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# Reorganization of the macrophage epigenome during sustained stimulation

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# 1 Abbreviations

| | |
|---|---|
| -me1 | monomethylated lysine residue |
| -me2 | dimethylated lysine residue |
| -me3 | trimethylated lysine residue |
| AGM | Aorta Gonads Mesonephros |
| AP-1 | Activating Protein-1 |
| ARG1 | Arginase 1 |
| ATP | Adenosine-5'-triphosphate |
| BCL6 | B-cell CLL/lymphoma 6 |
| BM | Bone Marrow |
| BMDM | Bone Marrow Derived Macrophages |
| bp | base pair |
| Brd4 | Bromo domain 4 |
| Brg1 | BRM/SWI2-Related Gene 1 |
| Brm | Protein Brahma Homolog |
| BSA | Bovine Serum Albumin |
| $CaCl_2$ | Calcium Chloride |
| CCR2 | CC-chemokine receptor 2 |
| cDNA | complementary DNA |
| CG | Cytosine Guanine |
| Chil3 | Chitinase-like 3 |
| ChIP | Chromatin Immunoprecipitation |
| ChIP-seq | Chromatin Immunoprecipitation-sequencing |
| CLRs | C-type Lectin Receptors |
| $CO_2$ | Carbonic acid |
| CpG | CG dinucleotide |
| CX3CR1 | CX3C-Chemokine Receptor 1 |
| Cxcl | Chemokine (C-X-C motif) ligand |
| DNA | Deoxyribonucleic Acid |
| dNTPs | Deoxynucleotides |
| DTT | Dithiothreitol |
| ECM | Extra Cellular Matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGR | Early Growth Response protein |
| EGTA | Ethylene glycol tetraacetic acid |
| ERK | Extracellular-signal-Regulated Kinases |
| eRNA | Enhancer /RNA |
| FBS | Fetal Bovine Serum |
| FDR | False Discovery Rate |
| FPKM | Fragments Per Kilobase Of Exon Per Million Fragments Mapped |
| GAPDH | Gliceraldeide-3-fosfato deidrogenasi |
| H2A | Histone 2A |
| H2B | Histone 2B |
| H3 | Histone 3 |
| H3-H4Ac | Histone 3 /4 Acetylation |

| | |
|---|---|
| H3K20me3 | Lysine 20 on Histone 3-me3 |
| H3K27Ac | Lysine 27 on Histone 3 Acetylation |
| H3K27me3 | Lysine 27 on Histone 3-me3 |
| H3K4 | Lysine 4 on Histone 3 |
| H3K9me3 | Lysine 9 on Histone 3-me3 |
| H4 | Histone 4 |
| HAT | Histone Acetyltransferase |
| HBSS | Henk's Balanced Salts Solution |
| HDAC3 | Histone DeAcetylase 3 |
| HDACs | Histone Deacetylases |
| HEPES | (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| Hepes-KOH | HEPES with potassium hydroxide |
| HP1 | Heterochromatin protein 1 |
| HSC | Hematopoietic Stem Cells |
| IFN | Interferon |
| IgG | Immunoglobulin G |
| IgG | Immunoglobulin G |
| IKK | kinase of IκB |
| IL- | Interleukin- |
| IL-1β | InterLeukin- 1 beta |
| IL-4 | InterLeukin- 4 |
| Il12b | Interleukin 12 beta |
| IRAK-M | IL1 Receptor-Associated Kinase M |
| IRAK3 | IL1 Receptor-Associated Kinase 3 |
| IRFs | Interferon Regulatory Factor |
| IκBα | Inhibitor of the nuclear factor of kappa light polypeptide gene enhancer in B-cells, alpha |
| JMJD2d | Jumonji Domain Containing 2d |
| JMJD3 | Jumonji Domain Containing 3 |
| JNK | c-Jun N-terminal kinase |
| KD | Knock Down |
| LB1 | Lysis Buffer 1 |
| LB2 | Lysis Buffer 2 |
| LB3 | Lysis Buffer 3 |
| LiCl | Lithium Chloride |
| LNs | Lymph Nodes |
| LPS | LipoPolySaccharide |
| LY6C | Lymphocyte antigen 6C |
| M-CSF | Macrophage Colony-Stimulating Factor |
| MDSCs | Myeloid-Derived Suppressor Cells |
| $MgCl_2$ | Magnesium Chloride |
| MHC II | Major Histocompatibility Complex II |
| miRNAs | micro-RNAs |
| MLL | myeloid/lymphoid or mixed-lineage leukemia |
| MMPs | Matrix Metalloproteinases |
| MPS | Mononuclear Phagocytic System |

| | |
|---|---|
| mRNA | messanger Ribo Nucleic Acid |
| NaCl | Sodium Chloride |
| NK | Natural Killer |
| NLRs | NOD (Nucleotide binding Oligomerization Domain)-like receptors |
| NO | Nitric Oxide |
| Nos2 | Nitric oxide synthase 2 |
| NP-40 | nonyl phenoxypolyethoxylethanol |
| o.n. | over night |
| P-TEFb | Positive Transcriptional Elongation Factor b |
| p300 | protein 300 |
| p38 | protein 38 |
| p65 | protein 65 |
| PAMPs | Pathogen Associated Molecular Patterns |
| PBS | Phosphate buffered saline |
| PCR | Polymerase Chain Reaction |
| PDGF | Platelet-Derived Growth Factor |
| PHF2 | PHD (plant homeodomain) finger 2 |
| PMSF | phenylmethanesulfonylfluoride or phenylmethylsulfonyl fluoride |
| PolII | Polymerase II |
| polyA | Poly-Adenylated |
| PRGs | Primary Response Genes |
| PRRs | Pattern Recognition Receptors |
| Ptgs1 | Prostaglandin G/H synthase |
| PU.1 | Purine-rich nucleic acid binding protein 1 |
| RefSeq | Reference Sequence |
| Rel/NF-kB | Nuclear Factor of kappa light polypeptide gene enhancer in B-cells |
| RETNLα | Resistin-like molecule alpha |
| RIPA | Radioimmunoprecipitation assay buffer |
| RLRs | Retinoic acid-inducible gene 1 (RIG1)-Like helicase Receptors |
| RNA | Ribonucleic Acid |
| RNA-seq | RNA-sequencing |
| Rpm | Revolutions per minute |
| RT | Room Temperature |
| RT-qPCR | Real Time- quantitative Polymerase Chain Reaction |
| SDS | Sodium Dodecyl Sulphate |
| SDS-PAGE | SDS- PolyAcrylamide Gel Electrophoresis |
| SHIP1 | SC homology 2 (SH2) domain-containing inositol-5 phosphatase 1 |
| shRNA | short hairpin RNA |
| SOCS1 | Suppressor Of Cytokine Signaling |
| SOD2 | Superoxide Dismutase2 |
| SP1 | Specific Protein 1 |
| SRGs | Secondary Response Genes |
| STATs | Signal Transducer and Activator of Transcription |
| SWI/SNF | SWItch/Sucrose NonFermentable |
| TAK1 | **TGF-β** Activated Kinase 1 |

| | |
|---|---|
| TAM | Tumor Associated Macrophages |
| TBP | TATA Binding Protein |
| TBST | Tris Buffered Saline with Twee |
| TE | Tris-EDTA |
| TFs | Transcription Factors |
| TGFβ1 | Transforming Growth Factor-β1 |
| TH1/2 | T helper type 1/2 |
| TIMPs | Tissue inhibitors of Metalloproteinases |
| TIR | Toll/IL-1R homologous region |
| TLR4 | Toll-Like Receptor 4 |
| TLRs | Toll-Like-Receptors |
| TNF | Tumor Necrosis Factor |
| TNFα | Tumor Necrosis Factor alpha |
| TRAF6 | TNF Receptor-Associated Factor 6 |
| Treg | regulatory T cells |
| Tris-HCl | Tris(hydroxymethyl)aminomethane chloride |
| TSA | Trichostatin |
| TSS | Transcription Start Site |
| VSV-G | Vesicular Stomatitis Virus- Glycoprotein |
| w/v | weight/volume |
| WCE | Whole-Cell Extract |

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# 6 Abstract

The chromatin of cells whose main function is to sense and react to environmental inputs, such as macrophages and other innate immune cells, undergoes rapid modifications in response to microenvironmental signals and provides general paradigms of how epigenomes are dynamically reorganized in a changing environment. A short exposure of macrophages to endotoxin (lipopolysaccharide, LPS) strongly activates transcription of hundreds of inflammatory genes. Conversely, a sustained stimulation results in a state of hypo-responsiveness to a subsequent microbial stimulation, which is commonly referred to as endotoxin tolerance. We used nascent RNA-seq and ChIP-seq to characterize genes and *cis*-regulatory regions that are differentially activated in unperturbed, LPS-stimulated and LPS-tolerized primary mouse bone marrow-derived macrophages (BMDM). We characterized promoter and enhancer states by mapping the methylation and acetylation states of associated histones and we identified differentially expressed genes by nascent RNA profiling. We clustered genes into different subsets based on their activity profiles and assigned to them enhancers with correlated dynamic behaviors. A detailed analysis of these datasets allowed us to dissect the mechanisms underlying functional switches in the macrophage gene expression program during sustained inflammation. Consistently with the RNA-seq data, genomic regions associated with transient genes were mainly characterized by transient acetylation of histone H3 lysine 27 (H3K27Ac), a mark of active transcription, while sustained genes were mainly associated with persistent acetylation. Conversely, the promoters of both transient and sustained genes maintained high levels of trimethylation of histone H3 at lysine 4 (H3K4me3) regardless of their transcriptional activity. Moreover, promoters of transient and sustained genes showed a distinct content of transcription factor binding sites. Sustained gene clusters displayed a significant enrichment in binding sites for Interferon Regulatory Factor (IRF) family transcription factors. The overlap between sustained genes and Interferon (IFN)-dependent genes,

confirm the dependence of this cluster on the IFN-β regulated feed-forward loop. In particular, IRF7 played a key role in the transcriptional regulation of sustained genes as indicated by the impact of its depletion. Transient gene clusters were mainly regulated by the Early Growth Response (EGR) and Nuclear factor-κB (NF-kB) family transcription factors, which are both downstream effectors of the TLR4 signaling pathway. Biochemical analysis of the key players of this signaling cascade revealed an almost complete exhaustion of the pathway after sustained LPS treatment, which correlated with reduced TRAF6 protein levels. These data suggest that the failure to reactivate those transcription factors that are involved in the transcriptional expression of transient genes is likely due to the hypo-responsive state of the TLR4 signaling pathway in cells exposed to LPS in a sustained manner.

# 7 Introduction

## 7.1 Inflammation

Inflammation is a complex and fundamental pathophysiological response to tissue malfunction or loss of tissue homeostasis, it is employed by both innate and adaptive immune systems to combat pathogenic intruders and involve relevant physiological roles, such as host defense, tissue remodeling and repair (Hotamisligil, 2006; Medzhitov, 2008). As it has been described in the first century by Celsus, the effects of these tightly regulated interactions culminate in the cardinal signs of local inflammation: *rubor* (redness), *calor* (heat), *tumor* (swelling), *dolor* (pain) and *functio laesa* (loss of function). Inflammation is a complex process and multiple control mechanisms must operate at different levels. The inflammatory response has to be regulated based on both the triggering stimulus and its dose, and it has to be deployed in a kinetically complex manner that suits the different phases of the inflammatory process. This complexity is achieved through multiple mechanisms that operate at different levels, including recognition of inflammatory stimuli by host receptors, regulation of signaling pathways activated by these receptors and chromatin-mediated regulatory changes that impact on gene expression (Medzhitov and Horng, 2009b; O'Byrne and Dalgleish, 2001). During the early phases of an inflammatory response, activated tissue-resident macrophages release specific chemokines (such as CXCL8/IL8), leading to the recruitment of neutrophils to the damaged tissue (Tester et al., 2007) (Tian et al., 2007). Following the extravasation and tissue infiltration, neutrophils release preformed granule proteins which, in turn, promote the recruitment and activation of inflammatory monocytes (Ley et al., 2007). This type of inflammation is normally a localized, protective response that follows trauma or infection. If the agent causing the inflammatory process persists for a prolonged period of time, the inflammation may become chronic. Chronic inflammation can result from a viral or microbial infection, environmental antigens (e.g., pollen), autoimmune reactions, persistent or repeated micro-

trauma and chemical irritants. Overall, *acute inflammation* is characterized by a rapid onset and a short duration. It is involved in the eradication of a bacterial, parasitic or viral infection while promoting wound healing. *Chronic inflammation* is characterized by prolonged duration with the formation of necrotic and fibrotic tissues in response to the sustained activity of lymphocytes and macrophages. Chronic inflammation also contributes to degenerative diseases such as atherosclerosis (O'Byrne and Dalgleish, 2001).

### 7.1.1 Tissue-resident and inflammatory macrophages, key players of the acute phase response

Macrophages are pivotal players of the inflammatory response but at the same time they may be involved in driving inflammatory diseases (Qiao et al., 2013). Macrophages are part of the mononuclear phagocytic system (MPS) and are generated by myeloid-committed hematopoietic stem cells located in the bone marrow (Doulatov et al.; Geissmann et al.). Haematopoietic progenitors generated in the yolk sac give rise to primitive macrophages, which populate the whole embryo to originate fetal primitive macrophages. In parallel, haematopoietic stem cells (HSCs), generated in the Aorta-Gonads-Mesonephros (AGM), give rise to progenitors that colonize the fetal liver and generate monocytes. Fetal liver-derived monocytes invade embryonic tissues, proliferate and differentiate into macrophages. Depending on the anatomical location, fetal liver-derived monocytes replace almost completely the population of yolk sac-derived macrophages. An exception is represented by the microglia, which arise mainly from yolk sac-derived macrophages (Ginhoux and Jung, 2014). Adult mouse circulating monocytes are a non-homogeneous population of cells that includes two main subpopulations identifiable by their cell surface markers: the first one is defined by high expression of CX3C-chemokine receptor 1 (CX3CR1), low expression of the myeloid marker lymphocyte antigen 6C (LY6C) and no expression of the chemokine receptor CC-chemokine receptor 2 (CCR2). These $Cxcr1^{+}Ly6C^{lo}Ccr2^{-}$ monocytes display patrolling

activity along the vascular endothelium. Instead, inflammatory monocytes are characterized by low level of CX3CR1, high level of LY6C and expression of CCR2, through which they sense the CCL2 chemokine that attracts them to inflamed tissues (Geissmann et al., 2003) (see Figure 5.1). The heterogeneity of macrophages reflects the functional specialization of these cells at different anatomical locations, as well as the capacity to acquire distinct phenotypes and physiological activities depending on the environment (Gordon and Taylor, 2005).

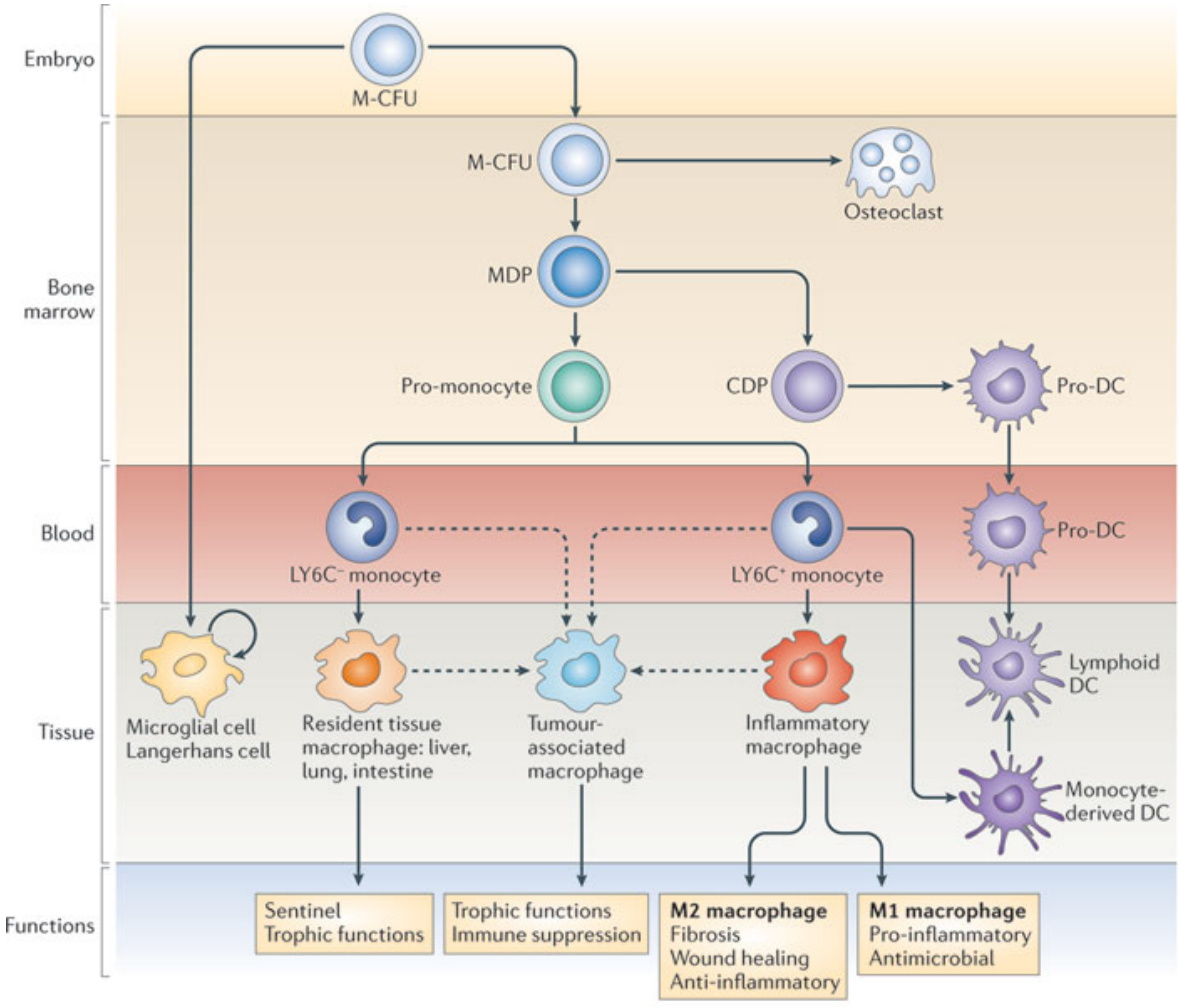


**Figure 7.1 Tissue Resident and Inflammatory Macrophages.** Monocyte's progenitors exit the bone marrow niche and differentiate in two different subtypes, according to their expression level of the surface marker LY6C. Tissue resident macrophages derive from LY6C- monocytes, while inflammatory macrophages are generated from LY6C+ monocytes. Depending on the local environment both tissue resident and inflammatory macrophages can be polarized toward a specific phenotype (Lawrence and Natoli, 2011).

Tissue-resident macrophages are present in most organs of the body where they can assume different phenotypic properties and transcriptional programs depending on the local milieu (Gosselin et al., 2014; Lavin et al., 2014) and contribute to tissue homeostasis. They either produce trophic factors and clear tissue debris, and also participate in tissue remodeling. They also attract other immune cells from the peripheral blood in case of infection and tissue damage (Murray and Wynn) (Gordon and Taylor, 2005; Krutzik et al., 2005; Murray and Wynn). Macrophages are sub-grouped according to their anatomical location and functional phenotype. Specialized tissue resident macrophages include for example alveolar macrophages, which reside in the lungs and have an important role in maintaining airway immune homeostasis (Hussell and Bell, 2014). The brain tissue resident macrophages (microglia) prune synapses during development (Lavin et al., 2014). Kupffer cells in the liver are the primary cells that encounter gut-derived toxins, such as LPS, and orchestrate immune responses within the liver (Nakamoto and Kanai, 2014). Moreover, macrophages of the spleen marginal zones silence innate and adaptive immunity to apoptotic cells (McGaha et al.) and sub-capsular sinus macrophages of the lymph nodes (LNs) eradicate viral agents drained in the lymph and begin the antiviral humoral immune response (Iannacone et al.; Junt et al., 2007) (see Figure 7.1).

### 7.1.2 The TLR4 pathway and the activation of the inflammatory response

Macrophages play a role in the acute phase of the inflammatory response by recognizing pathogen-associated molecular patterns (PAMPs), foreign bodies (such as asbestos and silica among others) and dead cells, through plasma membrane and cytoplasmic receptors. Macrophages express a broad set of Pattern Recognition Receptors (PRRs) (Takeuchi and Akira), including both intracellular and transmembrane Toll-Like-Receptors (TLRs) (Horng et al.), C-type lectin receptors (CLRs), scavenger receptors, retinoic acid-inducible gene 1 (RIG1)-like helicase receptors (RLRs) and NOD-like receptors (NLRs) (Geissmann

et al.; Gordon and Taylor, 2005; Kawai and Akira, 2009; Takeuchi and Akira). TLRs mediate innate immune defense by detecting the presence of microbial pathogens and activating the downstream intracellular signaling pathways that lead to phagocytosis, cellular activation and release of cytokines, chemokines and growth factors (Elinav et al.; Kawai and Akira, 2009; Osorio and Reis e Sousa). Once TLR4 is activated, its intracellular TIR (Toll/Interleukin1 Receptor)-domain creates a docking platform for an array of intracellular mediators, whose activity leads to the recruitment of TNF-Receptor Associated Factor 6 (TRAF6) and TGF-β Activated Kinase 1 (TAK1) (Horng et al., 2001; Lin et al., 2010) (Medzhitov et al., 1998; O'Neill, 2008; O'Neill and Greene, 1998) (Kawagoe et al., 2008) and eventually the activation of the Extracellular-signal-Regulated Kinase (ERK) (Herbert et al.), c-Jun N-terminal Kinase (JNK), and p38 pathways and transcription factors such as NF-κB and the Activating Protein-1 (AP-1) (Landstrom, 2010) (Barton and Medzhitov, 2003) (See Figure 7.2).

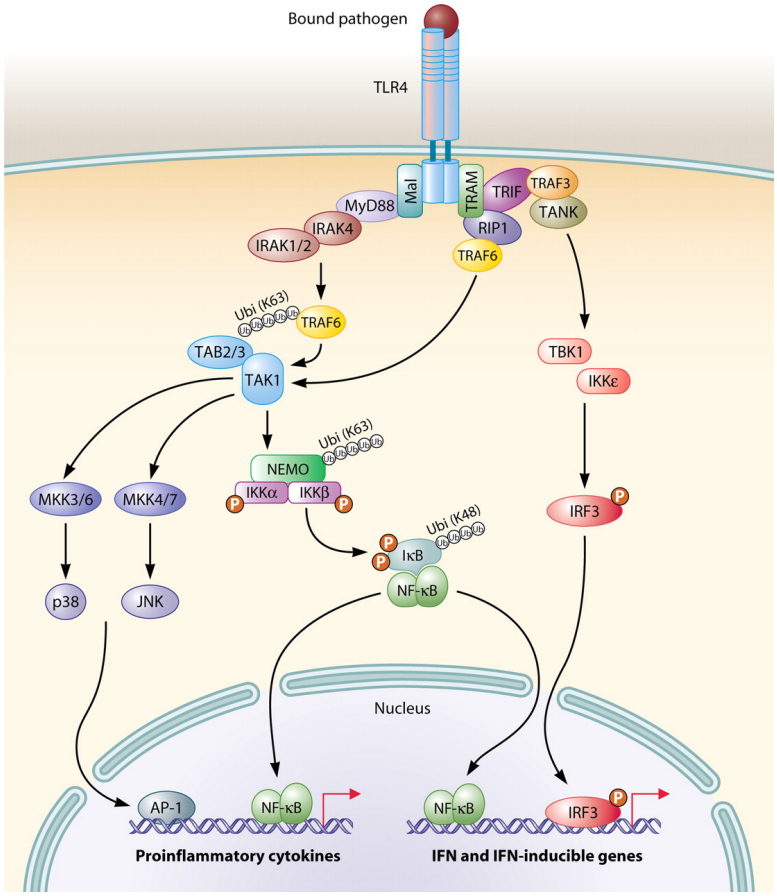


**Figure 7.2 The TLR4 pathway**. Pathogen recognition through the TLR4 receptor triggers both the MyD88-dependent and MyD88-independent pathway. The induced signaling cascade activates NF-kB, AP-1 and IRF3 transcription factors, resulting in the expression of both pro-inflammatory and IFN-dependent genes (Mogensen, 2009).

TLR4 engagement results in the activation of the IκB kinase (IKK), and the activated IKK complex phosphorylates IκBα on the serine32 and 36 (Ser32 and Ser36), leading to its poly-ubiquitination. The ubiquitinated IκBα is degraded via the 26S proteasome, thereby inducing nuclear translocation of p65:p50 as well as other less abundant Rel/NF-kB hetero- and homodimers (Karin and Ben-Neriah, 2000) and the resulting production of pro-inflammatory mediators, such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6). A precise and balanced production of these effectors is required to prevent pathologic inflammation (Green and Marshak-Rothstein, 2011). For this reason, normal inflammation is a self-limiting process eventually leading to restoration of homeostasis.

### 7.1.3 The resolution of the inflammatory response

Resolution of inflammation consists of an active process that involves several tightly regulated biochemical and cellular mechanisms. Many negative regulators are upregulated upon inflammatory stimulation. Two classes of transcriptional factors are involved in negative regulation and they exert basal and inducible repressor functions. p50, for example, belongs to the first category and it is constitutively expressed. p50 homodimers function mainly as a transcriptional repressors of NF-kB target genes because p50 lacks a transcriptional activation domain (Thanos and Maniatis, 1995). On the contrary, inducible repressors are normally not expressed or expressed only at low levels, but are transcriptionally induced after an inflammatory stimulus, indicating that they are part of a negative feedback mechanism that limits the inflammatory response. Usually inducible repressors block the expression of secondary response genes (SRG), whereas basal repressors inhibit the expression of primary response genes (PRG) associated with CpG islands, which otherwise would present high basal activity (Medzhitov and Horng, 2009b). A possible deregulation of the factors involved in this process may lead to excessive and/or sustained responses.

## 7.2 Macrophage polarization

Depending on their activation state, cytokine profile and functional properties, macrophages have been classified into M1 (classically-activated) and M2 (alternatively-activated) macrophages (Gordon, 2003; Mantovani et al., 2005; Mantovani et al., 2004). The M1/M2 definition is based on the cytokines that are associated with these macrophage phenotypes. The classical macrophage activating factor, produced by stimulated T helper 1 (TH1)- lymphocytes and natural killer (NK) cells, is interferon-γ (IFNγ) (Schroder et al., 2004), while the alternatively-activated macrophages are associated with TH2-type immune responses and they are activated by the cytokines interleukin-4 (IL-4) and IL-13

(Gordon, 2003). Classically- activated macrophages are strongly positive for the Major Histocompatibility Complex of class II (MHCII), they protect the host organism from infectious agents, such as bacteria, protozoa and viruses and they play a role in anti-tumor immune response. Tissue injury or infection related agents, such as the bacterial lipopolysaccharide (LPS), with the help of recruited natural killer secreting interferon-γ, polarize macrophages toward the M1 phenotype (Gordon and Martinez; Lawrence and Natoli; Mosser and Edwards, 2008; Sica and Mantovani). Cytokines such as interferon-β (IFN-β), tumor necrosis factor-alpha (TNF-α) and IL-1 also promote an M1 polarization of macrophages (Bosschaerts et al.; Mosser and Edwards, 2008; Serbina et al., 2003). Reactive oxygen species and nitrogen intermediates produced by activated macrophages are highly toxic for the invading agents but they are also potentially harmful for the tissues and organ where the infection takes place. M1 macrophages produce also IL-12 and IL-23, which are decisive in influencing the polarization of TH1 and TH17 cells, which further drive inflammatory responses (Serbina et al., 2008). For all the mentioned reasons pro-inflammatory and antimicrobial M1 macrophage responses must be finely regulated in order to avoid extensive collateral tissue damage to the host. Aberrant regulation of M1 macrophages has been associated to several chronic inflammatory and autoimmune diseases, such as Crohn's disease, multiple sclerosis, rheumatoid arthritis and autoimmune hepatitis (Kawane et al., 2006; Murphy et al., 2003; Smith et al., 2009). In contrast to pro-inflammatory M1 cells, M2-alternatively activated macrophages are involved in resolution of inflammation and tissue repair, facilitate wound repair and fibrosis and also exert anti-parasitic activities (Anthony et al., 2006; Biswas and Mantovani; Pesce et al., 2009a). M2 macrophages express factors promoting the growth of epithelial cells and fibroblasts, including Transforming Growth Factor-β1 (TGF-β1) and Platelet-Derived Growth Factor (PDGF) (Barron and Wynn). M2 macrophages are also specialized to engross and eradicate dead cells, debris and various extracellular matrix (ECM) components that would promote tissue-damage mediated by M1 macrophages. M2 macrophages are indeed able to

produce matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), which control ECM turnover (Barron and Wynn; Wynn, 2004, 2008). Moreover, M2 macrophages produce immunoregulatory proteins such as IL-10, resistin-like α (RETNLα), chitinase-like proteins (Chil3/Ym1), which suppress allergic inflammation, and arginase 1 (ARG1), in order to regulate pro-inflammatory functions of M1 macrophages and to start the wound healing process (Elias et al., 2005; Herbert et al.; Lee, 2009; Pesce et al., 2009b; Reese et al., 2007; Sutherland et al., 2009). Accordingly, while M1 macrophages promote a TH1, TH17 response, M2 macrophages are programmed to release chemokines recruiting fibroblasts, TH2 cells and regulatory T (TReg) cells (Curiel et al., 2004; Imai et al., 1999). M2 macrophages have also been proposed to suppress allergic inflammation by degrading or sequestering chitin, a potent and highly abundant allergen in the airway (Reese et al., 2007).

