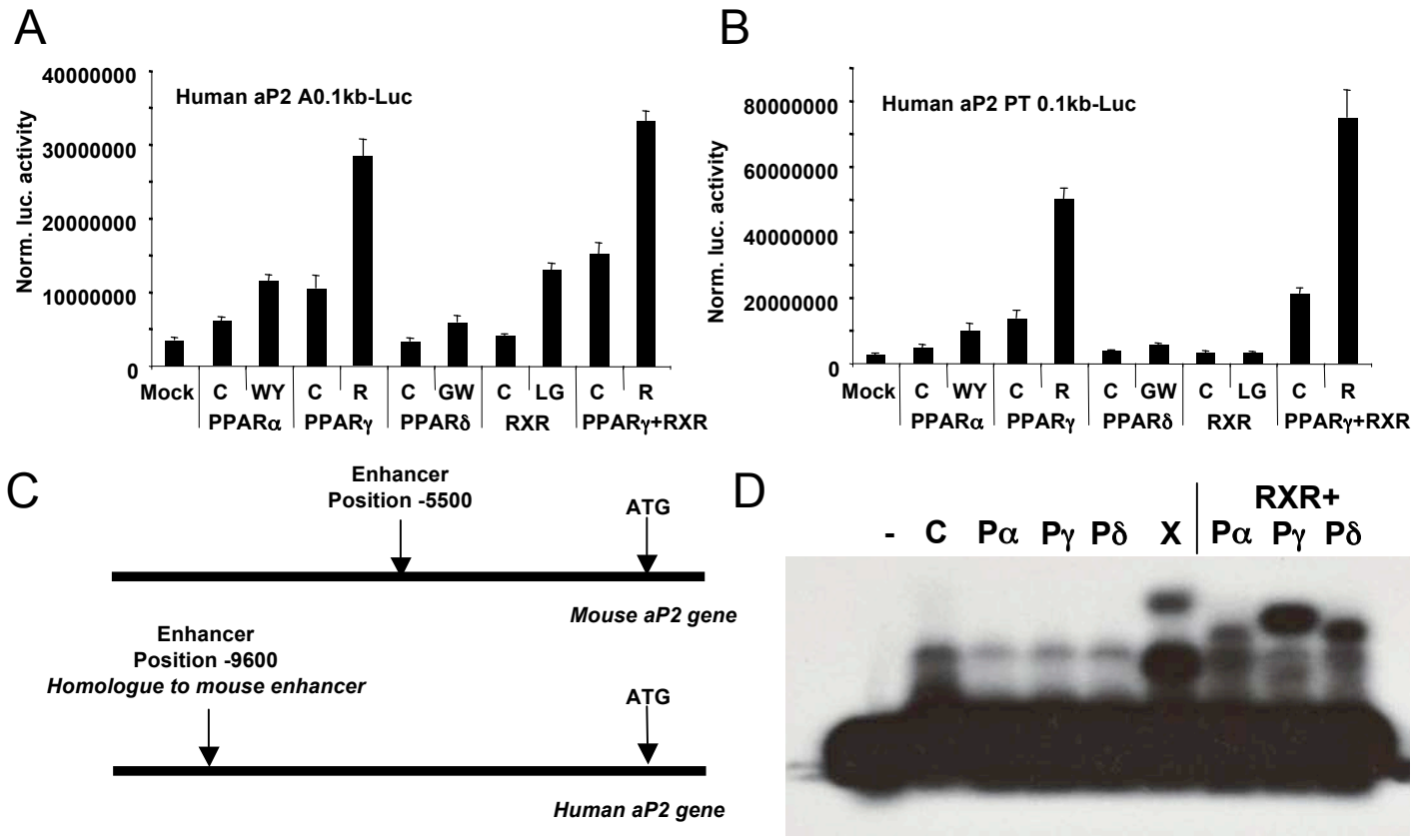
Supplementary Figure 3

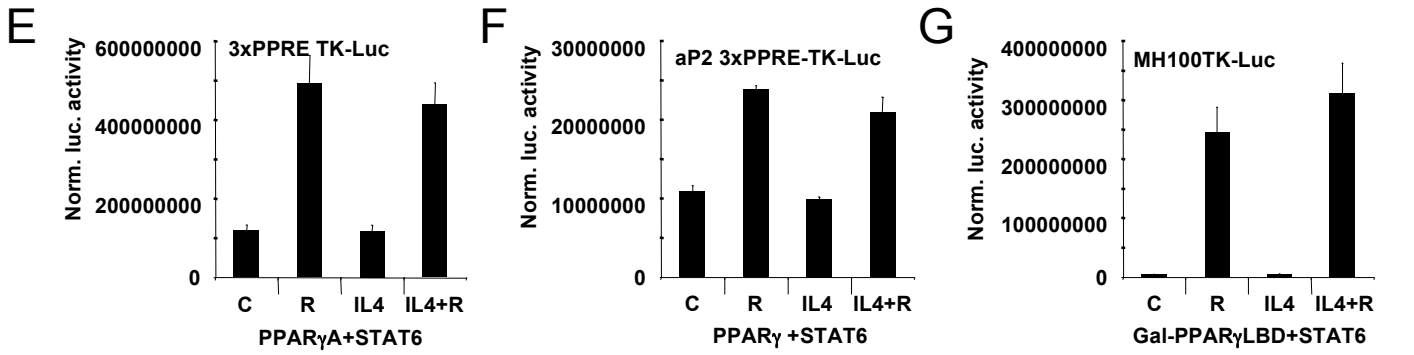


Supplementary Figure 4



Supplementary Figure 5





Supplementary Figure 6