Other macrophage subpopulations/activation states have been reported in the past: 'Regulatory' macrophages, similar to suppressive M2 cells, are induced by TLR agonists in the presence of IgG (immunoglobulin G), apoptotic cells and prostaglandins and secrete large amounts of interleukin-10 (IL-10) and TGF-β1 (Jenkins et al.; Sutterwala et al., 1997; Sutterwala et al., 1998). Tumor Associated Macrophages (TAMs) have been described with either protective or pathogenic roles in cancer (Sica and Bronte, 2007). Tumor M1 macrophages can activate killing ability and amplify TH1 response, providing a mechanism of antitumor response (Biswas and Mantovani), while TAMs and MDSCs (Myeloid Derived Suppressor Cells) suppress adaptive tumor-specific immune responses, promote tumor progression inducing tumor growing, invasion, metastasis, stroma remodeling and angiogenesis (Brower; Daurkin et al.; Erreni et al.; Laoui et al.; Mantovani; Quatromoni and Eruslanov; Richards et al.; Tang; Wang et al.; Wu et al.). It is important to point out that the M1/M2 dicotomy represents only the two extremes of a much larger series of phenotypes and functional states that macrophages can assume. It has

already been well documented that macrophages can undergo dynamic transitions between these two different functional states, underlying the great plasticity of these cells (Mosser and Edwards, 2008) and the spectrum of macrophage activation phenotypes is indeed much broader (Murray et al., 2014) (see Figure 7.3).

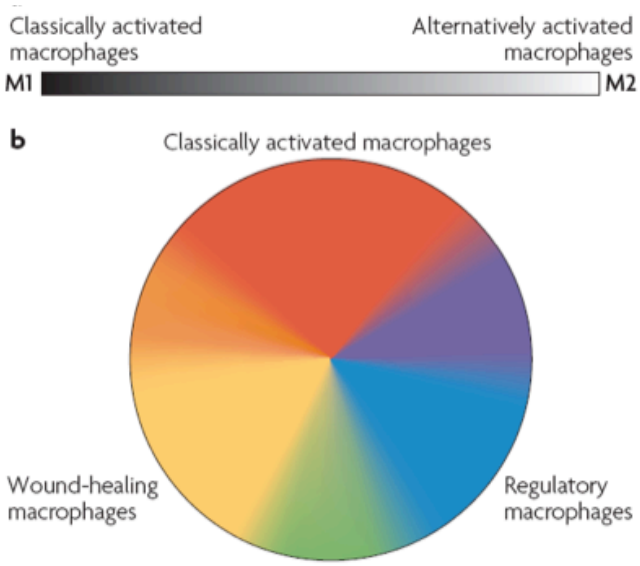


**Figure 7.3 Dynamic and complete view of macrophage phenotypes.** Each potential macrophage's phenotype is compared to a spectrum of colors on a color wheel. Macrophages can be "red" (classically activated macrophages), "yellow" (wound healing macrophages) and "blue" (regulatory macrophages). Macrophages can assume many other phenotypes, such as the combination of these three primary colors results in several others shades. Moreover, these colors can be interchangeable, meaning that wound-healing cells may potentially become inflammatory, or anti-tumor macrophages may become pro-tumor macrophages (Mosser and Edwards, 2008).

This kind of view may ensure that the number of "subsets" is absolutely higher than expected and underline the great plasticity of macrophages (Mosser and Edwards, 2008). Indeed, numerous studies have documented flexibility in their programming, with macrophages switching from one functional phenotype to another in response to the variable micro-environmental signals of the local milieu (Hagemann et al., 2008; Kawanishi et al.; Mylonas et al., 2009; Rutschman et al., 2001; Stout et al., 2005; Stout and Suttles, 2004) (Lavin et al., 2014) (Gosselin et al., 2014). Furthermore, a network analysis

performed on human macrophages demonstrated that that, depending on the stimulus they encountered, macrophages display a full spectrum of activation rather than a discrete M1 versus M2-polarization (Murray et al.) (Xue et al., 2014)

## 7.3 Transcriptional regulation in macrophages

Macrophage activation, regardless of the nature of the stimulus, leads to radical changes in gene expression programs (Ivashkiv; Lawrence and Natoli; Medzhitov and Horng, 2009b; Smale). The advent of genome wide technologies increased the possibility to unravel the fine macrophages' transcriptional program. Indeed, taking advantage of the recently advanced techniques and experimental approaches, mainly chromatin immunoprecipitation (ChIP) coupled with high-throughput sequencing (ChIP-seq) and RNA-seq as well as the computational tools for the deconvolution of genomic data sets, it is becoming clear that signaling cascades and transcription factors important for macrophage activation induce epigenetic changes in the chromatin states (Glass and Saijo; Medzhitov and Horng, 2009b; Smale). The following section will introduce general and basic concepts on the chromatin structure underlying the transition between active and inactive transcriptional states.

### 7.3.1 Epigenetic regulation of chromatin

Epigenetic mechanisms, broadly defined as regulatory mechanisms associated with chromatin modifications, include chemical modifications (methylation, acetylation, and phosphorylation) of histones, methylation and hydroxymethylation of CpG DNA dinucleotides and noncoding-RNA-mediated chromatin changes (Ernst et al.; Margueron and Reinberg; Mattick; Natoli; Probst et al., 2009; Zhou et al.).

The nucleosome is the basic building block of chromatin (Kornberg, 1974). Each nucleosome consist of 146-147 bp of DNA wrapped in 1.67 left-handed superhelical turns around a histone octamer, which is composed of two H2A-H2B dimers and a H3-H4

tetramer (Luger et al., 1997). Nucleosomes are connected with each other by the stretches of ”linker DNA” associated with the linker histone H1 that is not part of the core particle and controls chromatin compaction. It is now clear that nucleosomes are plastic and can acquire different properties (Zentner and Henikoff, 2013) through covalent post-translational modifications of their N-terminal domains (tails). Histone tails are modified at several positions and by several different chemical modifications. For example, acetylation of histone tails at specific amino acid residues relaxes the chromatin and act as an activating histone mark, regardless of its location on the tail. In contrast, methylation of histone tails can act as either an activating or repressing mark, depending on both the type and location of the modification (Figure 7.4).

These histone marks are ‘written’ and ‘erased’ by dedicated enzymes and impact on the activity of coactivators and co-repressors to determine the rates of transcription initiation and elongation. Common histone modifications of the N-terminal tail are lysine acetylation and methylation that are recognized by bromodomain and chromodomain or PHD family proteins, respectively. Acetylation has a general positive role in gene activation by neutralizing the positive charge of the lysine and thus inducing a chromatin decompaction. Instead, while the methylation of H3K4 is associated with gene activation, H3K9 methylation for example is recognized by HP1 (heterochromatin protein 1) that mediates chromatin compaction and repression, and H3K27 methylation is associated with repression by Polycomb proteins (Shilatifard, 2008) (Cockerill, 2011).

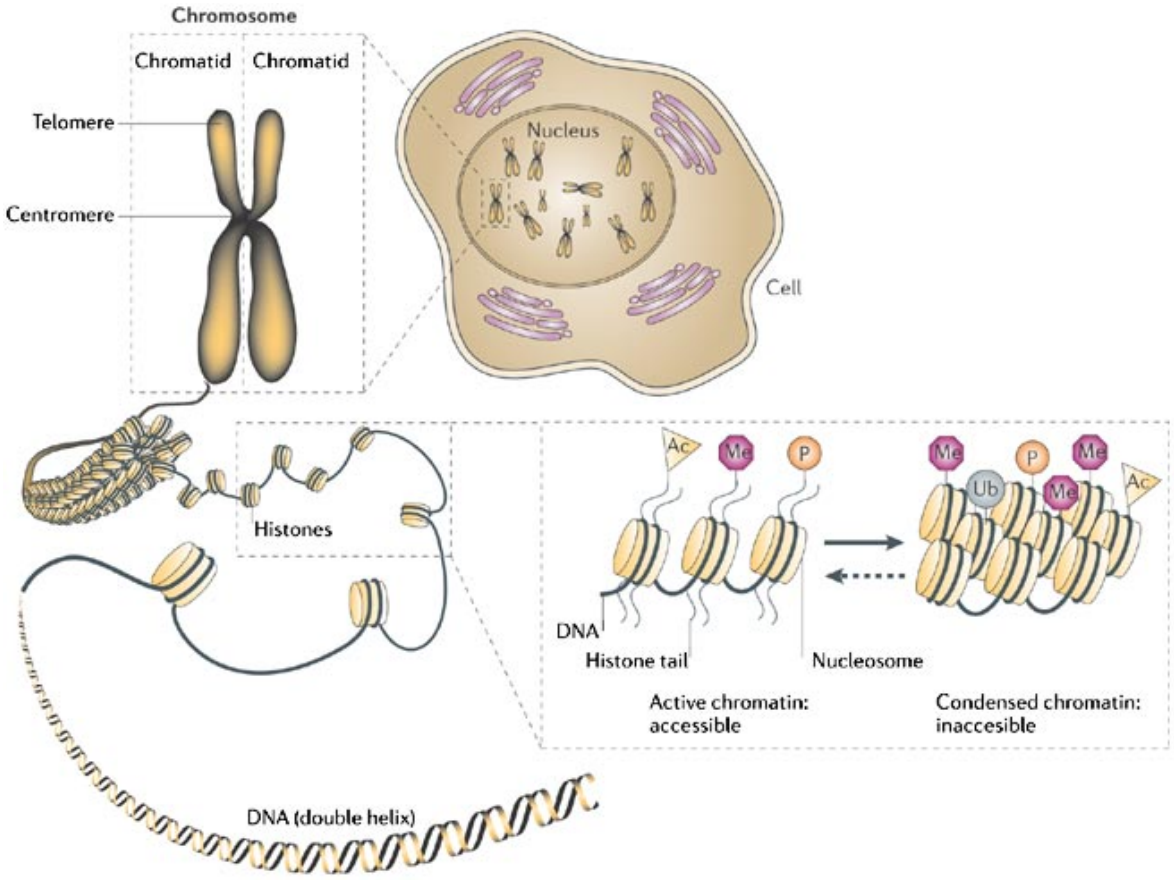


**Figure 7.4 Composition of nucleosomes**. Nucleosome is the building block of chromatin The N-terminal tails of histone are subjected to post-translational modifications that control nucleosome functions (Sparmann and van Lohuizen, 2006).

### 7.3.2 The *cis*-regulatory repertoire of macrophages

Macrophages treated *in vitro* with LPS activate hundreds of genes expressing mediators and actors of inflammation, each of them being regulated in a kinetically complex and specific manner. Primary response genes (PRGs) are immediately activated after the stimulus, while secondary response genes (SRGs) and some slowly activated primary response genes show delayed activation after the stimulus. Those genes that can be induced without de novo protein synthesis are formally defined as PRGs, while SRGs require new protein synthesis to be activated (Herschman, 1991). These two classes of genes have different activity states and modes of response due to specific sequence features of their promoters. Promoters of PRGs, tumor necrosis factor (*Tnf*) or superoxide

dismutase2 (*Sod2*) for example, contain regions highly enriched of CpG dinucleotides, named CpG islands (Deaton and Bird; Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). Unmethylated CpG dinucleotides are recognized by proteins containing the CXXC domain and by constitutively expressed transcription factors as for example SP1 (specific protein 1) (Ayton et al., 2004; Deaton and Bird; Lee and Skalnik, 2005). Furthermore, the SET1 and MLL complexes contain the CXXC domain, thus they recognize unmethylated CpG promoter regions and catalyze the deposition of H3K4me3, the best known activatory histone mark (Lee and Skalnik, 2008). Genome wide analysis have revealed that CpG island-containing promoters are characterized by relative depletion of nucleosomes, making them easily accessible to selectively induced transcription factors, and a precise nucleosome free region overlapping the RNA polymerase II peaks (Ramirez-Carrozzi et al., 2009; Valouev et al.). RNA polII constitutively associated with CpG islands synthesizes immature long unspliced transcripts in unperturbed conditions (Hargreaves et al., 2009). After stimulation with an inflammatory signal, RNA polII is phosphorylated on its serine 2 of the C-terminal domain repeats, allowing transcriptional splicing and therefore productive transcription (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). In this manner, promoters containing CpG islands can be rapidly activated in response to external stimuli by inducible factors that promote increasing on acetylation of histone H4. Acetylation is then recognized by the bromodomain-containing adaptor protein Brd4, that recruits the Positive Transcription Tlongation Factor (P-TEFb). Indeed, the cyclin dependent kinase P-TEFb promotes elongation and pre-mRNA processing through its ability to phosphorylate the C-terminal domain of RNA polII (Hargreaves et al., 2009). In summary, CpG island enriched promoters, being pre-bound by the RNA PolII and lacking of any nucleosomal barrier, undergo a fast binding of activated transcription factors (such as NF-κB, AP-1 and IRFs) and activation in response to exogenous stimuli (Lawrence and Natoli) (see Figure 7.5).

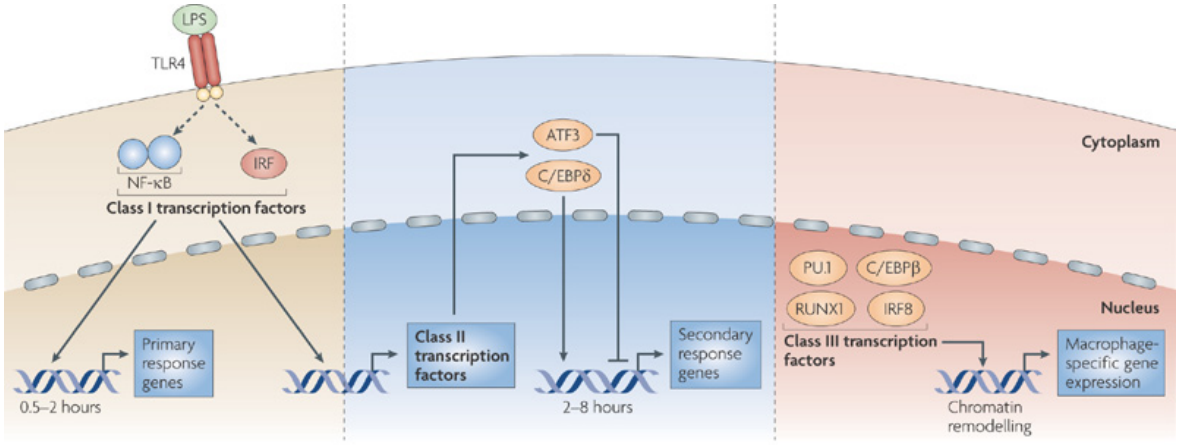


**Figure 7.5 PRGs and SRGs promoters, sequence and chromatin features.** Primary response genes are regulated mainly by nuclear factor-κB (NF-κB) and interferon-regulatory factor (IRF) proteins. Secondary response genes are mainly induced by transcription factors induced in the primary response, such as CCAAT/enhancer-binding protein-δ (C/EBPδ) (Medzhitov and Horng, 2009a).

Secondary response genes (SRGs), such as interleukin (*Il-6*), nitric oxide synthase (*Nos2*), interleukin-12b (*Il-12b*) and some of the primary response genes, usually the slowest responding to LPS, such as CC-chemokine ligand 5 (*Ccl5*), display well-positioned nucleosomes at their promoters. Promoters of SRGs do not contain CpG islands and require a stimulus-regulated chromatin-remodeling step for the recruitment of crucial transcription factors (such as NF-κB) and eventually RNA polII (Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009). Moreover, in a de novo protein synthesis dependent way, the multi-molecular complex SWI/SNF is required to remodel chromatin conformation and make genomic DNA more accessible to transcription factor binding sites (Clapier and Cairns, 2009; Weinmann et al., 1999). SWI/SNF uses the energy of ATP hydrolysis to catalyze changes in nucleosome conformation (Imbalzano et al., 1994; Kwon et al., 1994; Ramirez-Carrozzi et al., 2009; Ramirez-Carrozzi et al., 2006) and it is recruited in response to specific transcription factors (Ramirez-Carrozzi et al., 2006) able to recognize their binding sites also in a condensed chromatin and capable to interact with the SWI/SNF

complex (Ramirez-Carrozzi et al., 2009; Ramirez-Carrozzi et al., 2006). Retroviral delivery of short hairpin RNA against the core Brg1 and Brm ATPase subunits of the SWI/SNF complexes compromise only the induction of the secondary response genes and primary responsive ones with delayed kinetics (Ramirez-Carrozzi et al., 2006). Moreover, SWI/SNF-independent promoters also exhibited constitutively high histone acetylation and histone H3K4 trimethylation, as mentioned before, suggesting that these promoters have a chromatin conformation similar to the one found in promoters of active genes (Ramirez-Carrozzi et al., 2009; Ramirez-Carrozzi et al., 2006). Moreover, thanks to a temporal profiling of nascent transcripts in LPS stimulated macrophages, it has been recently confirmed that rapidly induced genes contain CpG-island promoters. However, 36–58% of SRG and PRG with delayed kinetics genes also contain CpG-island promoters, as well as high levels of H3K4me3, and RNA polymerase II association in unstimulated cells. Interestingly, non-CpG island genes are induced by a higher magnitude after stimulation respect to CpG island-containing genes, probably because of the control in the basal state operated by specific TFs (Bhatt et al., 2012). In LPS-stimulated cells, several promoters of the SWI/SNF dependent genes contain IRF3 consensus binding sites (Ramirez-Carrozzi et al., 2009). IRF3 is activated by Toll-like receptor 4 (TLR4) through TIR domain-containing adaptor protein inducing IFN-β (TRIF) (Ramirez-Carrozzi et al., 2009). In $IRF3^{-/-}$ mice, promoters of the same genes are insensitive to restriction enzyme accessibility, suggesting that IRF contribute to nucleosome remodeling at these genomic sites (Ramirez-Carrozzi et al., 2009). In conclusion, genes displaying different states and modes of response to stimulation are predetermined by specific sequence features of their promoters (see Figure 7.5).

Moreover, promoter states of inflammatory genes are regulated by additional mechanisms, including the post-translational modification of their nucleosomes. Refractory genes to acute induction by inflammatory stimuli are characterized by the presence of negative marks such as trimethylation of the histone 3 in lysine 9 and 27 (H3K9me3 and

H3K27me3), absence of positive marks, and inaccessibility of DNA at their promoters. Genes ready to be transcribed, but not in an active state are named poised and they are characterized by the presence of activating histone marks (H3K4me3, H3-H4Ac), a more relaxed chromatin conformation, and in some cases, a pre-bound RNA polymerase II (Pol II) is stalled near the transcription start site (TSS). Poised genes are kept transcriptionally silenced by co-presence of repressive histone marks, co-repressor complexes, and partially closed chromatin that requires additional positive histone marks and ATP-dependent nucleosome remodeling to provide full accessibility for transcription factors. In conclusion, actively transcribed genes are marked by active histone modifications, open chromatin configuration and ongoing transcription (Ivashkiv).

It has been recently demonstrated in LPS-treated macrophages that dynamic changes in chromatin modifications correlate with transcriptional regulation, thus determining accessibility and binding of LPS recruited transcription factors, such as NF-kB, STATs and IRFs (Ghisletti et al.; Natoli). The epigenetic landscape, established during macrophages differentiation by master transcription factors at these genetic loci, can be remodeled under pro-inflammatory and polarizing stimuli (as it will be explained in more detailed in the following section). Accordingly, the pre-established epigenetic landscape helps integrate signaling over time and underlies reprogramming of cells to alter their gene expression responses to subsequent stimuli (Ghisletti et al.; Heinz et al.; Ivashkiv; Jin et al.; Natoli; Natoli et al.; Pham et al.). Transcription of inflammatory cytokines is kept silenced until macrophages are not properly activated by pro-inflammatory stimuli. Gene loci are controlled by repressors such as B cell leukemia (BCL)6 and nuclear receptors that recruit co-repressor complexes containing histone deacetylases (HDACs) and histone demethylases, thus limiting the amount of positive histone marks (Barish et al.; Glass and Saijo). Moreover, as it has been mentioned previously, chromatin of silenced genes is in a closed conformation and it is marked by negative histone modifications (H3K9me3, H3K27me3 and sometime also by H3K20me3) (Ramirez-Carrozzi et al., 2009; Stender et

al.). Once macrophages are stimulated with a pro-inflammatory stimulus, negative histone marks are erased by demethylases such as JMJD3, JMJD2d, AOF1, and PHF2, repressors and co-repressors are released and, as in the case of secondary responsive genes, chromatin is remodelled by SWI/SNF complexes. All together, these events allow the binding of signaling transcription factors to their promoters, the increasing of positive histone marks (mainly H3K4me3 and acetylation of histone H4) and the releasing of elongating Pol II (Adelman et al., 2009; De Santa et al., 2009; Hargreaves et al., 2009; Ramirez-Carrozzi et al., 2009; Ramirez-Carrozzi et al., 2006; Stender et al.; van Essen et al.).

### 7.3.3 Enhancer regulation of inflammatory gene expression

Inducible genes are regulated by a promoter located immediately upstream to the Transcription Start Site (TSS) and by one or more enhancers. These genomic elements generate a kind of platform for specific DNA-binding proteins, which recognize specific DNA consensus sequences. In the past, many studies were focused on the analysis of promoters, since they can be easily identified, being close to the TSS of each gene. Thanks to the recent technological advances, distant enhancers have now been identified for a number of pro-inflammatory genes (Ghisletti et al., 2010b). Macrophage immune response is finely regulated by the collaboration between promoters and enhancers, through a physical contact mediated by specific transcription factors (Nolis et al., 2009).

Enhancers are distal regulatory elements located at variable distances from the transcription start site of the genes they regulate (Bulger and Groudine). They interact with the promoters of regulated genes and are major players in the tissue-specific gene regulation (Natoli). Technological advances in experimental techniques have been applied to identify and characterize enhancers. In particular, DNase I- hypersensitivity based approaches, or the formaldehyde assisted isolation of regulatory elements (FAIRE) assay, which allows the recovery of the soluble (i.e., nucleosome-free) fraction of the chromatin,

and the combination of chromatin immunoprecipitation (ChIP) with massively parallel short read high throughput sequencing (ChIP-seq), along with the progression of computational tools, allowed to have a genome wide mapping of the cis-regulatory elements, transcription factor (TF) functions and epigenetic processes involved in the regulation of gene transcription (Hesselberth et al., 2009; Kidder et al.; Zhou et al.) (Crawford et al., 2006) (Crawford et al., 2006; Gaulton et al.; Giresi et al., 2007; Mendenhall and Bernstein, 2008; Sabo et al., 2006) (Roy et al.) (Ernst et al.) (Rosenbloom et al.). The profiling of the genomic distributions of histone modifications revealed a peculiar chromatin signature of enhancers. Precisely, enhancers are characterized by high level of monomethylation of histone H3 lysine 4 (H3K4me1) and low level of trimethylation of the same residue (H3K4me3), which is instead high at promoters (Heintzman et al., 2007; Pennacchio et al., 2007; Visel et al., 2009). They are bound by tissues and signaling specific transcription factors (such as PU.1) and histone acetyl transferases, such as p300, and show active transcription of the so called eRNA (RNA associated to enhancers region) (De Santa et al.; Ghisletti et al.; Heinz et al.; Jin et al.; Natoli and Andrau). Furthermore, subsequent studies showed that H3K4me1 distributions are highly cell type-specific (Heintzman et al., 2009; Heintzman et al., 2007; Xi et al., 2007). These findings indicate the essential role of enhancers in driving cell-type-specific gene expression programs, rather than promoters, which tend to be invariant across cell types. Furthermore, other studies demonstrated that enhancers, along with the peculiar chromatin signature, are characterized by a distinctive nucleosomal structure, being enriched in non-canonical histone variants, mainly the H2A variant H2A.Z (Calo and Wysocka, 2013; Zlatanova and Thakar, 2008). Depending on their chromatin signature, enhancers have been classified as active, poised and intermediate (Creyghton et al.; Rada-Iglesias et al.). Active enhancers are characterized by a high level of H3K4me1 and H3K27ac, while inactive enhancers display H3K4me1 only. We can further distinguish inactive from poised enhancers, which display high levels of H3K4me1, sometimes

positive for H3K27me3 mark and negative for H3K27Ac, but they can be activated upon stimulation (Zentner et al.). In macrophages, histone acetyltransferases (like p300) are recruited to specific subsets of enhancers that are activated by LPS (Ghisletti et al., 2010a). Moreover, a recent genome-wide analysis confirmed that thousands of cis-regulatory regions gain H4 acetylation upon the pro-inflammatory stimulus LPS (Chen et al.). HDAC3 knock out mice show almost completely absence of LPS-induced histone acetylation, suggesting a role for the histone deacetylase HDAC3 in regulating acetylation levels at a subset of genomic regions (Chen et al.). ChIP-seq studies in macrophages showed that almost the entire repertoire of enhancers is constitutively bound by PU.1, a transcription factor constantly expressed at high levels in macrophages and required to induce and to maintain macrophage differentiation (Ghisletti et al.). Interestingly, although macrophages´ *cis*-regulatory regions are driven by Pu.1 and defined during differentiation, it has been recently shown that this landscape is plastic and can be expanded upon environmental insult (Ostuni et al., 2013). In this study, a new set of genomic regions that gained characteristics of enhancers *de novo* in response to stimulus were termed "latent enhancers" (Ostuni et al.). These results indicated that cells can acquire new functional properties and could incur in a partial remodelling of the available regulatory regions. These latent enhancers are distal regulatory elements unmarked in the unperturbed state, while they are marked by the master regulator PU.1 and stimulus-specific transcription factors only upon stimulation (Ostuni et al.). In this study, PU.1 can bind latent enhancers only by cooperating with other TFs activated by stimulation (e.g. STAT1 and STAT6), since these regulatory regions have a low affinity binding site for PU.1 (Ostuni et al.) (Figure 7.6).

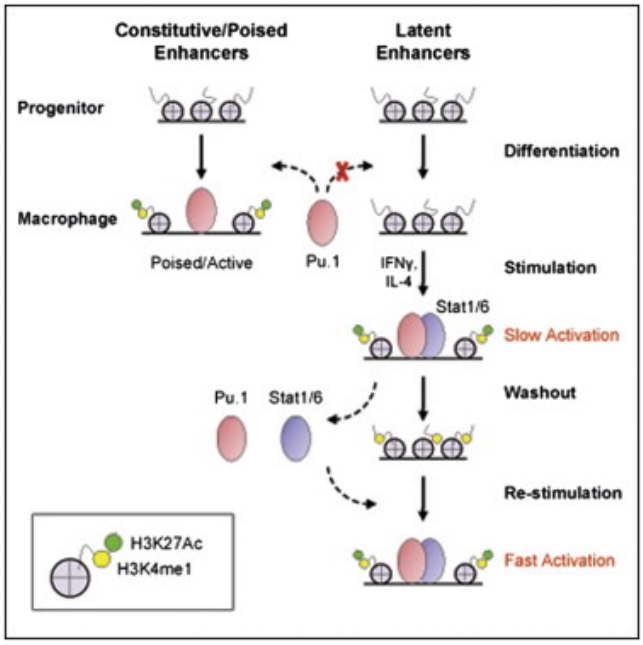


**Figure 7.6 Latent enhancers.** Latent enhancers are regulatory elements unmarked and unbound in differentiated cells that are generated by stimulus-dependent TFs. Latent enhancers may confer short-term memory of environmental exposure (Ostuni et al., 2013).

This suggests that cells benefit of a particular repertoire of enhancers specifically established to regulate the appropriate gene expression program under perturbed condition. These enhancers have a mechanism of induction and activity that is radically different from the one of classical enhancers, established during differentiation by cooperative binding of lineage-determining TFs. Moreover, *in vitro*, washout of the stimulus resulted in rapid loss of acetylation and Pu.1 occupancy, whereas residual H3K4me1 was sustained, thus providing an epigenomic memory of the initial perturbation (Ostuni et al.). Moreover, two very recent papers demonstrated that, despite their common origin, tissue resident macrophages gained a different set of enhancers in response to different microenvironmetal stimuli. Indeed, RNA-seq and ChIP-seq experiments demonstrated that tissue resident macrophages differentially use a common set of enhancers to induce the transcription of differential PU.1 partners, whose activity leads to the generation of an environmental specific repertoire of enhancers (Lavin et al., 2014) (Gosselin et al., 2014).

### 7.3.4 PU.1 is the master myeloid regulator that controls the genomic regulatory landscape in macrophages

PU.1 *(Purine-rich box 1)* is a tissue-specific ets-family member that is expressed exclusively in cells of the hematopoietic lineage (Scott R.McKercher, 1996). Pu.1 is characterized by three domains: the DNA binding domain (ETS domain), with 85 highly conserved amino acids that recognize a purine-rich DNA site containing the core sequence 5'-GGAA/T-3'; the transactivation domain, rich in glutamine and acidic residues, and the PEST domain, necessary for the protein-protein interaction and PU.1 turnover (McKercher et al., 1996; Scott et al., 1994). PU.$1^{-/-}$ mice die of severe septicemia within 48h from their birth, because of their lack of mature macrophages, neutrophils, B cells and T cells (DeKoter et al., 1998; Scott R.McKercher, 1996). Pu.1 is expressed from very early stages of hematopoietic differentiation (Back et al., 2005) and it affects the cell fate determination in a context- and dose- dependent fashion (DeKoter and Singh, 2000). Indeed, Pu.1 expression increases along the myeloid lineage, reaching its maximum level in monocytes. High levels of PU.1 favour macrophage development, whereas low levels of PU.1, about ten-fold lower than in macrophages, are associated with B-cell development (Bakri et al., 2005; Carotta et al., 2010; Dahl et al., 2003).

It has been demonstrated that PU.1 is not only fundamental for macrophage differentiation, but it defines also almost the entire macrophage's cistrome (Ghisletti et al., 2010). Genome-wide mapping of PU.1 occupancy revealed that its distribution is widespread in the macrophage genome (Ghisletti et al., 2010a; Heinz et al., 2010b). PU.1 binds thousands of H3K4me1 positive regions and displays a particular distribution in macrophages. Indeed, B cells show a completely different PU.1 localization, leading to different cell-type specific enhancer repertoires. (Ghisletti et al., 2010a; Heinz et al., 2010b). Considering the very early expression of PU.1, it has been proposed and then proved the hypothesis of PU.1 as transcription factor with pioneering activity. Pioneer factors are functionally

defined as sequence-specific DNA-binding proteins able to bind to their target sites when embedded in a nucleosomal context that is not permissive for binding of other TFs (Zaret and Carroll). Pioneer factors controls both the expression of inducible genes in mature cells and genes involved in the differentiation program of developing cells (Zaret and Carroll). It has been demonstrated that PU.1 can trigger a partial reprogramming of non-myeloid cells to macrophage phenotype. Fibroblast ectopic expression of PU.1 drives the deposition of H3K4me1 in regions originally devoiding of this mark (Ghisletti et al., 2010). Moreover, these data have been supported by an independent study, in which PU.1 was fused to the estrogen receptor ligand-binding domain. Upon cell treatment with tamoxifen, PU.1 positive sites gained H3K4me1 (Heinz et al., 2010). Very recently, it has been demonstrated that PU.1 is involved in the displacement of nucleosomes, thus generating accessible stretches of DNA sequences and the binding of other TFs that would otherwise be unable to invade nucleosomal DNA (Barozzi et al., 2014; Ghisletti et al., 2010b; Heinz et al., 2010a). Aforementioned PU.1 properties suggest its possible role in mediating looping between enhancers and promoters of the regulated genes. PU.1 regulates its own expression in hematopoietic stem cells by binding to its upstream regulatory region (Xi et al.) and triggering the formation of a chromosomal loop (Staber et al., 2013). Moreover, Pu.1 expression results to be necessary for inducing Irf8 production in dendritic cell progenitors. PU.1 is indeed involved in mediating the looping between a distant enhancer and the *Irf8* promoter (Schonheit et al., 2013). How PU.1 cooperates with other transcription factors in order to mediate its functions is not fully understood. However, a genome wide study on dendritic cells showed how 25 transcription factors are distributed upon LPS treatment and how they temporally bind their sites. Being broadly distributed, PU.1 results to be included in the first class of TFs. Due to their widespread distribution, these TFs are very likely the chromatin openers which mediate the binding of a second class of TFs, *primer* TFs. Primer TFs, such as Junb, Irf4 and Atf3 are constitutively bound to DNA and they may dispose for the inducible responses. Lastly, *effector* TFs, NF-kB,

IRF, AP-1 and the STAT family members among the others, are induced upon stimulation and they coordinate the expression of specific groups of pro-inflammatory genes (Biddie et al., 2011; Garber et al., 2012; Natoli, 2010). Therefore, the potential for pro-inflammatory genes to be transcriptionally induced is established before stimulation by binding of TF subsets to their genomic *cis*-regulatory regions (Garber et al., 2012).

## 7.4 Endotoxin tolerance, a complex transcriptional inflammatory response

After an inflammatory stimulus, the expression of several hundred genes is either induced or repressed in macrophages (Ramsey et al., 2008) (Ravasi et al., 2007). This complex transcriptional response consists of multiple gene sets, or transcriptional modules, which encode different functional programs and that are often controlled by dedicated transcription factors. This feature enables autonomous control of individual transcriptional modules, because the transcriptional regulators that control their expression can be differentially regulated by positive and negative signals.

One of the clearest examples of module-specific transcriptional regulation is provided by LPS tolerance. LPS/Endotoxin (ET) tolerance is a state of hypo-responsiveness to LPS (and other microbial stimuli) that is induced during conditions of sustained inflammation to limit inflammation-associated pathology. Edotoxin Tolerance has been described for the first time by Paul Beeson in 1946. He observed that repeated injection of typhoid vaccine in rabbits leads to a progressive reduction of fever induced by vaccination (Foster & Medzhitov, 2009). Similarly, mice, injected with a sublethal dose of lipopolysaccharide (LPS) were protected from a subsequent and otherwise lethal dose of LPS (Cavaillon & Adib-Conquy, 2006). Moreover, this study also demostrated that monocytes/macrophages are the principal cells responsible for the induction of ET in vivo.

Macrophages under the state of tolerance, meaning that they have been stimulated for some hours with a pro-inflammatory stimulus, are unable to express inflammatory genes, such as tumor necrosis factor alpha (TNFα), interleukin-1 beta (Il-1β), and interleukin-6 (IL-6), when re-challenged with a second dose of LPS. However, they are still capable of inducing the expression of other genes such as IL-10 and IRAK-M (or IRAK3, a negative regulator of the TLR4 pathway, which mediates LPS signaling) (Porta et al., 2009b) (see Figure 7.7).

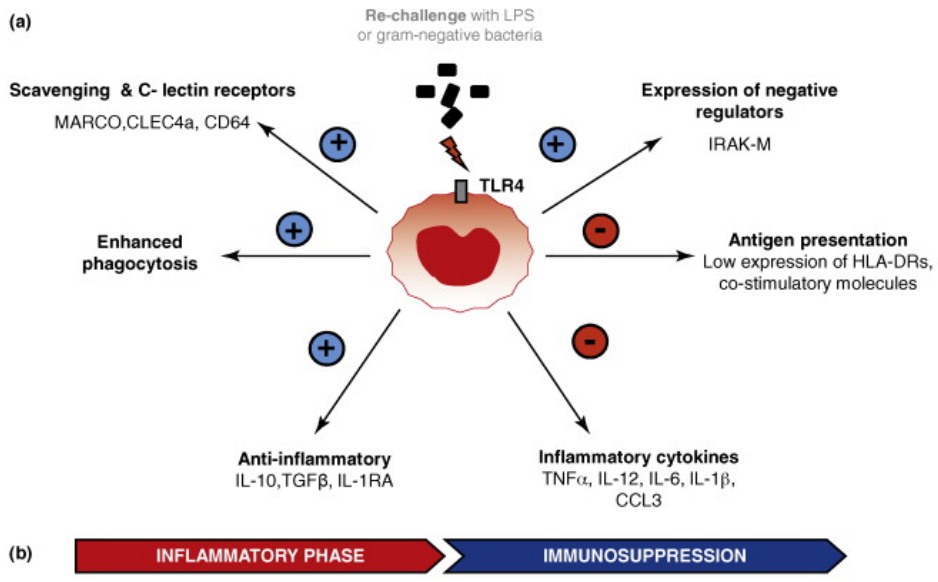


**Figure 7.7 Phenotype of endotoxin tolerant monocyte/macrophage.** Upon sustained LPS stimulation, macrophages display immunosuppression properties. From (Biswas and Lopez-Collazo, 2009).

This is an anti-inflammatory state under sustained exposure to bacterial products like lipo-polysaccharide (LPS), observed both *in vitro* and *in vivo* (Biswas and Lopez-Collazo, 2009). Macrophages from septic patients or from animal models of sepsis display alterations in the pro-inflammatory cytokines production (Valledor et al., 2010). Macrophages of septic patients enter into this sustained hypo-responsive state that has been suggested to favor secondary infections (Greisman and Hornick, 1975; McCall et al., 1993) (Rutenburg et al., 1965) (Valledor et al., 2010) (McCall et al., 1993). Moreover, endotoxin tolerance has been observed in several diseases, not only in monocytes of septic patients,

but also in patients suffering from diseases like cystic fibrosis, acute coronary occlusion and kidney ischemia (Biswas and Lopez-Collazo, 2009). In sepsis, endotoxin tolerance may contribute to limit the inflammatory reaction and to protect from shock, but at the same time it increases the risk of secondary infections, which are a major cause of death in patients recovering from sepsis. Finally, a state that is similar to endotoxin tolerance can be found in tumor-associated macrophages, which can be largely assimilated to alternatively activated macrophages with a poor ability to promote an anti-tumor inflammatory reaction (Mantovani and Sica).

### 7.4.1 The endotoxin tolerance mechanisms

The specific mechanisms underlying endotoxin tolerance are only partially understood. Despite that, it is clear that the mechanisms responsible for this phenomenon are multifactorial, and likely they involve negative feedbacks at multiple levels. Endotoxin tolerance may be partially explained by each one of the proposed following mechanisms: (Scott R.McKercher) the downregulation of the TLR4-mediated signaling pathway, (Scott R.McKercher) changing in the NF-kB subunit composition, (Scott R.McKercher) epigenetic modification of the regulatory elements involved in controlling the expression program and (Scott R.McKercher) microRNA-mediated regulation of the endotoxin tolerance phenotype.

Regarding the first point, several studies have indicated defects in the TLR4 pathway. Multiple levels of the signaling pathway may be involved, starting from the receptor, adaptors, signaling molecules, and transcription factors (Biswas & Lopez-Collazo, 2009). For example, the desensitization of TLR4 and decrease in TLR4-MyD88 complex formation have been linked to tolerized macrophages (Fan & Cook, 2004; Biswas & Tergaonkar, 2007). Moreover, others groups suggested the involvement of negative mediators of the TLR4 downstream cascade, including both degradation of proximal

signaling molecules, such as IRAK1, or upregulation of negative regulators, including IL-1 receptor-associated kinase M (IRAK-M), suppressor of cytokine signaling 1 (SOCS1), SC homology 2 (SH2) domain-containing inositol-5-phosphatase 1 (SHIP1), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBa) and the human Mitogen-activated protein kinase phosphatase 1 (MKP1), a dual-specificity phosphoprotein phosphatase (Kobayashi et al., 2002) (Beutler, 2004; Sly et al., 2004) (Nakagawa et al., 2002). Mice lacking IRAK-M, the inhibitor of the IRAK1/IRAK4 cascade, show an increased inflammatory response to bacterial infection and are unable to develop LPS tolerance upon sustained stimulation (Kobayashi et al., 2002).

An impairment of the canonical p65/p50 NF-κB activity has been demonstrated in endotoxin-tolerant murine macrophages and human monocytes (Porta et al., 2009a) due to over-expression of p50 NF-κB homodimers, which lack the transactivation domain and thus cannot trigger transcription when binding to gene promoters (Porta et al., 2009a). In agreement with this hypothesis, $p50^{-/-}$ murine macrophages cannot undergo to tolerization upon sustained treatment with LPS (Bohuslav et al.,1998; Wysocka et al., 2001).

Micro-RNAs (miRNAs) have emerged as key players in selectively silencing the intermediates of TLR signaling cascade. MicroRNAs are a class of endogenous non-coding RNAs highly conserved throughout evolution, which modulate the expression of specific genes by controlling the stability and/or the translation of target mRNAs (Monticelli et al., 2005) (Bartel, 2004) (El Gazzar and McCall, 2010) (Nahid et al., 2009). In the endotoxin tolerant state, miR146 is up-regulated and it has been suggested to be involved in targeting IRAK1 and TRAF6, thus preventing NF-κB activation (Nahid et al., 2009).

Some works have proposed the explanation that epigenetic mechanisms may explain the gene-specific nature of tolerance (Chen and Ivashkiv; Foster et al., 2007; Park et al.). Promoters of tolerized genes display a less grade of accessibility, due to a lower level of active histone marks and the reduced recruitment of Brg1 nucleosome-remodeling

complexes. By contrast, promoters of non-tolerized genes (genes that remain active) are characterized by a high level of active histone marks and an open chromatin conformation (Foster et al., 2007). This has been shown both *in vitro* and *in vivo* in peripheral blood monocytes from septic patients, where the promoters of IL-1β and TNFα are enriched in repressive chromatin marks, thus reducing the ability of the macrophage to respond to subsequent LPS challenges. In addition, the H3K9 histone methyltransferase G9a can bind the TNFα promoter during the induction of LPS tolerance and direct DNA methylation via the HP1-dependent recruitment of the DNA methylase Dmnt3a/b, restricting the transcriptional activation of those genes (Chen and Ivashkiv, 2010).

Of note, very recently it has been shown that also tryptophan catabolism is involved in the establishment of ET. LPS treatment leads to the activation of the ligand-operated transcription factor aryl hydrocarbon receptor (Sia et al.) and the hepatic enzyme tryptophan 2,3-dioxygenase, which downregulate early inflammatory gene expression. $IDO^{-/-}$ (indoleamine 2,3-dioxygenase 1 knockout mice) display impaired AhR ability to mediate long-term regulation of systemic inflammation, thus suggesting a collaborative role in mediating the tolerance state {Bessede, 2014 #657}.

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# 8 Aims of the work

Macrophage transition from pro-inflammatory to an anti-inflammatory phenotype, occurring in response to sustained microbial stimulation (endotoxin tolerance), is characterized by the reduced ability to produce inflammatory mediators. This property of macrophages is believed to have an important protective role in the short term, in that it reduces the local and systemic consequences of an excessive acute production of inflammatory molecules (Biswas and Lopez-Collazo, 2009; Medvedev et al., 2006). At the same time, since endotoxin tolerance is a long-lasting phenomenon, in some cases it amplifies the ability of the organism to defend from microbes thus favoring the frequent occurrence of secondary infections with a high mortality rate in patients recovering from sepsis. Therefore, understanding the molecular bases of macrophage reprogramming during the course of a sustained inflammatory response is a relevant objective that may have a major impact on the understanding of a whole group of disease states characterized by sustained inflammation.

The objective of this project is to understand how the macrophage genome is reprogrammed into an anti-inflammatory state in response to prolonged microbial stimulation. This will provide a glimpse onto the deepest molecular roots of endotoxin tolerance and macrophage reprogramming.

The project is based on the use of an *ex vivo* model of macrophage (bone marrow-derived macrophages) reprogramming in response to sustained endotoxin stimulation. We have applied cutting-edge genomic technologies to map regulatory regions (ChIP-seq), as well as nascent RNA-seq, to characterize *cis*-regulatory regions and genes that are differentially active in unperturbed, LPS-stimulated and LPS-tolerized primary mouse bone marrow-derived macrophages (BMDM). We characterized promoter and enhancer states by mapping methylation and acetylation of histones and we identified differentially expressed

genes by nascent RNA profiling. We clustered genes into different subsets based on their transcriptional profiles and assigned them to enhancers with correlated dynamic behaviors. Once genomic regulatory regions that displayed a different state of activity in alternatively stimulated macropahges have been identified, we computationally analyzed the data in order to identify the underlying genomic determinants of diversity, namely the specific composition of transcription factor (TF) binding sites that account for their diverse activity in different functional states. These approaches provided a characterization of the genomic bases for the activation of alternative gene expression programs from the same macrophage genome.

# 9 Material and Methods

## 9.1 Cells and Reagents

Animal experiments were performed in accordance with the Italian Law (D.L.vo 116/92), which enforce the EU 86/609 Directive. Bone marrow-derived macrophages (BMDM) were isolated from 8-10 weeks old C57/BL6N (Charles River) mice and generated as previously described (De Santa et al., 2007). Briefly, bone marrow cells were isolated from mice bones and red blood cells were lysed by osmotic shock (1 minute of 0.2% of NaCl followed by a minute of 1.6% NaCl, in order to restore the osmotic physiological condition). After two washings in cold PBS, BM cells were resuspended in BM medium (DMEM supplemented with 30% L929-conditioned medium containing M-CSF, 20% FBS, 2 mM glutamine, 100 U/ml penicillin 100 mg/ml streptomycin, 0.5% sodium pyruvate, 0.1% β-mercaptoethanol). Cells were then plated $1x10^6$ cells in 10 ml of BM-medium per 100 mm petri dish. Cells were harvested at day 7-8 of differentiation. Cells were then stimulated with 10 ng/ml of LPS from *E.Coli* serotype EH100 (Alexis) or with 100 ng/ml for the tolerization protocol. Cells were subjected to 1 hour of washing out before restimulation.

293T cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. Cells were incubated at 37°C in a 5% $CO_2$ humidified atmosphere.

P50-/- macrophages were obtained by *p50-/-* mice, gently given by A. Sica (Saccani et al., 2006)

## 9.2 Lentiviral mediated KD

### 9.2.1 Lentiviral Constructs and Production of lentivirus

pLKO.1 ShIRF7, kindly given by I. Amit, was used and IRF7-KD was validated with RT-qPCR, using expression primers for *Irf7* NM_016850

*FORWARD* CCAGTTGATCCGCATAAGGT

*REVERSE* GCAGAACCTGAAGCAAGAGG

293T packaging cells were co-transfected with 6 μg of psPAX8 (encoding viral proteins for packaging of viral particles), 3 μg of pMD2.G (encoding the VSV-G envelope protein and triggers the entry of the virus in the cell to infect) and 8 μg of either plko.1 scrambled luc-sequence or the vector containing the *irf7*-directed shRNA-sequence. 293T cells were transfected with the $CaCl_2$ strategy. DNA was mixed in 500 ul of water containing 62,5 ul of 2M $CaCl_2$ and dissolved drop by drop onto 500 ul of Henk's balanced salts solution (HBSS). 1 ml of transfection mix was added to each plate of 293T cells and left unperturbed for 6-8 hours. Supernatants from transfected 293T cells were collected at 48 h post-transfection and immediately used for infections.

### 9.2.2 Lentiviral Transduction of Bone Marrow-Derived Macrophages

On day 0, bone marrow cells were isolated and $1{,}5x10^6$ cells were seeded on a 100 mm plates in BM medium. On days 5 and 6 cells were infected for two consecutive days (1 infection per day). In detail, the BM medium was removed, and the lentiviral containing supernatants, supplemented with 8 μg/ml polybrene and 8 μl/ml of 1 M HEPES pH 7.5, were added. After 8 hours, the virus-containing medium was gently removed and replaced by fresh BM medium. Puromycin selection (3 μg/ml) started on day 7. Assays were carried out at day 9.

## 9.3 Chromatin Immunoprecipitation and Sequencing

## (ChIP-Seq)

The purpose of the ChIP assay is to determine whether a protein binds to a particular genomic region on chromatin. DNA-binding proteins can be covalently cross-linked to the chromatin, in order to fix a precise genomic localization of the protein. Cells are then lysed and the DNA is sheared into small fragments (around 250 bp) by sonication. Immunoprecipitation of Protein-DNA complexes is obtained by using specific antibodies and, after cross-linking reversion, the DNA can be purified. Immunoprecipitated DNA is identified either by PCR, using sequence-specific primers or by genome sequencing (ChIP-seq), leading to the entire genomic localization of the protein.

### 9.3.1 Chromatin Immunoprecipitation (ChIP)

ChIP was carried out using a protocol previously described in our lab (Ghisletti et al., 2010b). $20\times10^6$ (ChIP-seq for histone marks and Pu.1) or $100\times10^6$ (ChIP-seq for RNA Pol II) macrophages were fixed with 1% of formadehyde in BMDM-medium for 10 minutes. The cross-linking was stopped by the addition of Tris-HCl pH 7.6 for 5 minutes. Cells were washed three times with cold PBS, collected using a silicon scraper, pooled in a 50 ml Falcon tube and spun at 1,500 rpm for 5 min. Chromatin was obtained with a three step lysis as described in the paper cited above. Briefly, pelleted cells were resuspended in LB1 buffer (50 mM Hepes-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton X-100), kept 10 min on ice to lyse the plasmamembrane and centrifuged at 2500 rpm for 5 min. Pelleted nuclei were washed in LB2 buffer (10 mM Tris-HCl pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA) for 10 min at RT under gentle rocking to remove residuals of detergents and then centrifuged at 2500 rpm for 5 min. Finally, pellets were resuspended in LB3 buffer (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). The chromatin

was shared by sonication (amplitude 30%, 30 sec, 5-7 cycles) and the size of the DNA fragments was checked extracting DNA from a small aliquot (30 µl) of the suspension and loading it on a 1.5% agarose gel. After addition of 1% Triton X-100, the solution was centrifuged at max speed for 10 minutes, in order to remove cellular debris. A 50 µl aliquot of cell lysate was saved as whole-cell extract (WCE) and used as ChIP input. While, the remaining lysate was incubated overnight at 4°C with paramagnetic protein G Dynabeads (Invitrogen), previously coupled with 3–10 µg of antibody in PBS/BSA 0.5%. All the centrifugation steps were carried out at 4°C and all lysis buffers were supplemented with 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin; inibitors of the proteases. Ready-to-use beads were added to the cell lysate and gently mixed on a rotating platform o.n. at 4°C The day after, beads were collected using a magnetic and the supernatant was removed and discarded. The DNA-protein complex coupled to the beads was washed 6 times with 1 ml of Washing Buffer/RIPA modified buffer (50mM HEPES pH7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0,7% Na-deoxycholate) for 5 min on ice, and once with 1 ml TE/50 mM NaCl. All the procedures was performed on ice. Beads were then centrifuged at 3000 rpm for 3 minutes at 4°C and TE buffer was discarded in order to proceed with DNA-protein complex elution. DNA was eluted with 250 µl elution buffer (TE/SDS 2%) at 65°C for 15 minutes under shaking. After a centrifugation at maximum speed for 1 minute, beads were pulled down and the 240 ul of supernatant was collected and transferred to a new tube where DNA was decrosslinked overnight at 65°C in TE/2%SDS, along with the previously conserved WCE, supplemented with 3 volumes of elution buffer. Finally, DNA was diluted five-fold with PB buffer (Qiagen) and incubated 30 minutes at 37°C under shaking condition and purified with Qiaquick columns (Qiagen) and quantified with PicoGreen (Invitrogen), following manufacturer's instructions.

### 9.3.2 ChIP-qPCR

For ChIP-QPCR validation, 0,2 µl of purified DNA and 200 nM primers, diluted in a final volume of 20 µL in SYBR Green Fast Reaction Mix (Applied Biosystems), were used. Accumulation of fluorescent products was monitored for 40 cycles by real-time PCR using both the 7500 or 7900HT Fast Real-Time PCR System (Applied Biosystems). Each primer pair used in this study has been tested for linear amplification, on at least three 8-fold dilutions of genomic DNA and each PCR reaction generated only one specific amplicon, as revealed by the melting temperature profile of final products (dissociation curve). QPCR detection system was updated with the Fast SYBR Green Master Mix during the course of the presented work and the PCR cycle settings adjusted following manufacturers´ instructions.

### 9.3.3 ChIP-sequencing procedure

ChIP DNA was prepared for HiSeq 2000 sequencing system (Illumina) using the TruSeq ChIP preparation protocol (Illumina) consisting of blunting, addition of 3'-dA-overhangs, ligation of Illumina adapters and PCR amplification. ChIP-seq libraries were quantified with Agilent Bioanalyzer and cluster generation was performed hybridizing samples onto flow cells and amplifying them for later single-end multiplex-sequencing (36 nt) on the HiSeq 2000. Non-duplicated sequence-read tags with mapping quality higher than 20 were aligned to mm9 assembly using Fish-The-ChIP pipeline (Natoli et al., 2011). Briefly, short reads were subjected to quality controls and aligned to a reference genome. Obtained peaks were identified, annotated respect to the reference genome, and raw signal tracks were generated for visualization on the UCSC and IGV genome browsers.

### 9.3.4 Chip-seq Analyses

Non-duplicated sequence-read tags with mapping quality higher than 20 were aligned to the mm9 release of the murine genome using Bowtie v0.12.7 (Langmead et al., 2009).

Only unique alignments were retained, allowing up to two mismatches compared to the reference genome (options -m 1 -v 2). Peak calling was performed using MACS v1.4 (Zhang et al., 2008). Each IP was compared to input DNA derived from bone marrow-derived macrophages. Chromatin state were obtained using ChromHMM (Ernst et al., 2011) . ChromHMM is an approach for the learning and analysis of chromatin states using a multivariate Hidden Markov Model that explicitly models the observed combination of marks. We trained models using 4 to 16 chromatin states, separately for each time point. In all four cases, adding states further than 8 did not add any other significantly different state. We then used the 8-states model trained on the untreated data to derive a genome-wide segmentation for the other time points.

## 9.4 Nascent RNA-seq, Total RNA and miRNA RT-qPCR

### 9.4.1 Nascent RNA extraction

To mimic the acute phase response, macrophages were treated for 30 minutes, 1h, 2h, and 4h with 10 ng/ml of LPS while tolerization was obtained treating the cells for 24h with LPS at 100 ng/ml. Tolerized cells were then washed out of the conditioned medium and left resting for an hour, before re-challenging them with fresh media containing 10 ng/ml of LPS for the same time points as in the priming phase.

Chromatin associated RNA/nascent RNA was extracted in order to evaluate the transcriptional dynamicity of a specific gene. Nascent RNA is the immediately formed RNA, in which any post-transcriptional modification has occurred. Nascent RNA is obtained by cellular fractionation; cytosolic and nucleoplasmic fractions were discarded and only RNA associated to the chromatin fraction was retained and further analyzed.
Nascent RNA was extracted from $20x10^6$ cells for each time point according to the published protocol (Bhatt et al., 2012). Subcellular fractions were prepared as described

before (Pandya-Jones and Black, 2009), with minor changes. While petri dishes were kept on ice, cells were washed twice with 10 ml of cold PBS buffer. Cells were then scraped off with 4 ml of PBS containing RNase inhibitor (40 U/ml) and protease inhibitors. The cell suspension was centrifuged at 1500 rpm for 5 min at 4°C and supernatant was discarded. The obtained pellets were resuspended in 200 ul of CYTOPLASMIC LYSIS BUFFER (10 mM Tris-HCl pH 7.5, 150 mM of NaCl and 0.15% NP-40 supplemented with 200 U/ml of RNase inhibitor in water) using wide orifice tips, pipetting up and down 15 times and incubating on ice for 5 minutes. The lysate was gently layered on top of 500 ul of a chilled SUCROSE CUSHION (24% w/v of sucrose dissolved in the cytoplasmic lysis buffer without NP-40) and centrifuged at 13,000 rpm for 10 min at 4°C. Once centrifuged, the supernatant containing the cytoplasmic fraction was carefully removed. The remaining nuclei pellets were washed briefly once in PBS with RNase inhibitor and spun at 3,500 rpm for 5 min at 4°C. After centrifugation, supernatant was discarded and nuclei pellets were resuspended in 200 ul of pre-chilled GLYCEROL BUFFER (20 mM Tris-HCl pH 7.9, 75 mM NaCl, 0.5 mM EDT in glycerol 50% supplemented with 0.85 mM DTT and 200 U/ml RNase inhibitor) pipetting up and down 10 times. 200 ul of cold NUCLEI LYSIS BUFFER (20 mM HEPES pH7.6, 7.5 mM $MgCl_2$, 0.2 mM EDTA, 300 mM NaCl, 1M UREA, 1% NP-40 in water supplemented with 0.1 mM of DTT and Aprotinin, Leupeptin and PMSF (in order to block proteases activity and proceed with protein analysis) were added and carefully mixed pipetting up and down 4 times. Each tube was gently mixed on a vortex for 4 seconds and incubated on ice for 1 min. Once well resuspended, tubes were submitted to centrifugation at 13,000 rpm for 2 min at 4°C. The supernatant containing the nucleoplasmic fraction was discarded and the chromatin pellet was washed once in cold PBS with RNase inhibitor. The washed chromatin fraction was centrifuged and the supernatant was removed. 50 ul of cold PBS was added to the chromatin pellet, well mixed pipetting up and down and 5 ul were taken off for protein analysis. To the remaining 45 ul, 500 ul of TRIzol were added and mixed by vortex

vigorously. RNA extraction was obtained combining the standard TRIzol protocol and the usage of Qiagen RNA extraction Kit. 100 ul of chloroform was added to the 500 ul of TRIzol and well mixed. Tubes were incubated at room temperature for 5 min and centrifuged at 13,000 rpm for 15 min at 4°C, in order to separate all the three phases (acqueous- inter- and organic phase). The upper aqueous phase, containing RNA, was collected and the RNA was purified on Qiagen columns following the standard protocols with DNase treatment.

### 9.4.2 Nascent RNA sequencing procedures (libraries preparation)

RNA' integrity was verified by Bioanalyzer and libraries were prepared using conventional Illumina TruSeq RNA sample preparation Kit V2, with minor modification. Specifically, polyA enrichment was avoided and the protocol was initiated from the RNA fragmentation step and sequenced on a HiSeq 2000 following standard protocols (50 bp pair with a sequencing depth of 50M reads). Chromatin fraction purity was confirmed by immunoblot analysis of total histone H3 (enriched in the Chromatin fraction), Tubulin α, and HDAC1 to exclude cytoplasmatic and nucleoplasmatic contamination.

### 9.4.3 Nascent RNA-seq Analysis

After quality filtering according to the Illumina pipeline, paired-end reads were aligned to the mm9 reference genome and to the *Mus musculus* transcriptome (RefSeq genes) using TopHat (Trapnell et al., 2010). Transcript abundance was quantified using Cufflinks 2.0.2 then differentially expressed genes were identified using Cuffdiff 2.0.2 (Trapnell et al., 2010). For each time point, LPS-induced genes were defined as those showing a significantly higher FPKM compared to untreated macrophages ($p <= 0.05$). Tracks for the UCSC genome browser were generated using the uniquely alignable reads. Tracks were linearly rescaled to the same sequencing depth. Data visualization was achieved as custom tracks on the UCSC genome browser.

### 9.4.4 Heatmap of nascent RNA-seq

The heatmap shows the counts of the reads in each nascent RNA sample of the complete time course. The number of reads for each LPS-induced gene was normalized per kilobases of exons and millions of sequenced fragments (FPKM). For visualization purpose, those genes showing a FPKM of zero were set to the lowest expression level that could be measured (excluding zeros). All values were then log2-transformed and finally converted to z-scores on a gene-by-gene basis.

### 9.4.5 RNA-seq clustering

In order to avoid variability due to noise in lower counts, those genes showing a FPKM lower than 1 were set to 1. Log2-transformed values were used to hierarchically cluster the regions using average linkage and 1 minus Spearman Rank's Correlation to measure the distance among the dynamic changes of expression of each gene against any other throughout the time-course. The resulting dendrogram was cut dissected using the R package *dynamicTreeCut* (Langfelder, 2008) using method="hybrid" and minClusterSize=20, resulting in 12 clusters.

### 9.4.6 Total RNA extraction and cDNA synthesis

Total RNA was extracted using both TRIzol (Invitrogen) or RNeasy kit (Qiagen), according to manufacturers´ instructions. RNA was quantified by ND-1000 spectrophotometer (NanoDrop Technologies from ThermoScientific) and its quality was assessed by measuring $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios.

Complementary DNA (cDNA) was obtained by reverse transcription with the following protocol: 500 ng of RNA were reverse-transcribed using ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer´s protocol. RNA was

mixed to 500 ng of Random Primers in RNase free water, in a total volume of 5 ul. Each tube was heated at 70°C for 5 minutes, in order to denature secondary structures of RNA. Tubes were immediately chilled on ice and briefly spun. The RNA mix was added to a second reaction mix containing 1x of ImProm-II reaction buffer, 25 mM of $MgCl_2$, dNTPs (10 mM each) and 1 ul of ImProm-II Reverse transcriptase, for a total volum of 20 ul. After an incubation of 5 minutes at 25°C (annealing), the elongation was left to proceed at 42°C for 1 hour. The transcriptase was inactivated at 70°C for 15 min. 12 ng of cDNA were used for quantitative real-time PCR amplification on an ABI 7500 machine using SYBR Green (Applied Biosystem). Relative gene expression was calculated by normalizing for the expression of the housekeeping gene TBP.

Primers used for RT-qPCR are listed here:

*Ccl5* (NM_013653)

Forward ACCATATGGCTCGGACACCACT

Reverse ACCCACTTCTTCTCTGGGTTGG

Il6 (NM_031168)

Forward CCATAGCTACCTGGAGTACATG

Reverse TGGAAATTGGGGTAGGAAGGAC

TBP (NM_013684)

Forward CTGGAATTGTACCGCAGCTT

Reverse ATGATGACTGCAGCAAATCG.

### 9.4.7 Total RNA-sequencing (PolyA RNA libraries preparation)

mRNA-Seq library preparation from 2 µg of total RNA was performed with the TruSeq RNA Sample Prep kit V2 (Illumina) according to the manufacturer's instruction and sequenced on a HiSeq 2000 following standard protocols, with paired-end 50-bp. For total RNA-seq analysis see point 9.4.2.

### 9.4.8 miRNA analysis

For miRNA analysis, total RNA was extracted with TRIzol reagent (Invitrogen), and used to perform quantitative reverse transcription-PCR (qRT-PCR) using a miRNA reverse transcription kit and TaqMan miRNA assays from Applied Biosystems, following the manufacturer's instructions. Snor202 was used as control.

## 9.5 Western blot analysis

Cells were washed twice with PBS, collected in 1 ml PBS using a silicon scraper, and centrifuged at 1500 rpm 5 min at 4°C. (All centrifugation steps were performed at 4°C and 52 (?) protease inhibitors - 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin - were added to all the lysis buffers).

Pellets were resuspended in RIPA-modified buffer (50 mM Hepes-KOH pH 7.6, 500 mM LiCl, 1 mM EDTA, 1% NP-40, 0.7% Na-Deoxycholate) and lysed 10 min on ice. Cell lysates were sonicated twice for 15 sec, centrifuged 20 min at max speed to remove cell debris and supernatant was recovered as total protein extract. 30ug of total protein extracts were run on a 12% SDS-polyacrylamide gels and separated proteins were transferred to a nitrocellulose membrane. After blocking for 1 h in TBST containing 5% of milk, each membrane was incubated over night at 4°C with the primary antibodies listed below. Infrared conjugated antibodies from Biosciences were used to detect the specific band using LICOR technology. α –tubulin was used as loading control.

## 9.6 Antibodies

Antibodies against H3K4me1 (ab8895), H3K27Ac (ab4729), H3K9me3 (ab8898), total H3 (ab1791) and GAPDH (ab9485) were from Abcam. Anti-H3K4me3 (39159) and H4Ac (pan-acetyl) (39243) were from Active Motif. Anti-RNA Pol II (sc-899), and IkB-a (C21)

(sc-371), TRAF6 (H-274) (sc-7221), HDAC1 (H51) (sc-7872) were from Santa Cruz. Anti-pIRF3(Ser396) (49475), Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) were from Cell Signaling. Anti Tubulin-α (T9026) was from Sigma-Aldrich. The anti-Pu.1 rabbit polyclonal antibody was generated in-house against the N-terminus of Pu.1 (aa. 1-100; NP_035485.1). IRDye 800 and 680CW secondary antibodies were used for WB experiment.

## 9.7 Gene ontology analysis

Gene ontology analysis was performed for each time point. We interrogated DAVID (Huang da et al., 2009) and we considered all the terms significantly enriched (Benjamini-Hochberg corrected p-values $\leq 0.05$) for the following ontologies: GO Biological Process and GO Molecular Function. FDRs were log10-transformed and the results represented as histograms.

## 9.8 Motifs Enrichment Analysis

In order to identify over-represented motifs corresponding to known TF binding sites, pscan (Zambelli et al., 2009) was run on the promoter regions (-500, +250 base pairs from the annotated TSSs) of the genes belonging to each cluster. Statistically significant over-represented PWMs were identified using the TSSs (defined on the same window) of the entire set of RefSeq genes.

# 10 Results

## 10.1 Clusters of sustained and transient gene activation identified in tolerized macrophages by RNA-seq analysis

In order to define and mechanistically dissect how the switch in the macrophage gene expression program during sustained inflammation *in vivo* is controlled by the global reorganization of the macrophage genomic landscape, we used the endotoxin tolerance model. Endotoxin tolerance is a long-lasting phenomenon, in which a sustained inflammatory response leads to a global rearrangement of the macrophage gene expression profiling (Foster et al., 2007). To characterize genes that are differentially expressed in unperturbed, LPS-stimulated and LPS-tolerized primary mouse bone marrow derived macrophages (BMDM), we used an *ex vivo* model of endotoxin tolerance, in which cells were left untreated, stimulated for 4 hours with 10 ng/ml of LPS or treated for 24 hours with 100 ng/ml of LPS and then challenged with a new dose of LPS (10 ng/ml) (Figure 10.1).


**Figure 10.1 LPS tolerance model scheme.** Experimental design: no treatment (0); no pre-treatment (4h of LPS), LPS tolerization /sustained stimulation (24h), corresponding; pretreatment (24h) and rechallenge with LPS (+4h).

We verified the endotoxin tolerance distinct expression pattern by analysing two representative genes (with known expression behavior (Foster and Medzhitov, 2009)) by

quantitative polymerase chain reaction (qPCR). Consistent with published data on LPS-induced endotoxin tolerance, pretreatment with LPS almost completely inhibited *Il-6* production after secondary LPS challenge in primary macrophages. LPS-treated BMDMs displayed transient expression of *Il-6* upon sustained treatment, while a sustained expression of the *Ccl5* gene was observed. *Ccl5* was highly expressed after 24 hours of LPS treatment, and was further induced upon secondary stimulation (Figure 10.2).

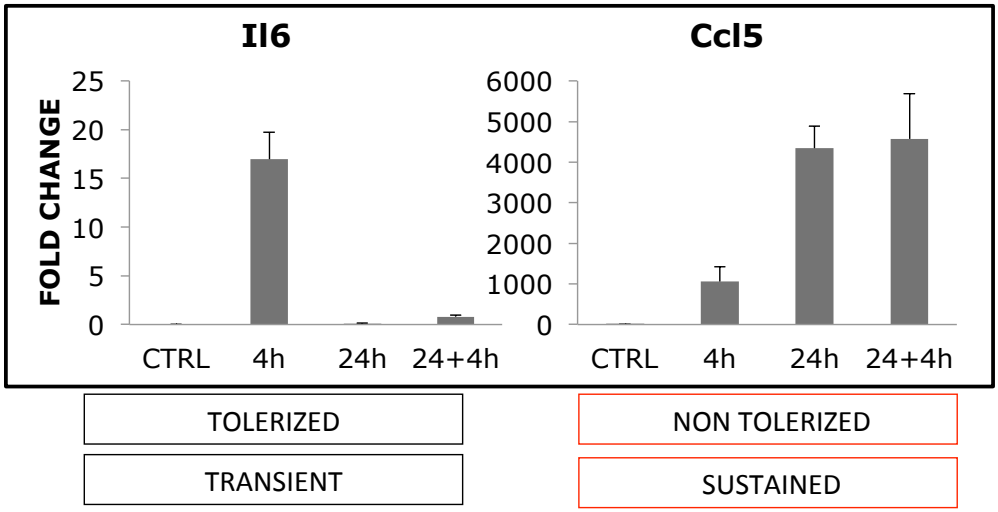



**Figure 10.2. Induction kinetics of *Il-6* and *Ccl5* genes**. *Il-6* and *Ccl5* expression is normalized relative to the housekeeping gene *Tbp*. Cells were stimulated with LPS as indicated in Figure 10.1. Error bars represent standard deviation of three independent experiments.

We named *TRANSIENT GENES*, those genes whose transcription at the secondary stimulation is at least three fold lower than upon the primary challenge with LPS (e.g. *Il-6*); *SUSTAINED GENES,* those genes that are induced during the first stimulation and maintained a high level of expression upon sustained stimulation and/or upon rechallenge with LPS (e.g. *Ccl5*). Sustained genes include both genes that are never completely turned off (*Ccl5*) and genes that are turned off and then reactivated upon secondary stimulation (*Il-1a*) (Figure 10.2 and Supplementary Figure 1).

We then moved to in-depth profiling of the *in vitro* model using nascent RNA-seq analysis. Since total RNA is affected by stabilization and degradation events, we decided to analyze chromatin associated RNA (Bhatt et al., 2012), to focus on the transcriptional events associated with LPS-induced gene expression in unperturbed, stimulated and tolerized cells. We fractionated cells to isolate chromatin-associated transcripts and we increased the kinetics adding several intermediate time points (see Figure 10.3). The purity of the chromatin fractions was assessed by Western blot analysis of β-tubulin, HDAC1, and histone H3, to confirm respectively the enrichment of the chromatin fraction and the absence of contamination from nucleoplasmic and cytosolic fractions (Supplementary Figure 2). Chromatin associated RNAs were analyzed by high-throughput sequencing, in order to obtain a comprehensive view of the kinetics of transcriptional induction, rather than what is provided by a conventional mRNA analyses. In Figure 10.3, a representative snapshot of the RNA-seq experiment showing the *Ccl5* and *Il-6* transcripts is reported. Analysis of nascent/chromatin-associated RNA confirmed the induction of *Il*-6 gene expression only during the primary stimulation and the sustained activation of *Ccl5* gene (Figure 10.3).

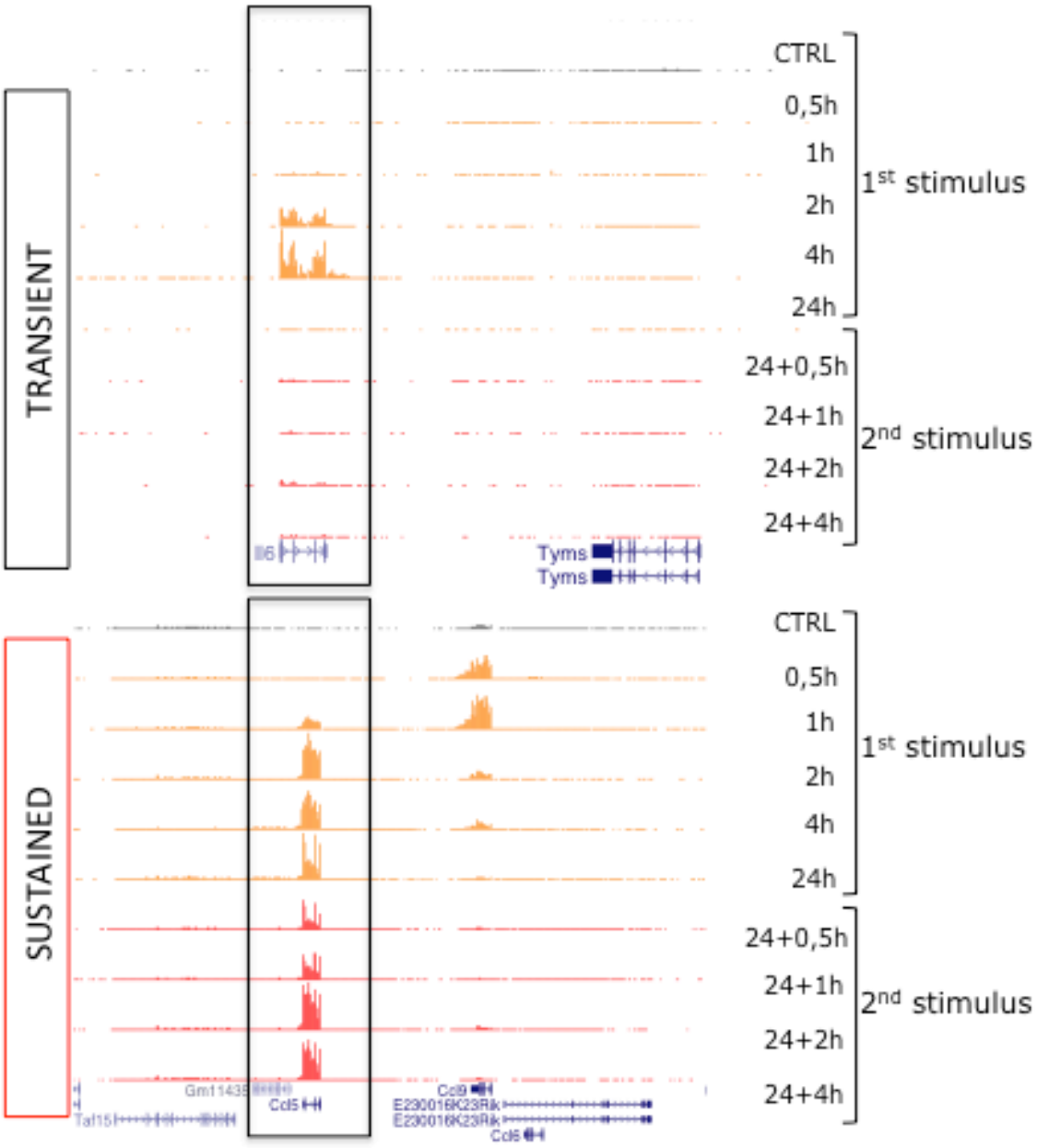



**Figure 10.3 Nascent RNA-seq in LPS-mediated macrophages activation and tolerance**. Chromatin associated RNA snapshots of the *Il-6 (*upper panel*)* and *Ccl5* gene locus (lower panel). First stimulation is depicted in yellow, the re-stimulation is depicted in red.

Moreover, with newly transcribed RNA profiling, we could confirm that sustained genes consist of both persistently transcribed genes (such as *Ccl5*) and genes being reactivated with a secondary stimulation, such as *Il-1a* (See Supplementary Figure 1). A heatmap of the chromatin associated RNA-seq profiling is reported in Figure 10.4.

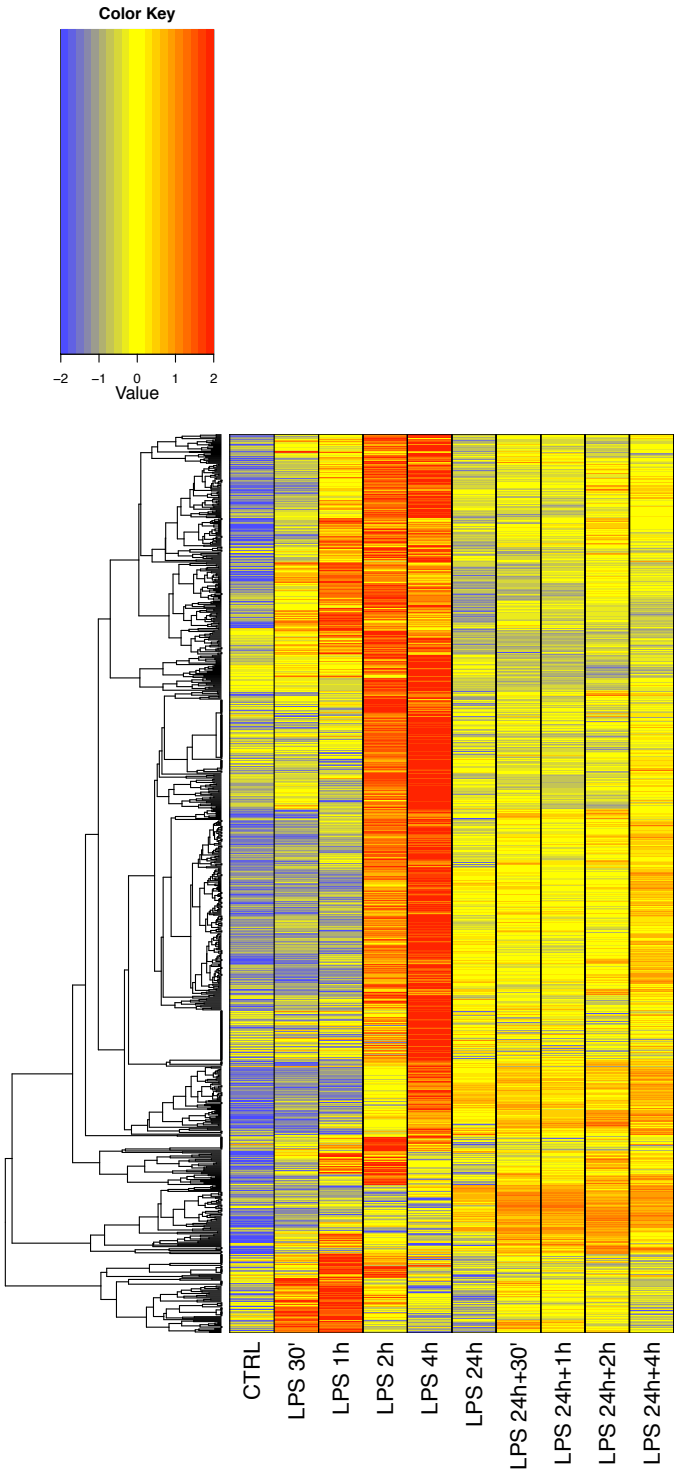



**Figure 10.4. A comprehensive view of nascent RNA-seq in LPS-mediated macrophages activation and tolerance.** High-throughput sequencing of chromatin associated RNA samples are reported in the heatmap. Each row corresponds to a single time point of the described experimental setting. The color code indicates from the lowest (blue) to the highest (Gazzaniga et al.) expressed gene. Differentially expressed genes were clustered according to their FPKM values. After log2-transformation, the values for each gene were converted to z-scores and genes were then hierarchically clustered.

A large number of genes was found to be mainly up-regulated upon 4 hours of LPS and the vast majority of those genes were then turned off, such as in the case of *Il-6,* and consistently with previously reported data (Foster et al., 2007). We then divided chromatin associated RNAs into twelve clusters based on their behaviors along the time course. The defined clusters were grouped in two subsets, according to their gene expression level at 24 hours of LPS treatment. We considered as sustained those genes that maintained at least 25% of the maximal level of transcription recorded during the primary stimulation. We called them *clusters with sustained transcriptional activity* (Figure 10.5).

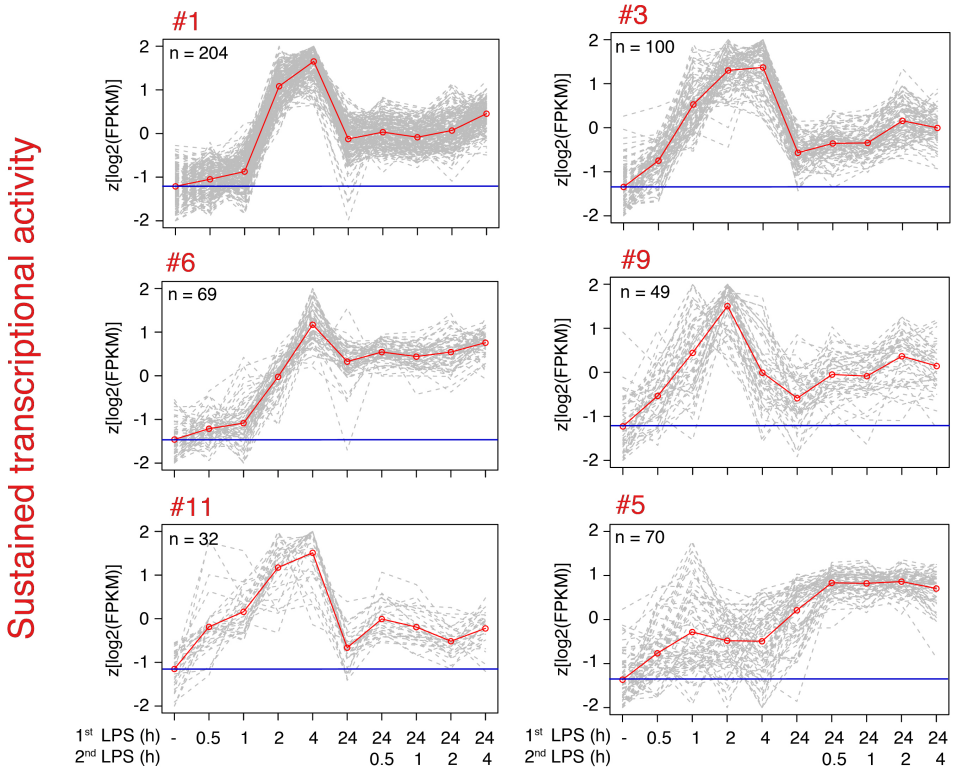



**Figure 10.5 Sustained transcriptional activity clusters.** Sustained gene clusters are shown. Genes are clustered according to their expression level calculated as log2FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped. Blue bar indicates the level of expression at point zero, took as reference. Sustained clusters have at least the 25% of the maximal level of transcription recorded during the primary stimulation. Clusters are numbered according to the amount of genes they include. Each grey line reproduce the kinetic of a single gene, while the red line stand for the median behavior of all the genes.

Genes displaying a level of expression lower than 25% compared to the maximum level at the primary stimulation were grouped in *clusters with transient transcriptional activity* (Figure 10.6)

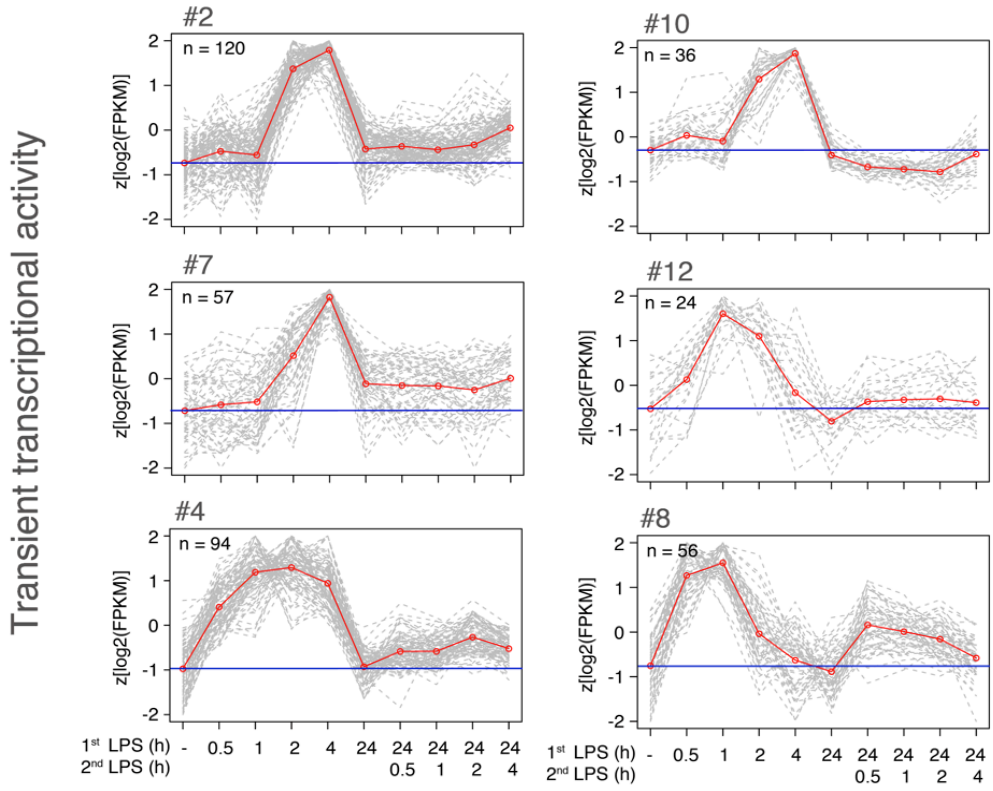



**Figure 10.6 Transient transcriptional activity clusters.** Transient gene clusters are shown. Genes are clustered according to their expression level expressed as log2 FPKM (Fragments Per Kilobase Of Exon Per Million Fragments Mapped). Blue bar indicates the level of expression at point zero, took as reference. Transient clusters have less than the 25% of the maximal level of transcription recorded during the primary stimulation Clusters are numbered according to the amount of genes they include. Each grey line reproduce the kinetic of a single gene, while the red line stand for the median behavior of all the genes.

Clusters are numbered according to the amount of genes they include. Only upregulated genes have been taken into account. Clusters#1 and #2 are the biggest, they include respectively 204 and 120, while clusters #12 include only 24 genes. Each grey line reproduces the kinetic of a single gene, while the red line stand for the median behavior of all the genes. Genes belonging to each cluster are listed in the Supplementary Table 1. In conclusion, cluster analysis of nascent RNA-seq datasets defined two different behaviors

of gene expression, sustained and transient transcriptional activity. Six different clusters were grouped in the big family of sustained transcriptional activity, while other six in the transient ones. We next analyzed properties and potential mechanism underlying the differential gene expression program for all the defined clusters.

## 10.2 Chromatin modifications are not correlated with loss of transcriptional activity at tolerized genes

Since it has been previously shown that chromatin modifications play an important role in selective gene expression in endotoxin tolerance (Foster et al., 2007) (Foster and Medzhitov, 2009) (Smale, 2010), we performed ChIP-seq experiments to differentially characterize gene-specific chromatin modifications associated with transient or sustained gene regulation. We characterized *cis*-regulatory regions that are differentially active in unperturbed, LPS-stimulated and LPS-tolerized BMDMs. Figure 10.7 reports a snapshot of *Il-27* and *Ccl5* promoter and enhancer regions.

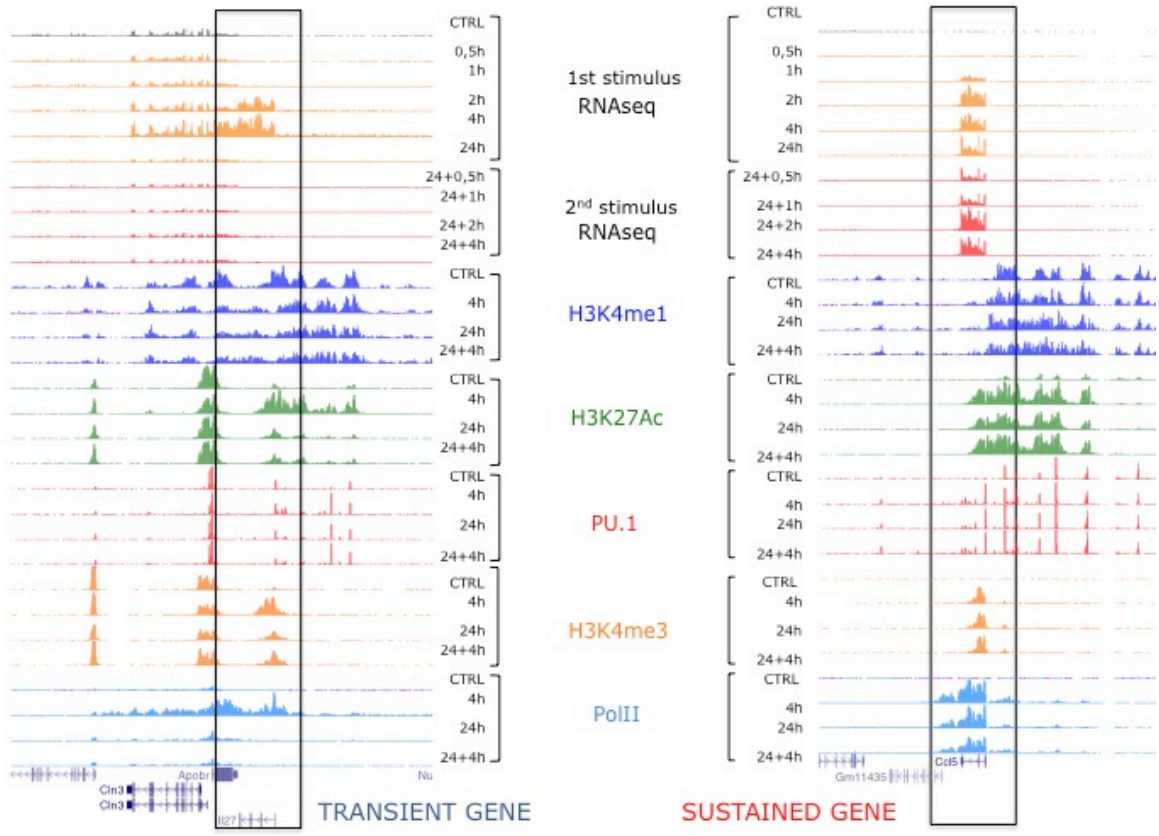



**Figure 10.7 Regulatory regions characterization.** *Il-27*/transient gene (left panel) and Ccl5/sustained gene (right panel) genomic regions are shown. Starting from the upper section of both transient and sustained panel, RNA-seq profiling, H3K4me1, H3K27Ac, PU.1, H3K4me3 and PolII occupancy are represented.

ChIP experiments for Histone H3 -Lysine 4 trimethylation (H3K4me3) and –Lysine 27 Acetylation (H3K27Ac) were performed along with Polymerase II (PolII) occupancy in order to identify active promoters, while Histone H3 -Lysine 4 monomethylation (H3K4me1) and H3K27Ac along with PU.1 transcription factor occupancy was used to map active enhancers, as previously described (Cheng and Zhu, 2011) (Ghisletti et al., 2010b). Figure 10.7 shows the genomic region surrounding *Il-27*. This region is positively marked by acetylation of H3K27 only upon 4 hours of LPS treatment, while losing the activation state upon 24 hours and 24+4 hours. In agreement with the transcriptional activity of the PolII in *Ccl5 locus,* H3K27Ac is gained upon treatment and remain present up to 24 hours and even higher upon re-stimulation (Figure 10.7). Both *Il-27* and *Ccl5* gained H3K4me3 only upon stimulation (Figure 10.7). Indeed, these results are in

agreement with previously reported data showing that late primary response genes (such as *Ccl5* and *Ccl2*) and secondary response genes (such as *Nos2*, *Il-12b*, and *Il-6*) activation require chromatin remodeling events (Ramirez-Carrozzi et al., 2006) (Hargreaves et al., 2009) (Ramirez-Carrozzi et al., 2009). Interestingly, regardless of their transcriptional activity (showed by total Pol II occupancy, light blue in Figure 10.7) both *Ccl5* and *Il-27* genes maintained high level of H3K4me3 in the late phase of the response. Genome wide analysis confirmed that, consistently with RNA-seq data, genomic regions associated with transient genes were mainly characterized by transient acetylation of H3K27, while sustained genes were mainly associated with persistent acetylation both at distal (Figure 10.8 A) and proximal (Figure 10.8 B) regulatory regions. Figure 10.8 reported the H3K27Ac analysis for the two largest clusters, sustained cluster#1 and transient cluster#2. Analysis of all the other clusters is reported in Supplementary Figure 3 (proximal regions) and 4 (distal regions).

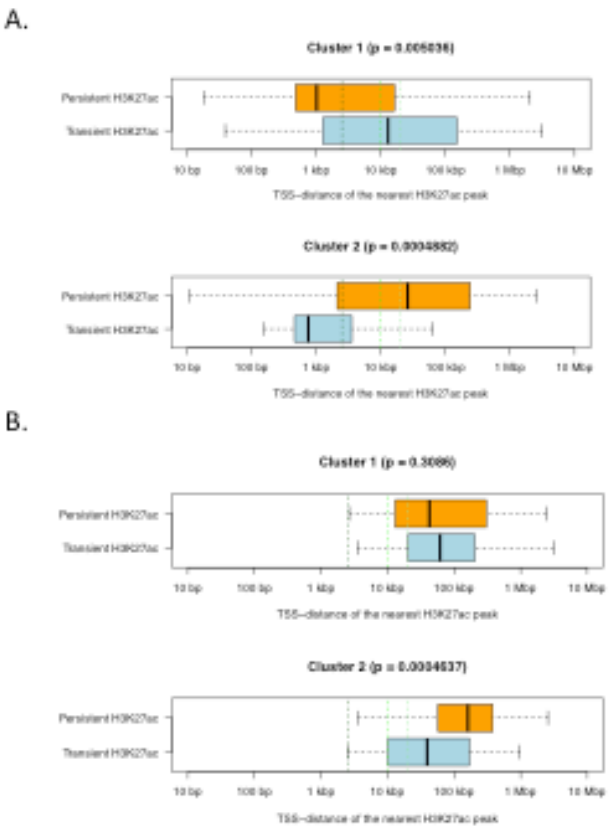



**Figure 10.8 Active chromatin modification levels at proximal and distal regulatory regions near transient and sustained genes**. **A** Proximal H3K27Acetylation level near transient and sustained gene (analysis of cluster 1 and 2 are shown) on the left. **B** Distal to TSS-H3K27 Acetylation level. Each gene was assigned to the nearest persistent or transient H3K27 acetylation peak. Transient genes are significantly closer to transient acetylation than sustained ones while the contrary is observed for the sustained ones (analysis of cluster 1 and 2 are shown).

A comprehensive analysis of the histone modifications characterizing sustained and transient gene promoters is reported in Figure 10.9. We used a machine-learning approach to identify the prevalent combinatorial patterns of histone modifications describing the complexity of chromatin profiles observed in sustained and transient promoters (Zhou et al.). We used antibodies for histone H3 lysine 4 tri-methylation (H3K4me3), a modification associated with promoters; H3K4me1, preferentially associated with enhancers; lysine 27 acetylation H3K27ac and pan acetylation of H4, associated with active regulatory regions. H3K27me3 and H3K9me3, associated with repressed regions.

We performed the analysis with both H3K27Ac (not shown) and total H4 Acetylation, in combination with H3K4me3 and H3K9me3. We obtained six different states, describing strong or weak promoters. At genome wide level, promoters of both transient and sustained genes maintained high levels of H3K4me3 regardless of their transcriptional activity, and not any histone modification associated with transcriptional repression such as H3K27me3 (data not shown) or H3K9me3 was gained at transient genes promoters, indicating that these modifications did not correlate with loss of activity at these genes (Figure 10.9 and Figure 10.10).

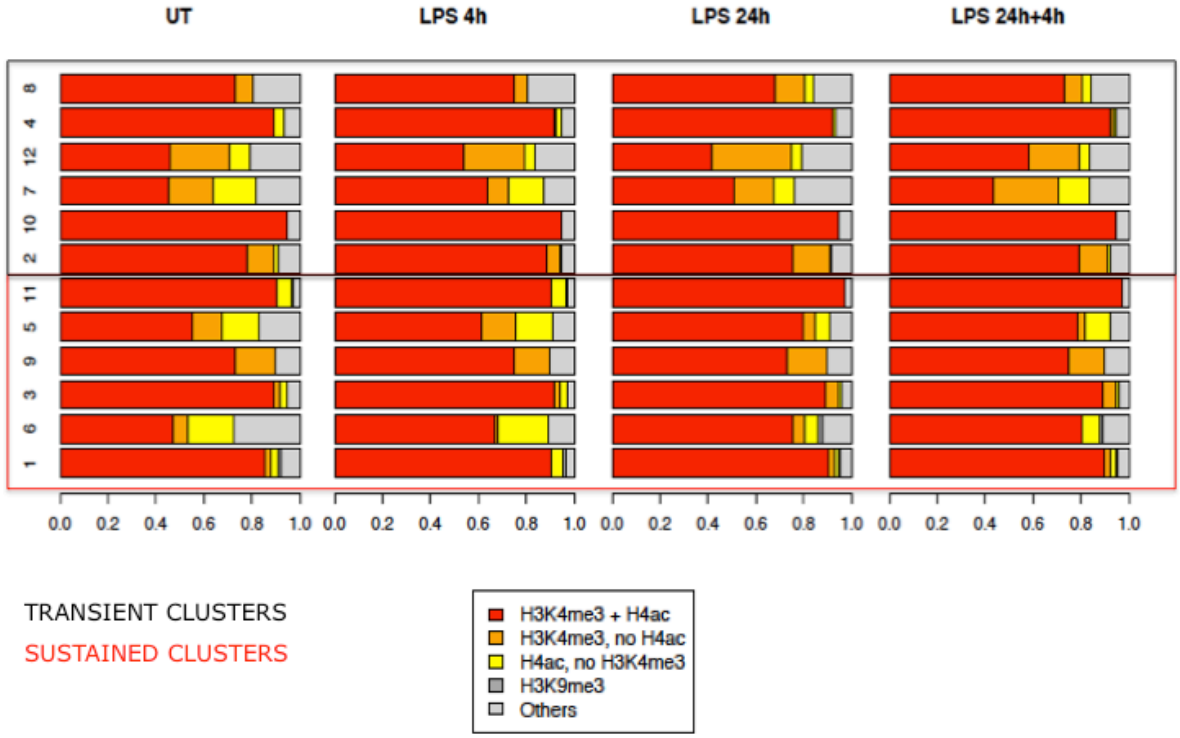



**Figure 10.9 Chromatin mark combinations associated with each cluster**. Each row shows the specific combination of marks associated with each chromatin state of each cluster. H3K4me3, H4Ac and H3K9me3 are considered in combination among each other. Genomic regions from -500 to +250 bp from the TSS have been considered.

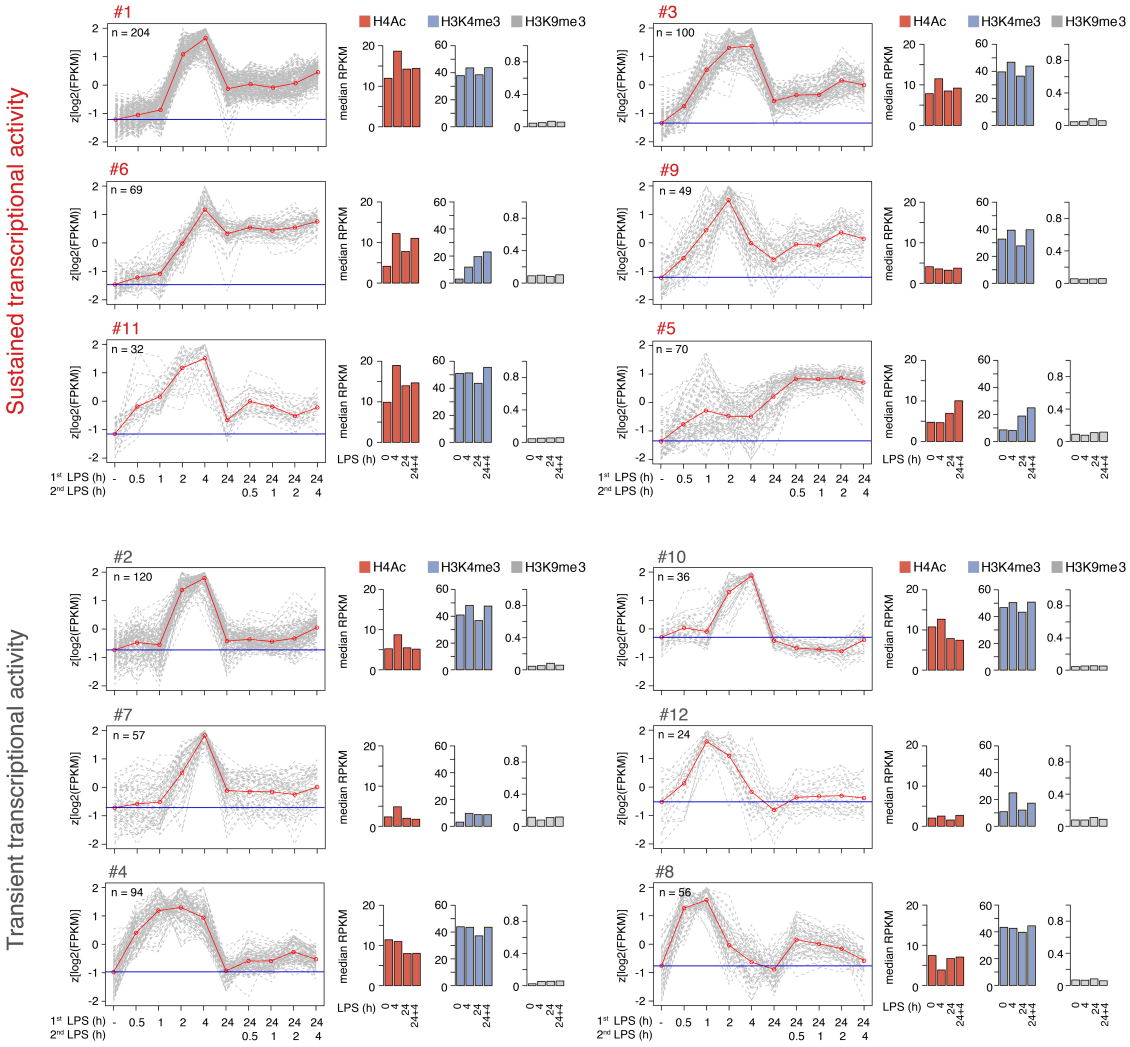



**Figure 10.10 Chromatin marks at promoters of genes belonging to transient and sustained clusters.** H3K4me3, H4 Acetylation, and the H3K9me3 signal at promoters of genes has been calculated as median FPKM for each cluster.

To confirm these findings, we next used an opposite approach, grouping all the clusters by their promoter chromatin properties. PCA analysis, based on the two component H3K4me3 and H4 Acetylation levels, did not clearly discriminate clusters according to their expression behaviors (Figure 10.11).

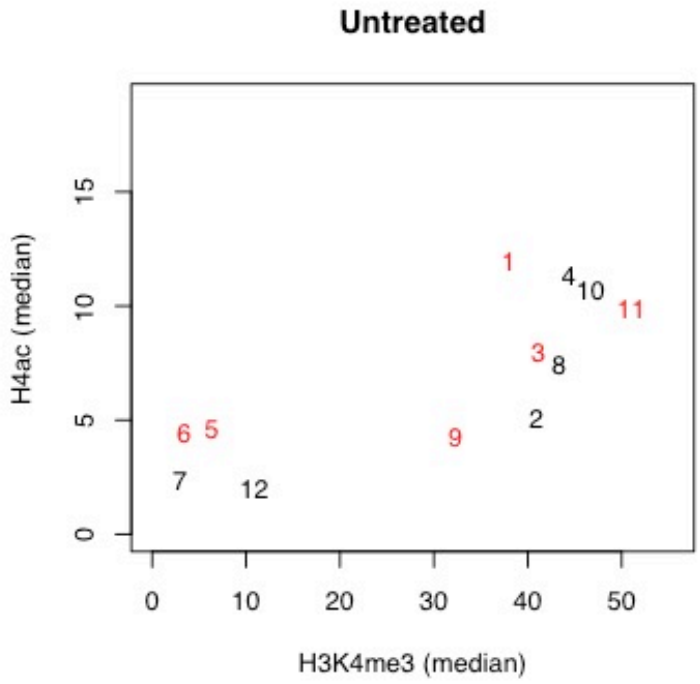



**Figure 10.11 Positive epigenetic marks based PCA analysis of sustained and transient clusters.** Each cluster is represented with its number, red numbers correspond to sustained clusters, and black numbers correspond to transient clusters.

The same result has been obtained by drawing a trajectory for the activatory epigenetic mark at TSS of each cluster. Whether the chromatin modification is involved in the regulation of a specific gene expression program, a "walking" of the modification level along the time course may be envisioned. Considering each time point, we calculated the median FPKM of H3K4me3 and H4 Acetylation at TSS of three representative clusters. While a difference in H4Ac trajectory was appreciable, likely due to the PolII transcriptional activity, we could not find any significant difference in H3K4me3 level among sustained and transient clusters (Figure 10.12).

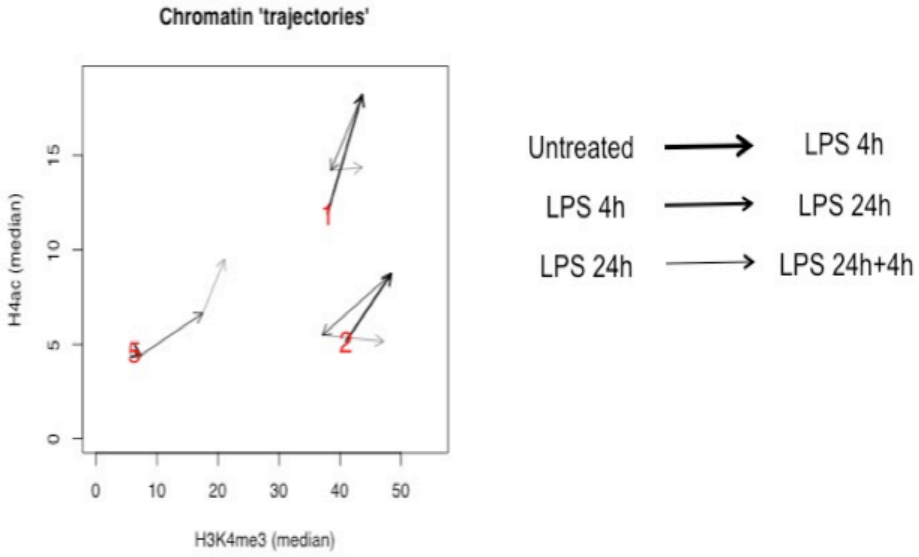



**Figure 10.12 Chromatin trajectories at TSS of transient and sustained clusters.** Median H3K4me3 level (calculated as FPKM) is plotted versus median level of H4Ac for clusters #1, #2, #5. Each arrow represents the "walking" from a time point to the subsequent, the thickest arrow from UT to 4h of LPS, the middle one from 4h to 24h and the thinnest from 24h to 24+4h. The three clusters taken into consideration are reported in the lower left corner of the figure.

Moreover, despite having a completely different expression profiling, cluster #1/sustained and cluster #2/transient showed almost the same level of H3K4me3, either in the unperturbed state or upon 24 hours of LPS treatment and upon re-stimulation. All together, these results suggested a chromatin independent mechanism underlying the gene expression program of transient and sustained genes.

## 10.3 Transient and sustained genes clusters have different functional features

We have demonstrated so far that the transcriptional activity of sustained and transient clusters cannot be explained by differences in epigenetic modifications. ChIP-seq data collected for both sustained and transient clusters did not justify such a big difference in their transcriptional behavior upon sustained treatment. To investigate the mechanisms underlying these transcriptional changes, we grouped the clusters previously defined by RNA-seq analysis according to their behaviors in terms of transcriptional activation. As a second step, motif discovery analysis was performed on promoters of genes belonging to these subsets in order to identify the transcription factors involved in their transcriptional regulation. Finally, gene ontology analysis was performed in order to identify different functional classes in the two subsets of genes.

### 10.3.1 Clusters characterization

First of all, according to their behavior, we assigned to all the clusters four indices describing four properties (Figure 10.13).

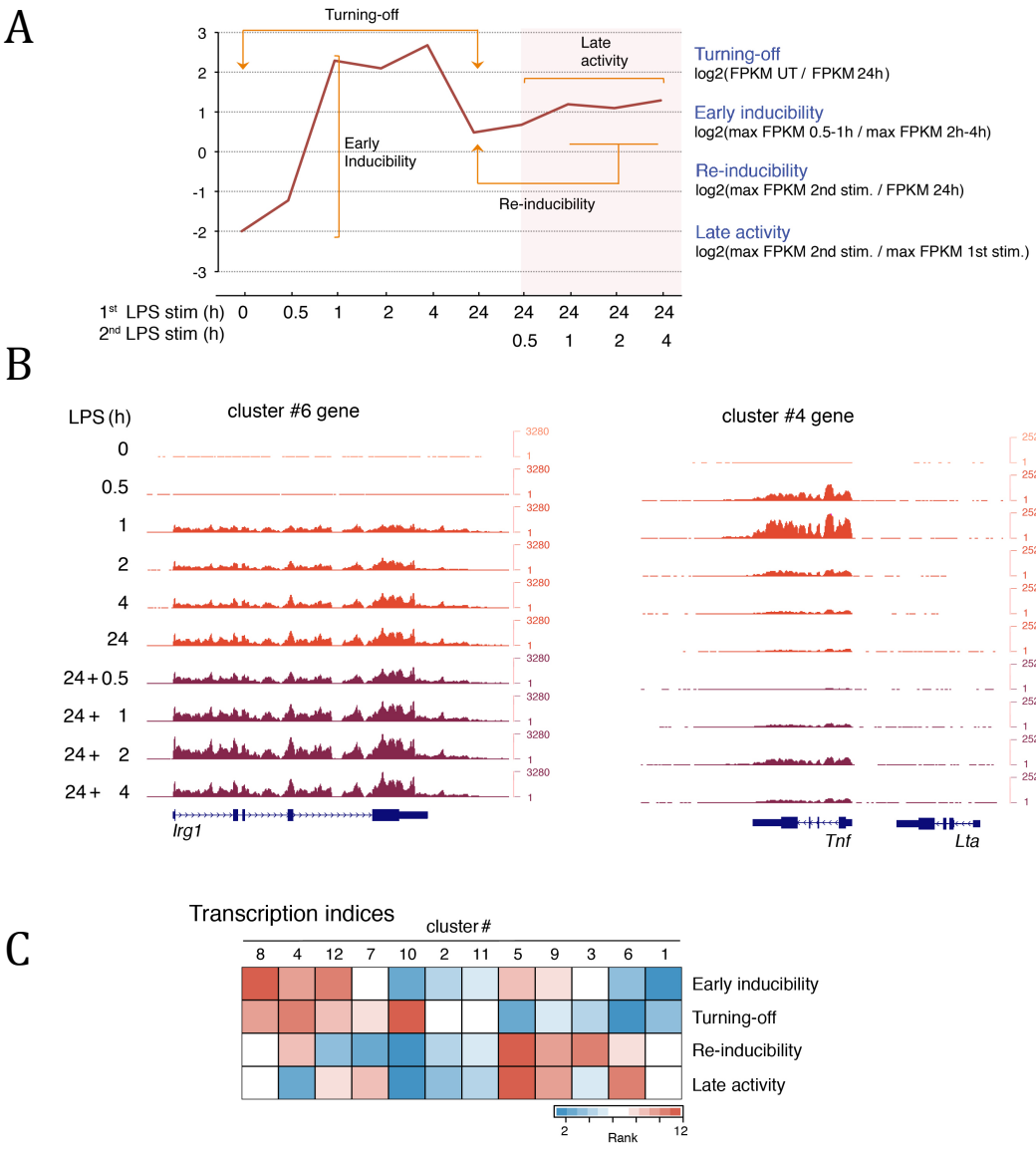



**Figure 10.13 Clusters characterization.** A scheme of the calculated index is reported in panel A. Two representative genomic regions showing *Irg1* (sustained) and *Tnf* (transient) genes are reported in B. A scheme of all the groups of genes clustered according to their rank is shown in C.

*Turn off index* formally described the persistence or transiency of the clusters. When gene expression at 24 hours of LPS (calculated as FPKM) was higher than gene expression at point zero the Turn off index was negative (clusters #1, #3, #5, #6, #9, #11), closed to zero when gene expression at 24 hours was similar to the one at unperturbed state and positive when lower than UT (clusters #2, #4, #7, #8, #10, #12). Transient and sustained clusters were clearly discriminated by Turn off index. Figure 10.13 B shows two representative

RNA-seq snapshots, *Irg1* belonging to cluster #6 (as example of sustained activity) has a negative turned off index, while *Tnf (*belonging to cluster *#4,* as example of transient gene) has a positive turned off index (Figure 10.13). For each cluster an index for their kinetic was then calculated (*early inducibility index*). Comparing maximum level of expression at 30 minutes-1 hours with the maximum level reached at 2-4 hours we could distinguish early and late induced genes. According to the definition of this index (log2 FPKM 30'-1 hours/FPKM 2-4 hours), sustained clusters were composed of late inducible genes, while transient clusters of both early (clusters #4, #12, #8) and late inducible genes (clusters #2, #7, #10) (Figure 10.13). We next calculated the *re-inducibility index,* comparing gene expression level at every re-stimulation time point with the one at 24 hours of LPS treatment (log2FPKM $2^{nd}$ stimulation/FPKM 24h). Transient clusters had an index value closer to zero than sustained clusters, formally proving the inability of these genes to be re-induced upon restimulation (Figure 10.13). Lastly, we calculated a *late activity index.* Comparing gene expression levels at the second stimulation with those at the first stimulation levels (log2 maxFPKM $2^{nd}$ stimulation/max FPKM $1^{st}$ stimulation), we could differentiate clusters being mostly expressed in the late phase than in the primary one (Figure 10.13).

## 10.3.2 Motif discovery analysis

We next characterized the promoters of transient and sustained clusters of genes. First of all, we scanned promoter regions using the PSCAN algorithm in order to find over-represented transcription factor binding site motifs. Transient and sustained gene clusters were well discriminated in their binding sites composition (Figure 10.14). PWM analysis of three representative clusters is shown in Figure 10.14. Among the others, transient gene clusters showed high enrichment in EGR- and NF-kB Position weight matrices (PWMs). Specifically, early inducible transient genes (cluster #4) were mainly enriched in NF-kB PWMs, while EGR- transcription factors PWMs were mainly over-represented in late

inducible transient genes promoters. Sustained clusters displayed instead a high statistically significant enrichment in binding sites for IRF family transcription factors (See Figure 10.14), as shown for the case of cluster #1 (Figure 10.14). Moreover, in agreement with already published data (Bhatt et al., 2012), more or less 80% of transiently expressed gene promoters was associated with CpG islands, while only 50% of sustained genes promoters contained CpG islands (expected CpG island content is 65%, as obtained by overlapping all the transcription start sites of RefSeq genes with all the identified CpGi (Illingworth, 2010 #184) (Table 1).

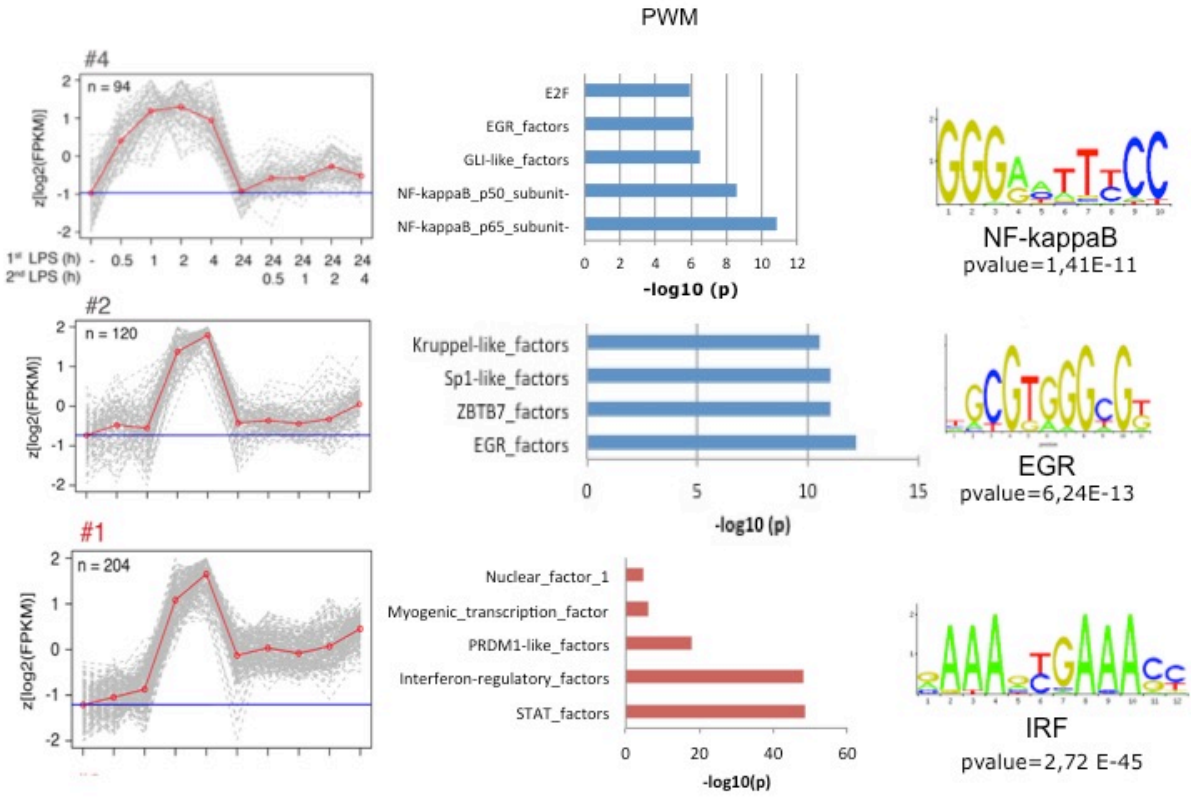



**Figure 10.14 PWM analysis of the transient and sustained clusters.** Position weight matrix analysis is reported for three representative clusters. Overrepresentation of transcription factors binding sites is ordered according to statistics (p-value). Position-specific sequence logo showing the binding sites for NF-kB, EGR and IRF are reported. The relative frequency of each nucleotide is shown as height of each letter.

| Cluster | Overlapping | Not Overlapping | Obs. fraction | Exp. Fraction | Enriched | Chisq. pvalue |
|---|---|---|---|---|---|---|
| 1 | 117 | 87 | 0,57 | 0,65 | 0 | 0,02 |
| 2 | 98 | 22 | 0,82 | 0,65 | 1 | 0,00 |
| 3 | 69 | 31 | 0,69 | 0,65 | 1 | 0,41 |
| 4 | 71 | 23 | 0,76 | 0,65 | 1 | 0,03 |
| 5 | 34 | 36 | 0,49 | 0,65 | 0 | 0,00 |
| 6 | 24 | 45 | 0,35 | 0,65 | 0 | 0,00 |
| 7 | 30 | 27 | 0,53 | 0,65 | 0 | 0,05 |
| 8 | 42 | 14 | 0,75 | 0,65 | 1 | 0,12 |
| 9 | 35 | 14 | 0,71 | 0,65 | 1 | 0,35 |
| 10 | 32 | 4 | 0,89 | 0,65 | 1 | 0,00 |
| 11 | 24 | 8 | 0,75 | 0,65 | 1 | 0,24 |
| 12 | 17 | 7 | 0,71 | 0,65 | 1 | 0,56 |

**Table 1 CpG islands overlapping with the promoters of the genes in each cluster.** CpG islands content located in promoters of genes for each cluster has been calculated. The number of genes overlapping with CpG islands and the observed fraction (Obs. Fraction) is reported along with expected fraction (Exp. Fraction). A binary description has been associated to enriched (Scott R.McKercher) versus not enriched (0) clusters and a p-value for each of them has been calculated with Chi-Square test.

### 10.3.3 Gene ontology analysis

Since the two different clusters of genes are regulated by different transcription factors, we analyzed whether transient and sustained genes belong to different functional categories. Using the DAVID algorithm, we found that generally, both clusters of genes are enriched in immune response mediators but sustained genes are mainly involved in viral immune responses. Gene ontology analysis for clusters #4 and #1 is reported in Figure 10.15.

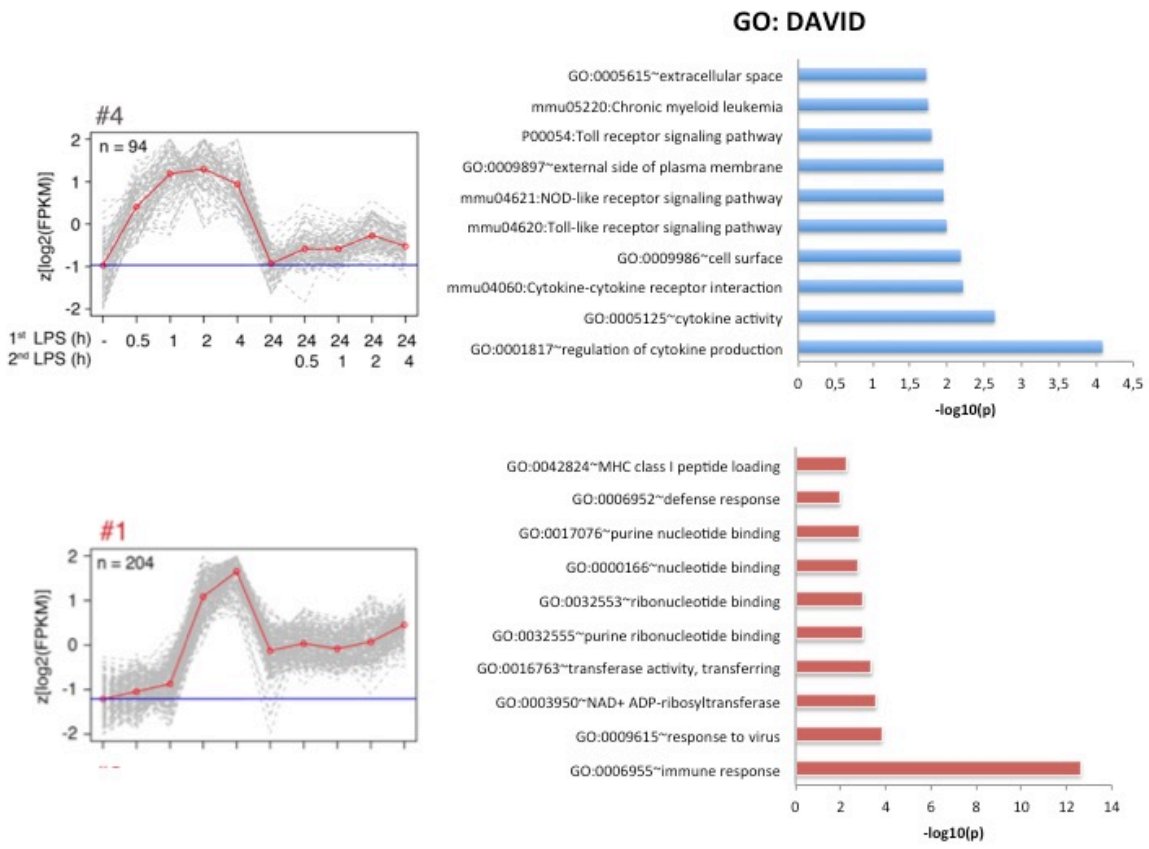



**Figure 10.15 Gene Ontology analysis on the transient and sustained clusters.** The two histograms represent the negative logarithm of the p-value evaluating the significance of gene ontology terms for genes belonging to Cluster #4 (transient) and Cluster #1 (sustained). The top 10 annotation clusters are listed as derived from the DAVID bioinformatics tool.

Since sustained genes resulted to be enriched in IRF transcription factors binding sites and involved in the anti-viral response, we crossed these data with previous reports on genes down-regulated in *Irf3*$^{-/-}$ (Table 2) and in IFN-β-response-impaired BMDM (Table 3) (Christine S, 2001; Ogawa, 2005). We found a significant overlap with sustained gene clusters #1, #6 and #11 and IRF3 dependent genes, and a significant overlap with sustained genes clusters #1, #3 and #11 and IFN-β dependent genes, thus suggesting the dependence of these clusters on the IFN-β -regulated feed-forward loop.

| Cluster | Overlapping | Not Overlapping | Obs. fraction | Exp. Fraction | Enriched | Chisq. pvalue |
|---|---|---|---|---|---|---|
| 1 | 58 | 146 | 0,28 | 0,14 | 1 | 7E-09 |
| 2 | 15 | 105 | 0,13 | 0,14 | 0 | 6E-01 |
| 3 | 12 | 88 | 0,12 | 0,14 | 0 | 5E-01 |
| 4 | 12 | 82 | 0,13 | 0,14 | 0 | 7E-01 |
| 5 | 5 | 65 | 0,07 | 0,14 | 0 | 9E-02 |
| 6 | 11 | 58 | 0,16 | 0,14 | 1 | 7E-01 |
| 7 | 5 | 52 | 0,09 | 0,14 | 0 | 2E-01 |
| 8 | 2 | 54 | 0,04 | 0,14 | 0 | 2E-02 |
| 9 | 2 | 47 | 0,04 | 0,14 | 0 | 4E-02 |
| 10 | 2 | 34 | 0,06 | 0,14 | 0 | 1E-01 |
| 11 | 6 | 26 | 0,19 | 0,14 | 1 | 5E-01 |
| 12 | 0 | 24 | 0,00 | 0,14 | 0 | 5E-02 |

**Table 2 Irf3 dependent genes overlapping with transient and sustained clusters.** The list of genes downregulated in *Irf3* KO mice derived macrophages has been crossed with genes belonging to each cluster. The number of genes overlapping with *Irf3* dependent genes and the observed fraction (Obs. Fraction) is reported along with expected fraction (Exp. Fraction). A binary description has been associated to enriched (Scott R.McKercher) versus not enriched (0) clusters and a p-value for each of them has been calculated with Chi-Square test (Cheng et al., 2011) (Ogawa et al., 2005)

| Cluster | Overlapping | Not Overlapping | Obs. fraction | Exp. Fraction | Enriched | Chisq. pvalue |
|---|---|---|---|---|---|---|
| 1 | 101 | 103 | 0,50 | 0,26 | 1 | 4,7E-15 |
| 2 | 38 | 82 | 0,32 | 0,26 | 1 | 1,3E-01 |
| 3 | 28 | 72 | 0,28 | 0,26 | 1 | 5,8E-01 |
| 4 | 21 | 73 | 0,22 | 0,26 | 0 | 4,7E-01 |
| 5 | 5 | 65 | 0,07 | 0,26 | 0 | 4,1E-04 |
| 6 | 13 | 56 | 0,19 | 0,26 | 0 | 2,0E-01 |
| 7 | 7 | 50 | 0,12 | 0,26 | 0 | 2,1E-02 |
| 8 | 1 | 55 | 0,02 | 0,26 | 0 | 4,5E-05 |
| 9 | 1 | 48 | 0,02 | 0,26 | 0 | 1,6E-04 |
| 10 | 3 | 33 | 0,08 | 0,26 | 0 | 1,8E-02 |
| 11 | 14 | 18 | 0,44 | 0,26 | 1 | 1,8E-02 |
| 12 | 1 | 23 | 0,04 | 0,26 | 0 | 1,6E-02 |

**Table 3 IFNb dependent genes overlapping with transient and sustained clusters.** The list of genes downregulated in IFN-β-impaired response derived macrophages has been crossed with genes belonging to each cluster. The number of genes overlapping with *IFNb* dependent genes and the observed fraction (Obs. Fraction) is reported along with expected fraction (Exp. Fraction). A binary description has been associated to enriched (Scott R.McKercher) versus not enriched (0) clusters and a p-value for each of them has been calculated with Chi-Square test (Cheng et al., 2011) (Ogawa et al., 2005)

In summary, as reported in Figure 10.16, we formally described all the clusters according to their turning off property, kinetics and re-inducibility. We then demonstrated that promoters of transient and sustained genes were well discriminated in their binding sites composition. Transient genes promoters were mainly enriched in EGR, and NF-kB binding sites, as well as they were enriched in sequences containing CpG islands. On the contrary, sustained gene clusters displayed a high statistically significant enrichment in binding sites for IRF family of transcription factors and their promoters were depleted in CpG island content. Moreover, gene ontology analysis suggested a specific function for sustained genes in mediating anti-viral response and the overlapping between sustained genes and IFNb dependent genes, confirmed the dependence on the IFNb-regulated feed-forward loop.

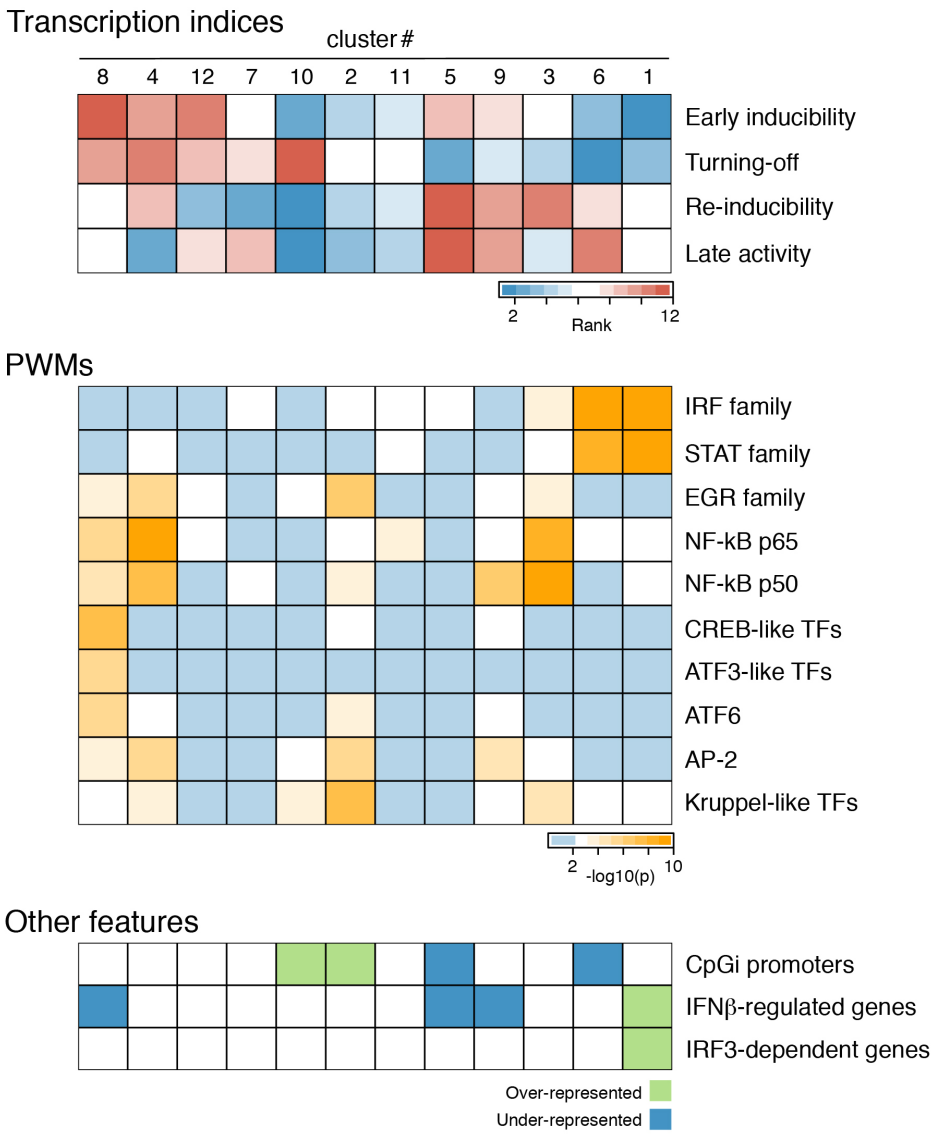



**Figure 10.16 Clusters features.** Transcription indices for each cluster of genes, Position Weight Matrix (PWM) and other features of their promoters are clustered according respectively to their rank, p-value and over-representation. Clusters are then manually ordered.

## 10.4 IRF7 controls sustained genes transcriptional expression

Since IRF transcription factors were found involved for sustained gene regulation, the expression levels of IRF transcription factors in stimulated and tolerized BMDMs were analyzed. As shown in Figure 10.17, chromatin associated RNA-seq data indicated that

among the expressed IRFs, only IRF7 was highly induced upon LPS treatment and maintained significant expression upon sustained stimulation. Q-PCR analysis from total RNA confirmed this result (Figure 10.17 lower panel), suggesting that IRF7 may have a key role in the transcriptional regulation of sustained genes.

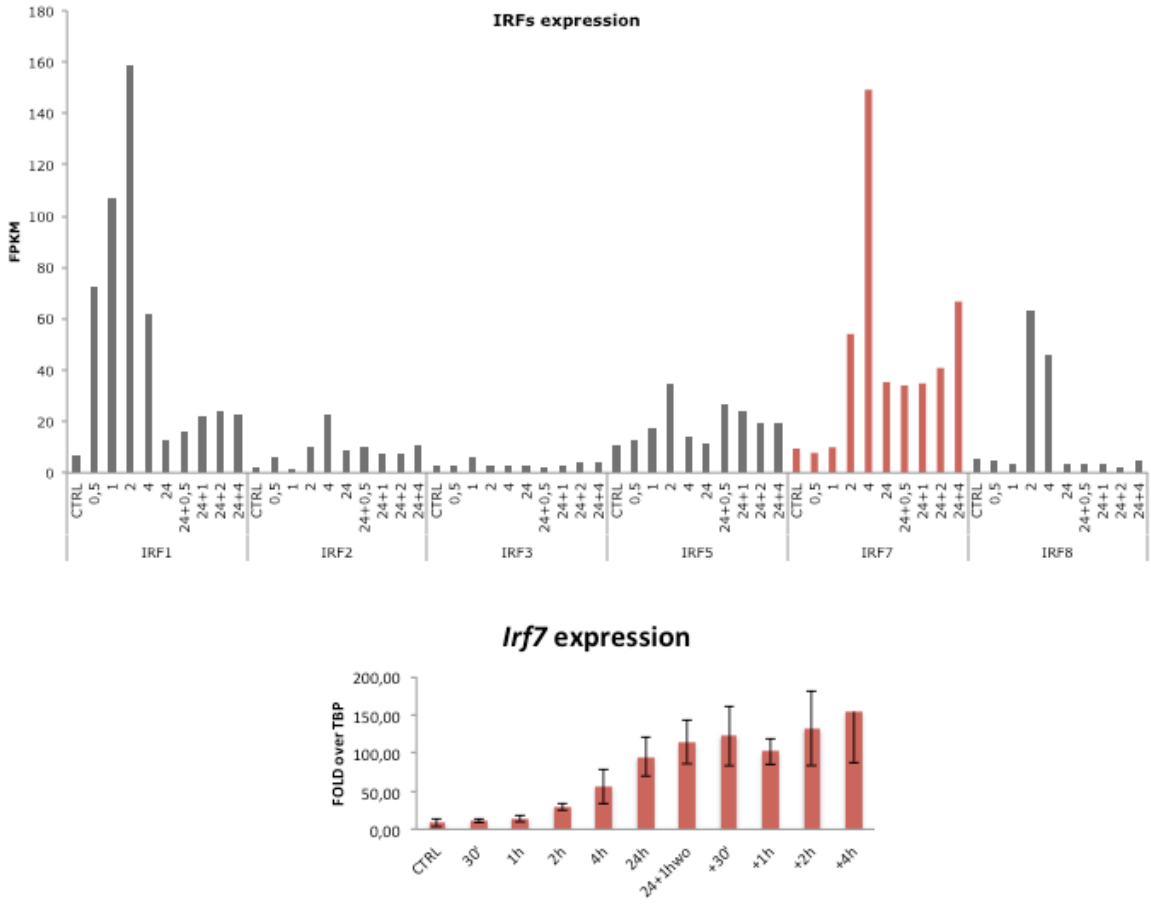



**Figure 10.17 IRFs expression. A.** IRF- family members expression in BMDMs. Chromatin associated RNAs for IRFs family members are reported. Expression has been calculate as FPKM and reported for each time point of the designated experimental setting. B. IRF7 mRNA expression. For each time point, mRNA expression was measured by Q-PCR and expressed as fold change relative to TBP (Tata Binding Protein).

Previous studies already showed that IRF7 is upregulated upon LPS stimulation, due to the autocrine loop created by IFN-β (Oganesyan et al., 2006) (Doyle et al., 2002) and a very recent publication reported a role for IRF7 in the transition from M1 to M2 phenotype in brain derived macrophages (Cohen et al., 2014). Considering the similarity between M2 macrophages and LPS tolerized macrophages, a potential role of IRF7 in the regulation of

sustained genes may be envisioned. A possible working model is that IRF7 may promote its own promoter activity, thus sustaining its own expression (Figure 10.18). Sustained *Irf7* expression may thus be sustained even in the absence of active signaling.

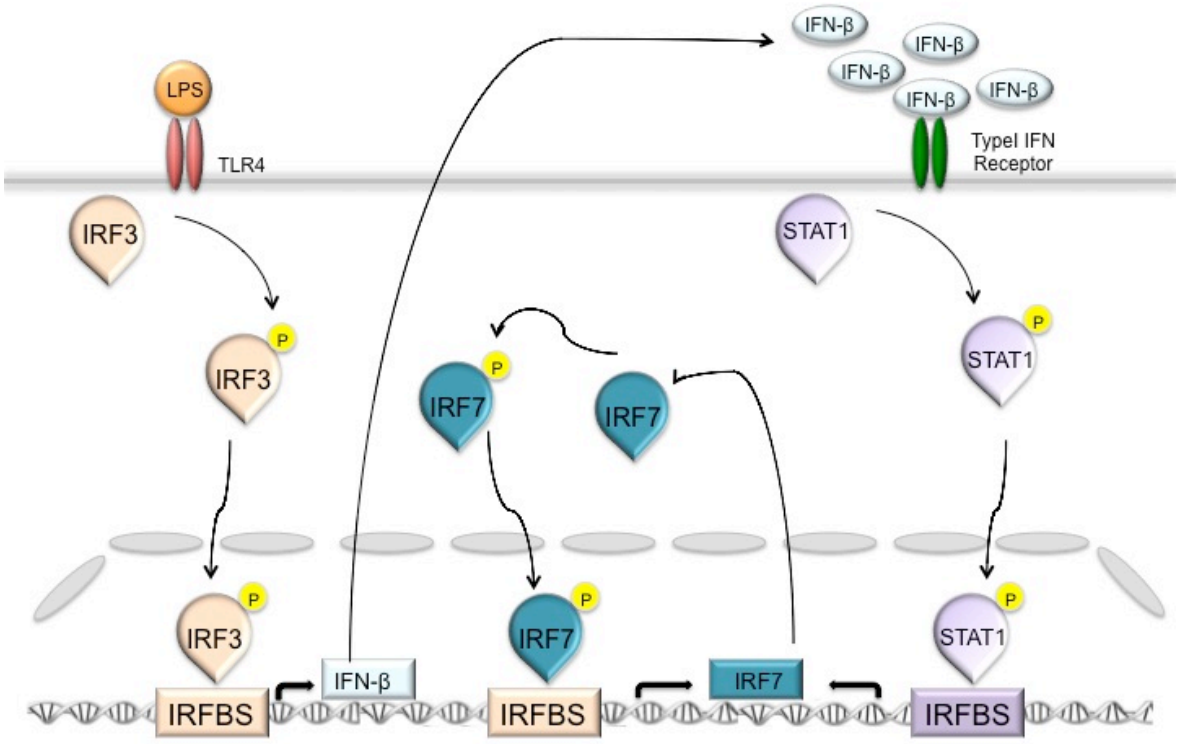



**Figure 10.18 IRF7 feed forward loop.** A scheme of the IRF7 feed forward loop activity is reported. In response to LPS, IFN-β is induced via a pathway dependent on the adapter TRIF, which controls the activation of the transcription factor IRF3 (29). IRF3 directly controls transcription of the *Ifn-β* gene, whose product is rapidly released to activate an autocrine and paracrine loop that is ultimately responsible for a secondary wave of gene induction that includes classic IFN-β–regulated genes (30). Therefore, Irf3-dependent genes include direct IRF3 targets (e.g., *Ccl5*) (30) and IFN-β–regulated genes such as *Irf7*. The latter are activated by a trimeric complex composed of Stat1, Stat2, and Irf9, which was initially indicated as IFN-stimulated gene factor 3 (*Isgf3*). Irf7 binds both its own promoters, thus leading to an autocatalytic control of its gene expression, and promoters of its direct target containing the specific trimeric matrix.

To validate this hypothesis we depleted *Irf7* by lentivirus-mediated shRNA delivery. We used lentiviral vectors expressing either *Irf7*-specific or control shRNAs (pLKO.1sh*Irf7* and pLKO.1shLuc, respectively). Depletion resulted in a ca. 80-90% reduction in *Irf7* mRNA level, as quantified by qPCR analysis of the transcript (Figure 10.19).

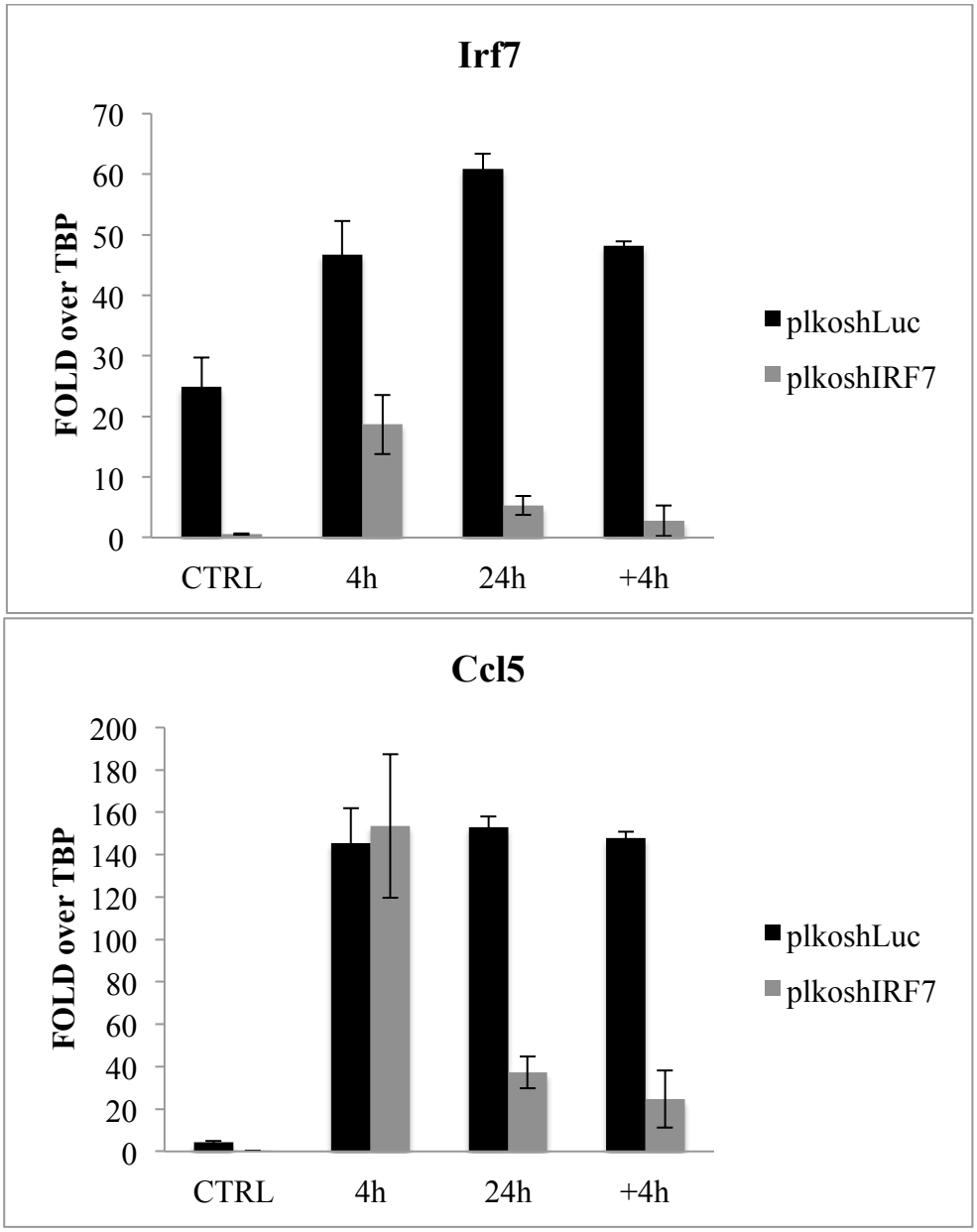



**Figure 10.19 Irf7 depletion affects** *Ccl5* expression in the late phase response. BMDM were infected either with lentiviruses expressing an shRNA anti *Irf7* (plkoshIRF7/red) or a scrambled sequence anti luciferase (plkoshLuc/blue). A. *Irf7* depletion. Irf7 knockdown is assessed by qPCR analysis. B. *Ccl5* expression. *Ccl5* expression has been verified for each of the three experiments in both plkoLuc and plkoIRF7 conditions. Both qPCR data are reported as fold change relative to TBP (Tata Binding Protein) and data are representative of three independent experiments. Error bar denotes the standard deviation of the mean.

To evaluate the impact of *Irf7* depletion at a genome wide level, total RNA-seq experiment was performed in untreated bone marrow derived macrophages, LPS-stimulated and LPS tolerized macrophages. A snapshot of four genomic regions is reported in Figure 10.20. The transcriptional activity of sustained genes was affected by *Irf7* depletion, as demonstrated by *Ccl5* and *Cxcl2* expression, respectively belonging to sustained cluster #1

and #cluster8. Despite that, some of the very late sustained genes (such as *Mmp9*) were not changed in their kinetics after Irf7 depletion. Most importantly, the effects of Irf7 removal were established only in the late phase of the response, while not affecting the expression of both sustained and transient genes at 4 hours of LPS. Figure 10.20 shows an example of a transient gene (*Sdc4),* whose transcriptional program was unperturbed by Irf7 depletion.

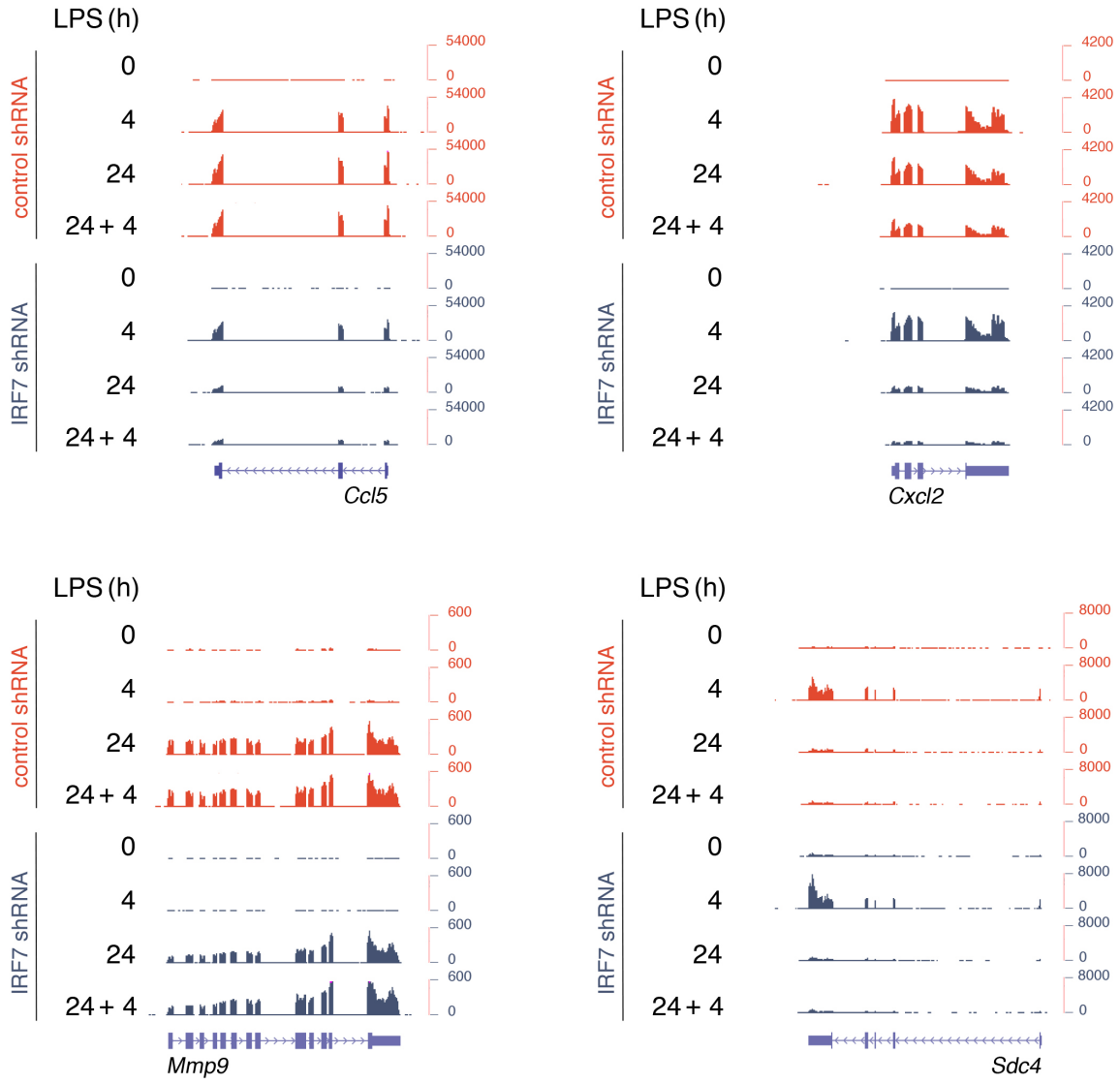



**Figure 10.20 Irf7 controls sustained transcriptional activity of sustained genes.** Total RNA-seq was performed in unperturbed, 4h, 24h and 24+4h of LPS stimulation. The distribution of RNA-seq reads at the *Ccl5, Cxcl2, Mmp9* and *Sdc4* locus is shown. Time points are indicated at the left and scale on the right. In red scramble shLuc, in blue sh*Irf7* transcripts are reported.

In Figure 10.21 the global quantification of Irf7 KD impact is reported. Genes belonging to sustained cluster #1 and transient cluster #2 showed different impact of Irf7 deficiency. We calculated the median expression of the transcripts in both shLUC and sh*Irf7* conditions. The log2ratio of FPKM at 4 hours and 24 hours of LPS treatment versus FPKM at unperturbed state is plotted in the presented box plot (Figure 10.21). As expected, only sh*Irf7* median expression of sustained cluster #1was statistically significantly reduced compare to shLuc (Figure 10.21).

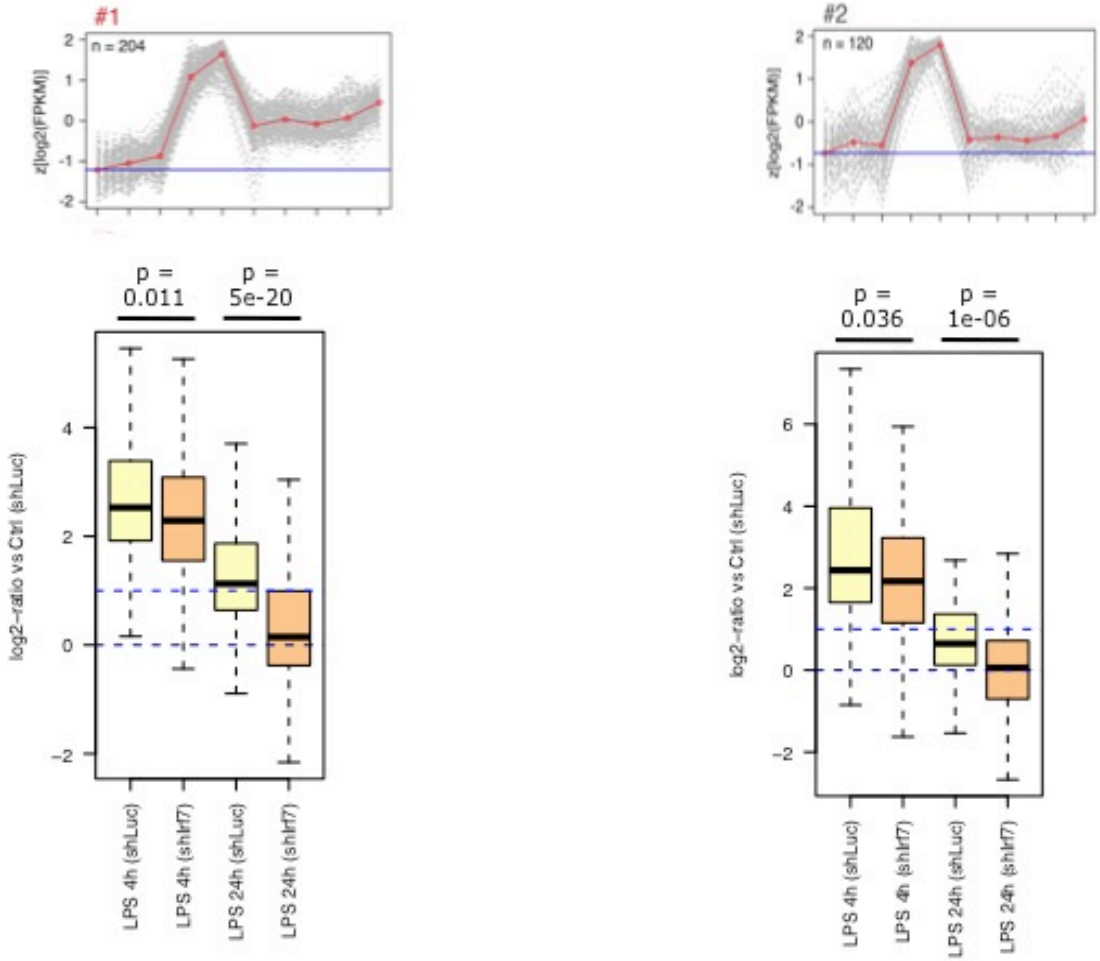



**Figure 10.21 IRF7 KD decreases cluster #1 genes transcription upon sustained stimulation.** The box plots indicate the log2 ratio between the designated time point and conditions and control expression.

All together these results suggested a role of IRF7 in mediating the long lasting transcriptional activity of sustained genes, while not affecting the acute phase response.

## 10.5 Transient genes are controlled by a multifactorial mechanism

As previously reported in Figure 10.14 and 10.16, Position Weight Matrix analysis (PSCAN) indicated that transient genes were mainly regulated by EGR- and NF-kB transcription factors, downstream effectors of the TLR4 signaling pathway. Biochemical analysis of the key players of this signal cascade revealed an almost complete exhaustion of the pathway after sustained LPS treatment (Figure 10.22).

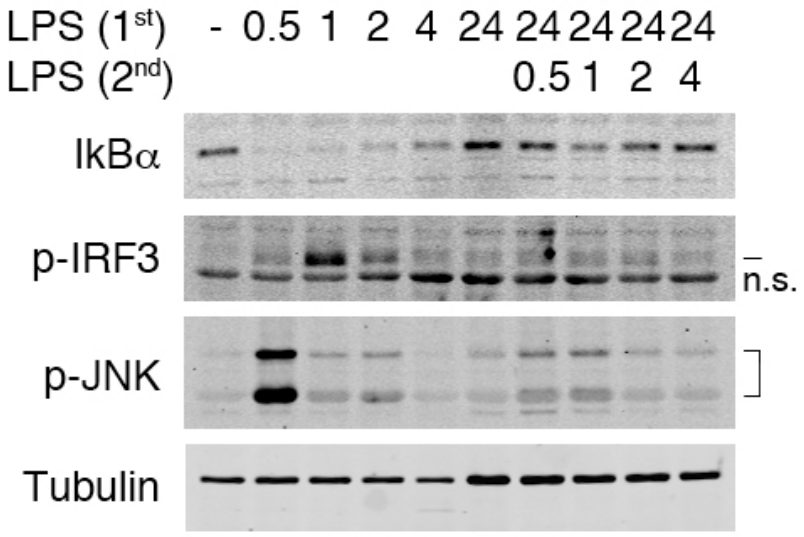



**Figure 10.22 Effects of sustained LPS treatment on the TLR4-mediated signaling pathway. I**mmunoblot analysis of total IκBα and phosphorylated (p-) IRF3 and JNK in mouse primary macrophages stimulated for 24 h with LPS (100 ng/ml) and challenged for various times with LPS (10 ng/ml). Tubulin was used as an internal loading control. The data represent one representative experiment of three independent replicates.

Phosphorylation of JNK, IRF3 transcription factors and degradation of IkB-α were completely downregulated at 24 hours post-LPS treatment. Restimulation of tolerized macrophages with LPS did not reactivate these pathways, or at least not at the same level as in the primary stimulation. Non-tolerized BMDMs responded to LPS with a robust and prolonged degradation of IκBα and transient phosphorylation of the MAPKs, JNK and the

transcription factor IRF3. In LPS tolerized marophages, LPS-induced degradation of IκBα was abrogated almost completely and activation of MAPKs was much lower, as well as activation of IRF3 protein (Figure 10.22), thus suggesting the exhaustion of the TLR4 pathway. TNF Receptor-Associated Factor 6 (TRAF6) degradation has been reported to be involved in the termination of TLR4 firing, as show in (Zhao et al., 2012). We then examined the involvement of TRAF6 in the LPS tolerance establishment. In our experimental setting, TRAF6 was down-regulated at protein level (Figure 10.23), both at sustained stimulation (up to 96 hours of LPS treatment) and upon restimulation of tolerized cells.

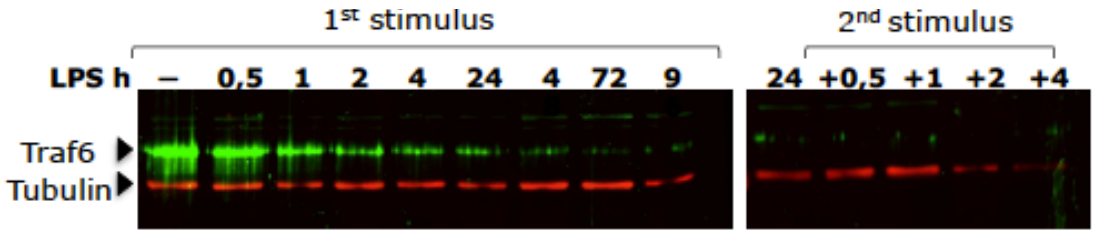



**Figure 10.23 Effects of sustained LPS treatment on TRAF6 degradation.** Degradation of TRAF6 is reported. Macrophages were treated with LPS for the designated time. Tubulin was used as an internal loading control. The data represent one representative experiment of three independent replicates.

As it has been proposed in the literature, TRAF6 is a primary target of miR-146a, which is induced upon LPS through NF-kB activity (Taganov et al., 2006). We thus envisioned a possible role for miR-146a in our experimental settings. First of all, we checked miR-146a level upon LPS treatment, both in acute and late sustained stimulation. miR-146a was highly upregulated by LPS treatment (Figure 10.24). Most importantly, miR-146 expression was maintained at a high level up to 72 hours after LPS removal (Figure 10.24 lower panel). This suggested that the failure of re-activation of those transcription factors that are involved in the transcriptional expression of transient genes is likely due to the hyporesponsive state of the TLR4 signaling pathway due to miR-146a activity.

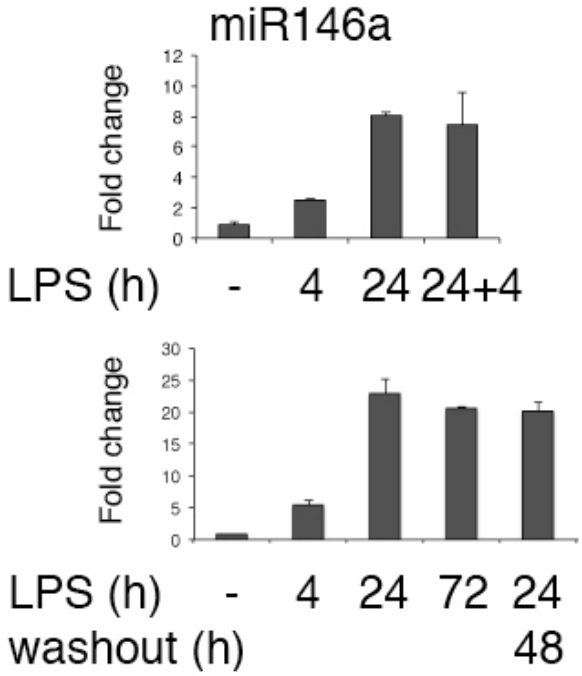



**Figure 10.24 miR-146a expression upon sustained stimulation.** RT-PCR analysis monitoring miR-146a expression in BMDM upon LPS treatment at the designated time point. In the lowe chart, miR-146a expression is evaluated upon LPS treatment and removal of the stimulus for 48h. Data are reported as fold change relative to sno202.

As it has been published, *p50*$^{-/-}$ mice cannot undergo to tolerization upon sustained treatment with LPS (Porta et al., 2009b), and it has been already demonstrated that p50$^{-/-}$ T cell cannot upregulate miR-146 upon stimulation (Yang et al., 2012). We then analysed miR-146 expression in p50$^{-/-}$ macrophages (Figure 10.25). As expected, p50$^{-/-}$ macrophages were unable to upregulate miR-146a upon LPS treatment, thus suggesting its involvement in the maintenance of the tolerization state.

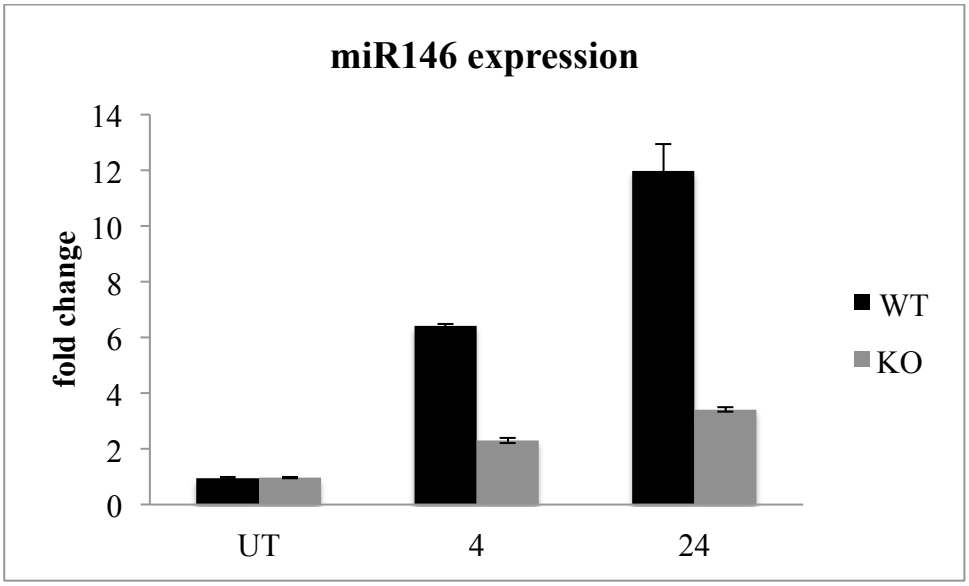



**Figure 10.25 miR-146a expression in p50$^{-/-}$ macrophages. Q**uantitative RT-PCR (qRT-PCR) analysis of miR-146a levels in BMDM derived from WT mice or p50$^{-/-}$. microRNA levels are reported as fold change relative to snoRNA202.

Our data suggest that the transcriptional control of transient genes is obtained by complex and tightly regulated events, probably involving more than one mechanism. Proteins specifically induced in the late phase of the LPS response may be involved in the transient transcription of these genes. To this purpose, we searched for pathways enriched in the cluster #5, which included lately activated sustained genes. Gene Set Enrichment Analysis (GSEA) revealed a statistical significant enrichment for proteins belonging to the TGF-β pathway. Indeed, Clusters #5 included some of the SMAD family members and some Id proteins (Figure 10.26), which are known to be component of the TGF-β pathway (Derynck and Zhang, 2003).

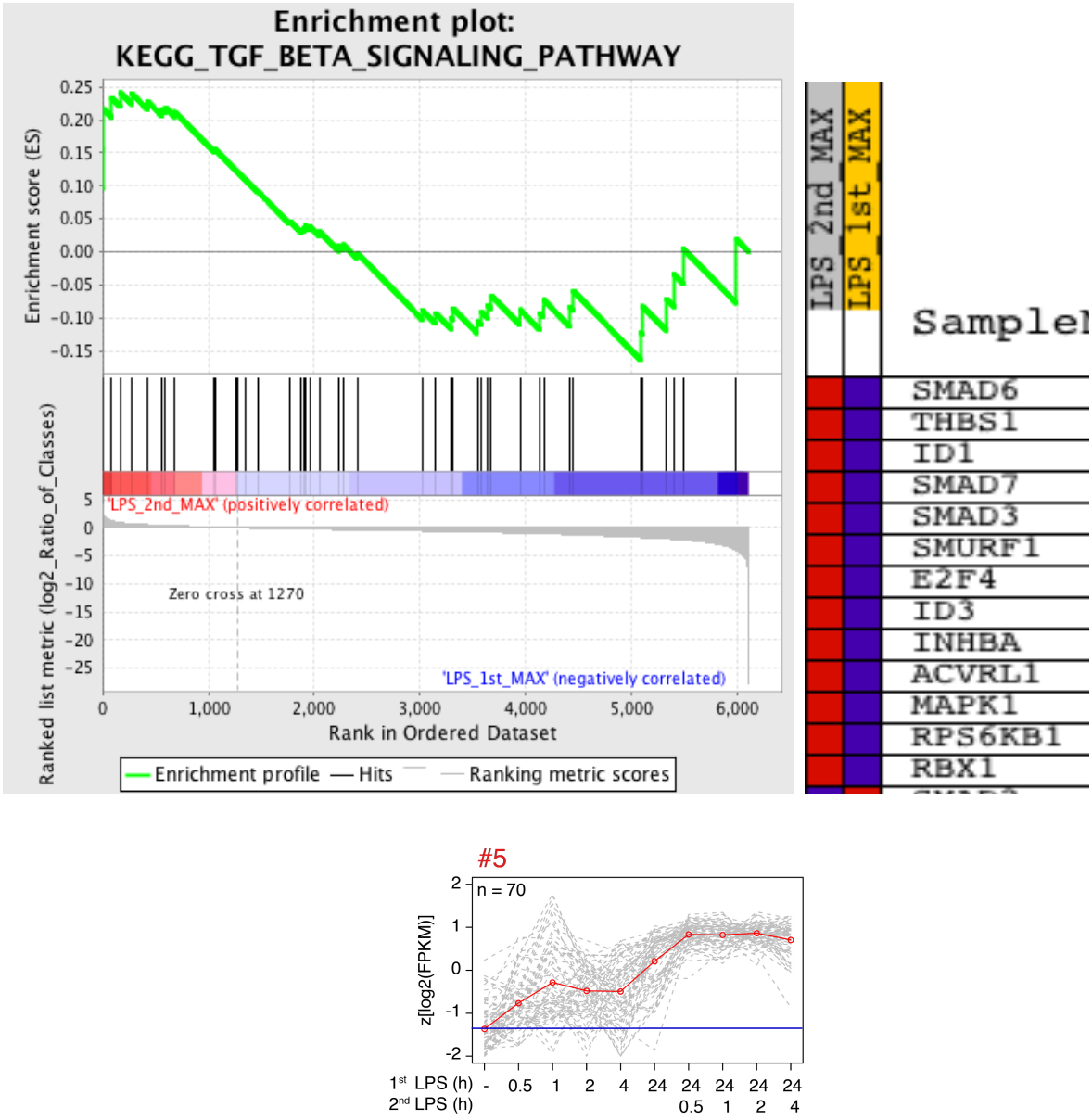
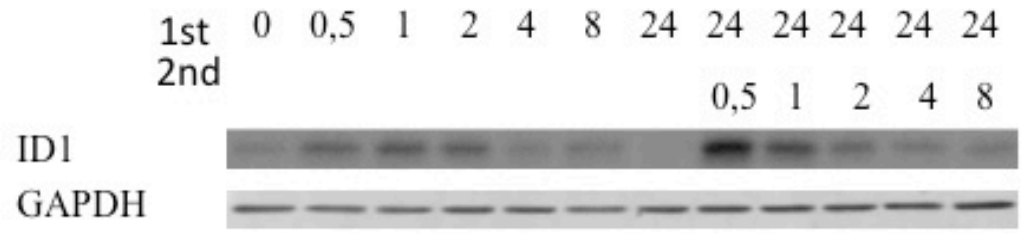



**Figure 10.26 Cluster #5 is enriched in proteins encoding genes belonging to the TGF-β signaling pathway**. Gene set enrichment analysis in genes belonging to cluster #5. Genes were sorted (x axis) from left to right based on their relative level of expression in the second stimulation vs. first LPS treatment. Genes up-regulated in the secondary stimulation, belonging to cluster #5 and included in the enriched TGF-β pathway are listed in the diagram on the right.

As already demonstrated, Id proteins are strongly induced by TGF-β (Gratchev et al., 2008). Moreover, they act as inhibitors of transcription by binding E-box transcription factors, thus preventing E-box mediated transcriptional activation of CpG island-promoter containing genes (Mantani et al., 1998). Moreover, since transient genes are enriched in

CpG islands containing promoters, we envisioned a possible role for Id proteins in their control. In order to validate this hypothesis, we checked the protein level of the Id. (Figure 10.27)




**10.27 Id1 is upregulated upon LPS restimulation.** Upregulation of Id1 is reported. Macrophages were treated with LPS for the designated time. GAPDH was used as an internal loading control. The data represent one representative experiment of three independent replicates.

Id1 is potently upregulated upon restimulation, even more than upon primary LPS treatment. These preliminary data suggested a possible role of Id1 in mediating the turning off of transient genes.

In summary, a multifactorial mechanism is responsible for the LPS tolerance establishment and attenuation of the expression of pro-inflammatory genes. We have demonstrated that the key players of the TLR4 signaling cascade revealed an almost complete exhaustion of the pathway after sustained LPS treatment due to TRAF6 degradation by miR-146 activity. This suggests that the failure of re-activation of those transcription factors that are involved in the transcriptional expression of transient genes is likely due to the hyporesponsive state of the TLR4 signaling pathway. Moreover, a role for TGF-β pathway may be envisioned and the involvement of Id proteins will be object of future research.

# 11 Discussion

Innate immune response is necessary to defend multicellular organisms against pathogen invasion, but in case of either too strong or too long response, innate immunity can be deleterious, causing the pathological manifestations of both acute and chronic inflammatory disorders. The inflammatory response must be tightly regulated, due to the potential dangerous effects of pro-inflammatory mediators, such as reactive oxygen species (Park et al., 2009) (Takeuchi and Akira, 2010). Thus, cells of the immune system must engage a multilayered control system to keep innate immunity and inflammatory responses under control. In both the human and mouse, macrophages exert different roles during a canonical inflammatory process, like the one elicited by exposure to microbial products or by necrosis of tissues. During the initial phases, macrophages recruited at the inflammation site acquire a typical inflammatory profile consisting in the release of several soluble mediators of the inflammatory response, while in case of sustained tissue damage macrophages start to express very low levels of inflammatory cytokines and chemokines but high levels of anti-inflammatory molecules and proteins involved in tissue repair. A similar transition from an inflammatory to an anti-inflammatory state is observed, both *in vitro* and *in vivo*, after prolonged exposure to bacterial products like lipo-polysaccharide (LPS) (Biswas and Lopez-Collazo, 2009). Macrophages that have been stimulated with LPS for a few hours are unable to reactivate the expression of inflammatory genes when exposed to a second LPS dose. However, they are still able to induce the expression of many other genes, including *Il-10* and *Irak-M* (or *Irak3*, a negative regulator of the TLR4 pathway, which mediates LPS signaling). This state of relative refractoriness to LPS re-stimulation is commonly referred to as endotoxin tolerance and results in a global and persistent switch of the gene expression program. The precise mechanisms underlying endotoxin tolerance are only partially known. It is clear that some inflammatory signal transduction pathways are strongly down-regulated in endotoxin-tolerant macrophages

(Biswas and Lopez-Collazo, 2009). However, this does not suffice to explain the stability over several days, and even weeks, of the reprogrammed state. Moreover, the vast majority of the published works have pointed the attention on the mechanisms explaining how the immune response is attenuated upon sustained stimulation, while ignoring the events underlying the maintenance of the expression of a big set of genes involved in the anti-inflammatory response.

In this study, we show that LPS sustained stimulation triggers the activation of distinct classes of genes, transient genes and sustained genes. Transient genes are up-regulated upon a few hours of LPS treatment and they are switched off upon sustained stimulation. On the contrary, sustained genes remain active even under long lasting stimulation or they are switched off upon sustained stimulation, but they can be reactivated when re-challenged with LPS. Genome wide profiling of chromatin associated RNAs allowed us to elucidate the mechanisms underlying both transient and sustained inflammatory gene expression. We experimentally analyzed stimulated and tolerized macrophages treated with LPS at different time points and we clustered all the upregulated genes according to their transcriptional activation behavior. Of note, the vast majority of sustained genes is upregulated upon LPS and maintains their transcriptional activity for long time, while relative few of them turn off transcription at 24 hours of treatment and they reactivate it upon re-challenge with LPS.

Until now, the most likely explanation for the memory of the tolerant state seemed to reside in a stable epigenetic reprogramming whereby inflammatory/transient genes are persistently shut down and anti-inflammatory/sustained genes are conversely made accessible and active (Foster et al., 2007). Since a genome wide study on epigenetic changes in tolerized macrophages was still lacking, we spotted *cis*-regulatory genomic regions to understand how the genomic information is alternatively used in different conditions. A simple model is that the gene expression switching observed both *in vivo* and

*in vitro* in tolerance states, reflects a stable change in the genomic regulatory landscape and specifically a stable change in the accessibility and usage of the available genomic regulatory information when cells shift from a phenotype to the other. We used ChIP-seq technology to map *cis*-regulatory regions of unperturbed, LPS treated and LPS tolerized macrophages. Genome wide analysis revealed that both transient and sustained genes maintain an accessible chromatin state, regardless their inactive or active transcription. In the late phase of the LPS response, we noticed an overall decreased acetylation in promoters and enhancers surrounding transient genes, while a persistent acetylation can be found at *cis*-regulatory regions of sustained genes. These results suggest an expected and logical correlation between acetylation of histones and polymerase II (Pol II) activity. On the contrary, methylation of histone H3 lysine 4 is maintained in the long lasting response, regardless of the transcriptional activity of the genes. H3K4me3 presence in the promoter of transient genes should generate an accessible chromatin configuration, allowing the reactivation of the gene in case of stimulus re-challenge. Despite that, transient genes are not reactivated in the presence of fresh stimuli, at least in our experimental conditions. We then analyzed two histone marks, H3K27me3 (trimethylation on Lys 27 of histone 3) (data not shown) and H3K9me3 (trimethylation on Lys 9 of histone 3), known to be associated with transcriptional repression (Kim and Kim, 2012). The promoters of transient genes do not acquire any negative histone marks in the late phase of the response. Thus, we exclude that the deposition of negative epigenetic marks play a role in the silencing of transient genes. Overall, our data suggest that chromatin modifications do not fully explain the complex transcriptional response of tolerized macrophages or at least we do not yet have the write marks tested.

To define the mechanisms underlying the transcriptional changes in the tolerant state, motifs discovery analysis was performed on promoters of transient and sustained genes.

Analysis of position weight matrix enrichment was able to discriminate sustained and transient genes in their binding site composition. Indeed, promoters of transient genes are mainly enriched in NF-kB and EGR- transcription factor binding sites, while sustained genes mainly in IRF/STAT matrices. These results suggest a role for different specific transcription factors in the fine regulation of the two subsets of genes.

Since Position Weight Matrix analysis indicated that transient genes were mainly regulated by EGR- and NF-kB transcription factors, downstream effectors of the TLR4 signaling pathway, we checked the TLR4 pathway members activity. As reported before, sustained LPS treatment leads to a complete exhaustion of the signaling cascade, thus explaining the impairment in activation of downstream transcription factors. Genes under control of TFs with an impaired transcriptional activity due to the signaling cascade exhaustion are not re-expressed, even in the presence of an open chromatin conformation. Our data indicate that this state of persistent unresponsiveness is generated and controlled by the degradation of the TRAF6 mediator, which is degraded by a parallel upregulation of its inhibitory miRNA, miR-146a. However, we did not formally demonstrate the involvement of miR-146 in the establishment of the refractoriness state of tolerized cells. In order to validate the possible involvement of miR-146a on the reduction of TLR4 signaling, we will test the effects of miR-146a antagonists on the secondary response to LPS in macrophages.

The endotoxin tolerance phenotype is multifactorial and involves the interaction of many regulatory pathways (Biswas and Lopez-Collazo, 2009). We checked for pathways specifically involved in the secondary response to LPS. Sustained cluster #5 (cluster of genes mainly upregulated in the secondary response) show a peculiar upregulation of several component of the TGF-β pathway. In particular, several Id proteins (Ids) are upregulated upon sustained stimulation, suggesting a possible role for the Id family members in the maintenance of the tolerance state. Impairment of upregulation of Id proteins, will probably deregulate the Id- mediated inhibition of E-Box factors on CpG

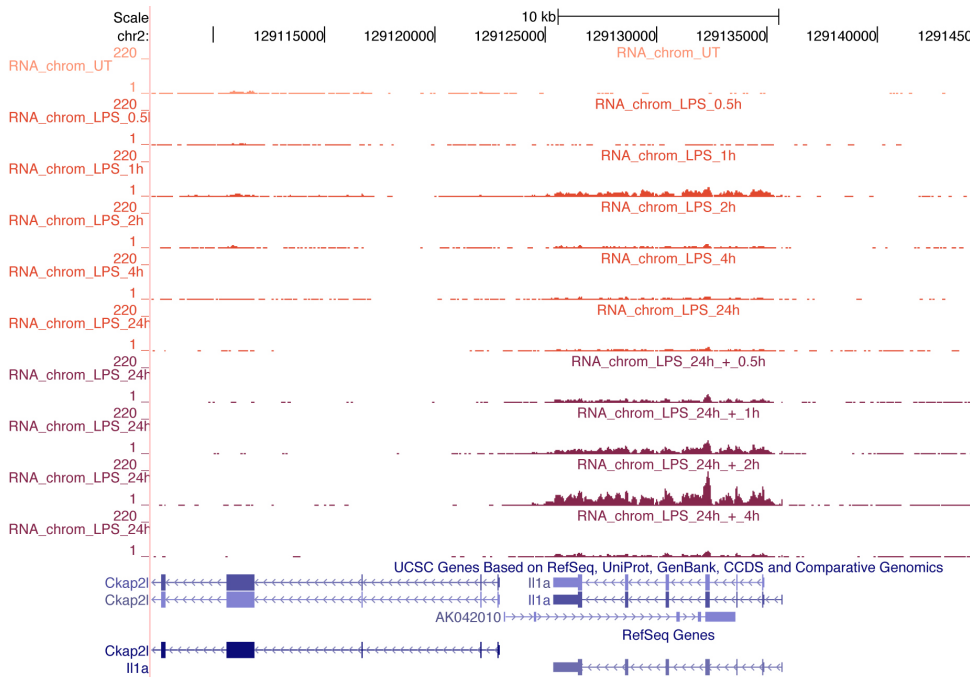
island containing promoters, thus suggesting the possibility that transient genes (enriched in CpG islands) may be transcriptional reactivated in case of re-challenge. Therefore, in the sustained stimulation, Id proteins may be under TGF-β control (being TGF-β an activator of Id proteins (Gratchev et al., 2008)) and may be involved in the negative control of CpG islands containing genes. In order to validate this hypothesis, we will deplete Id proteins in BMDM tolerized macrophages and we will test their impact on the transcription program of transient genes.

Finally, IRF transcription factors were found enriched in the promoters of sustained genes. We checked the gene expression level of all the IRFs in LPS stimulated and tolerized macrophages and we found that IRF7 is the only one to be upregulated and sustained at high levels in the late phase of the response. Moreover, recent finding proposed IRF7 as a player in the pro- to anti- inflammatory phenotype transition (Cohen et al., 2014). Since tolerized macrophages display overall an anti- inflammatory phenotype (Pena et al., 2011), we considered IRF7 as a good candidate for the control of expression of sustained genes. We propose that sustained genes can retain the ability to be expressed even in the absence of active signaling by TLR4 because of an IRF7-dependent feed forward loop. Once activated, IRF7 may keep high its own transcription by regulating its own promoter, thus generating an autocatalytic mechanism of sustainment. This could be a simple strategy developed by innate immune cells to keep anti-microbial responses active while preventing damage to the host organism, by switching off the production of pro-inflammatory mediators. In order to assess the involvement of IRF7 in expression of the sustained genes, we evaluated by RNA-seq the impact of IRF7 depletion on persistently expressed genes and we found out that removal of IRF7 had a dramatically impact on transcription of sustained genes.

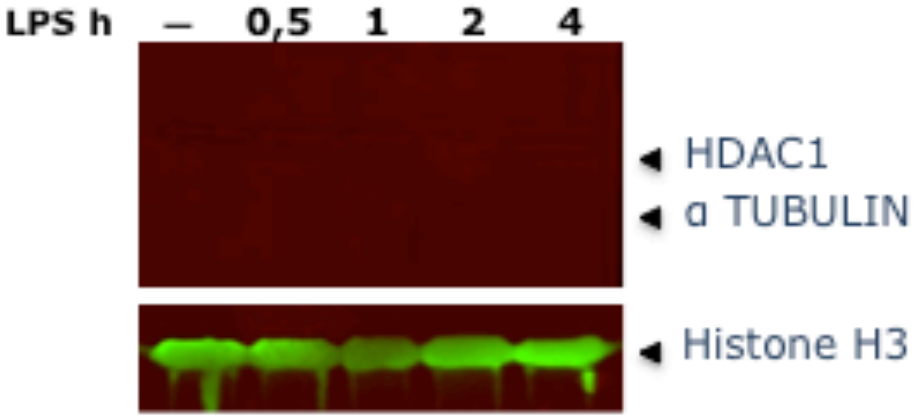
In conclusion, this study suggests that the differential expression of LPS-regulated genes during sustained LPS stimulation might be simply determined by activation and

availability of sequence specific transcription factors controlling specific subsets of genes rather than being dictated by epigenetic, chromatin-mediated mechanisms.

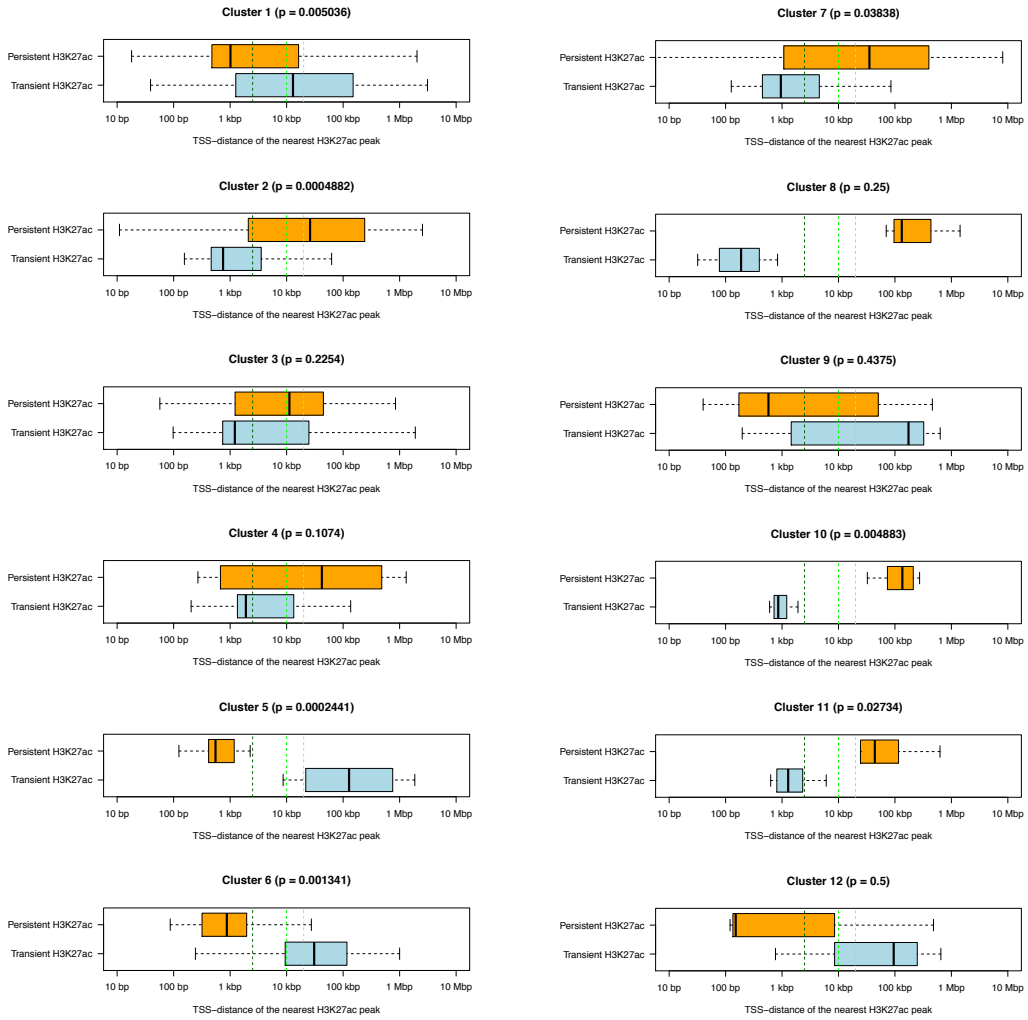
# 12 Supplementary Figures



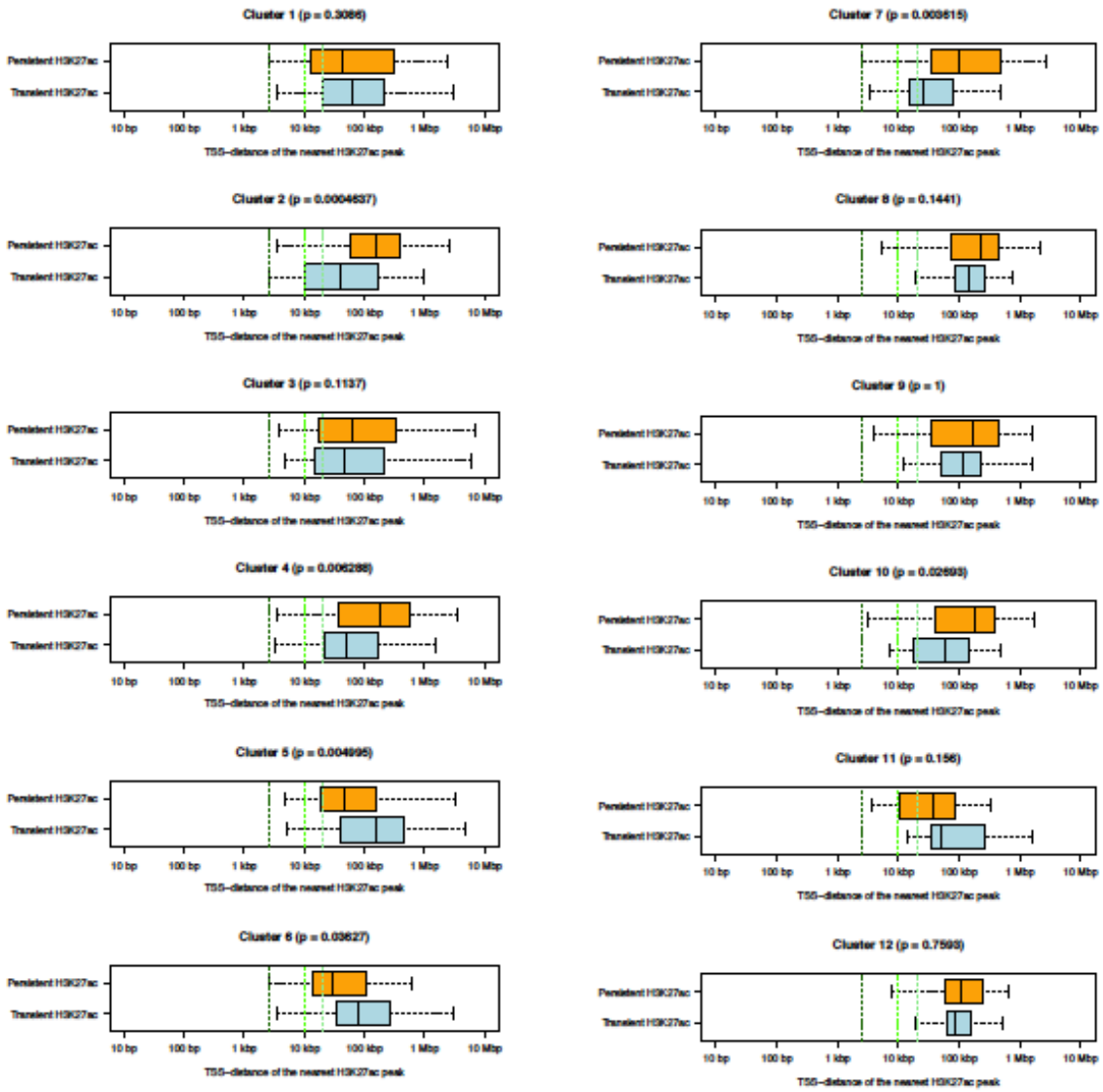

**Supplementary Figure 1. *Il-1a* locus**. Chromatin associated RNA snapshots of the *Il-1a* locus.




**Supplementary Figure 2. Chromatin fraction quality control.** HDAC1, Tubulin and histone H3 are reported. Macrophages were treated with LPS for the designated time. The data represent one representative experiment of three independent replicates.




**Supplementary Figure 3**. **Active chromatin modification levels at proximal regulatory regions near transient and sustained genes**. Proximal to TSS-H3K27 Acetylation level. Each gene was assigned to the nearest sustained or transient H3K27 acetylation peak. Transient genes are significantly closer to transient acetylation than sustained ones while the contrary is observed for the sustained ones.




**Supplementary Figure 4. Active chromatin modification levels at distal regulatory regions near transient and sustained genes**. Distal to TSS-H3K27 Acetylation level. Each gene was assigned to the nearest sustained or transient H3K27 acetylation peak. Transient genes are significantly closer to transient acetylation than sustained ones while the contrary is observed for the sustained ones.

# 13 Supplementary Tables

## Supplementary Table 1. List of genes in the various clusters

| CLUSTER 1 | CLUSTER 2 | CLUSTER 3 | CLUSTER 4 | CLUSTER 5 | CLUSTER 6 |
|---|---|---|---|---|---|
| 1110018G07Rik | 2310004I24Rik | 6530402F18Rik | 2010002N04Rik | 1300002K09Rik | 1600014C10Rik |
| 1110038F14Rik | 4632415L05Rik | 9030625A04Rik | 3110003A17Rik | 1700071M16Rik | A230028O05Rik |
| 1810029B16Rik | 4921513D23Rik | Apobr | 5930434B04Rik | 1700123I01Rik | Adora2b |
| 2010106G01Rik | 4932438H23Rik | Arhgef3 | 6330409N04Rik | 4933433H22Rik | Aoah |
| 2810474O19Rik | 4933412E12Rik | BC006779 | Atm | 9130014G24Rik | Apol9b |
| 5730508B09Rik | 4933426M11Rik | Batf | Bach1 | Acsl1 | Arid5b |
| 6230427J02Rik | 9430076C15Rik | Bckdhb | Bcl2a1b | Ankrd24 | B430306N03Rik |
| 9330175E14Rik | AW011738 | Bcl2a1a | Bcl2a1d | Antxr1 | Bcl6 |
| A130040M12Rik | Adamts4 | Bcl3 | Ccl2 | Art2a-ps | Casp3 |
| A530032D15Rik | Adora2a | Birc2 | Ccl3 | Bst1 | Cfb |
| AA467197 | Anxa6 | Birc3 | Ccl4 | Bzrap1 | Clec2d |
| AI504432 | Arhgap23 | Car13 | Ccl9 | Cd1d1 | Clic5 |
| AW112010 | Arid1a | Casp4 | Ccrl2 | Cd38 | Ddx60 |
| Abtb2 | Arid4a | Ccng2 | Ccrn4l | Cebpb | Dst |
| Adap2 | Armcx6 | Cd40 | Cd14 | Clec4d | Dusp28 |
| Agrn | Asb13 | Cpd | Cd83 | Cxcl16 | F830016B08Rik |
| Arhgef12 | Atp10a | Cxcl10 | Cdk6 | Cxcl3 | Fam116b |
| Ascc3 | Atp11a | Cybb | Cdkn1a | D330045A20Rik | Fcgr1 |
| Atad1 | BC016423 | D8Ertd82e | Cited2 | D730048J04Rik | Fmnl2 |
| BC013712 | Bambi-ps1 | Dcbld2 | Clic4 | Ell2 | Fpr2 |
| BC094916 | Casp7 | Dgkh | Csf1 | Fbxo40 | Gbp10 |
| Batf2 | Ccdc86 | Dyrk2 | Csrnp1 | Flrt2 | Gbp11 |
| Bcl9 | Ccnd2 | E330016A19Rik | Denr | Flrt3 | Gbp4 |
| Bfar | Ccnj | Ebi3 | Dnaja2 | Ggct | Gbp6 |
| Bst2 | Cd164 | Ehd1 | Dpep3 | Glrx | Gm11435 |
| Bzw2 | Cd69 | Ext1 | Dse | Gm14023 | Gm12185 |
| C130026I21Rik | Cdyl2 | Ezr | Dusp1 | Gm5150 | Gm14085 |
| C3 | Cenpj | Fas | Dusp16 | Grina | Gm14446 |
| Calcrl | Ch25h | Flnb | Dusp2 | H2-M2 | Gm16675 |
| Casp1 | Cish | Fnbp1l | Errfi1 | H2-Q6 | Gm4841 |
| Ccdc25 | Cnn3 | Foxp4 | Fam129a | Hmox1 | Gm4951 |
| Ccl5 | Col18a1 | Gbp5 | Fam133b | Id1 | Gm5424 |
| Ccnyl1 | Col27a1 | Gch1 | Fam177a | Id3 | Gm5506 |
| Cd274 | Cst7 | Gm11428 | Fchsd2 | Il1a | Gm6034 |
| Cept1 | Cxcl9 | Gpr84 | Fosl2 | Inpp5j | Gm7609 |
| Cmpk2 | Cycs | Gpr85 | Gadd45b | Itga5 | Gpr31c |
| Crlf3 | Daam1 | Hat1 | Gm12216 | Jag1 | Has1 |
| Csf3r | Dll1 | Hivep1 | Gm614 | Klra2 | Hcrt |
| Ctsc | Dync1i2 | Hk2 | Gm6377 | LOC100038947 | Il18bp |
| D14Ertd668e | Edn1 | Hspbap1 | Gpr132 | Ly6a | Inhba |
| Daxx | Enpp4 | Ifi203 | Gtf2f1 | Ly75 | Irg1 |
| Dck | Fam26f | Ifih1 | Hdac1 | Mdm2 | Itgal |
| Dcp2 | Fam82a2 | Igsf6 | Icam1 | Met | Kcnh4 |
| Ddx58 | Gca | Il1b | Icam4 | Olfr1445 | Lcn2 |
| Diap2 | Gm6524 | Il2rg | Icosl | Pcdh7 | Lrrc16a |

| | | | | | |
|---|---|---|---|---|---|
| Dram1 | Gnb4 | Jazf1 | Il10 | Pde5a | Lrrc8c |
| Epsti1 | Gng12 | Katna1 | Irf1 | Pdpn | Mefv |
| F730043M19Rik | Gpr126 | Klf7 | Itgav | Pilrb1 | Mmp14 |
| Fam102b | Gypc | Lcp2 | Junb | Pla2g7 | Ms4a4c |
| Fcgr2b | Hap1 | Malt1 | Klf6 | Ppap2b | Ms4a4d |
| Fcgr4 | Hdc | Mcm10 | Maff | Ptger2 | Nos2 |
| Fgl2 | I830077J02Rik | Med26 | Mapkapk2 | Rab20 | Phyh |
| Gbp2 | Ikzf1 | Mllt6 | Marcksl1 | Rasgrp1 | Pion |
| Gbp3 | Il13ra1 | Mrpl39 | Mmp13 | Saa3 | Pla2g4a |
| Gbp7 | Il6 | Myd88 | Mt1 | Sh2b2 | Prdx5 |
| Gbp9 | Inpp5b | Nfkb1 | Mtmr14 | Siglece | Procr |
| Glipr2 | Insl6 | Nfkb2 | Ndrg1 | Sirpb1a | Ptges |
| Gm12250 | Itga4 | Nfkbie | Nfkbia | Sirpb1b | Pydc3 |
| Gm4902 | Klf8 | Nod2 | Nfkbib | Slamf9 | Pydc4 |
| Gm5431 | Kpna3 | Nupr1 | Nfkbiz | Slc22a4 | Sipa1l1 |
| Gm6904 | Kremen1 | Ogfr | Nfxl1 | Slc7a11 | Slc28a2 |
| Gm6907 | Lap3 | Optn | Niacr1 | Slfn4 | Slc7a2 |
| Gm7030 | Larp1 | Otud5 | Nudt9 | Smad6 | Slfn1 |
| Gpd2 | Lcat | Pde4b | Pabpc1 | Smad7 | Smpdl3b |
| Gphn | Lhx2 | Pgap2 | Phxr4 | Snx18 | Stxbp3a |
| Gsdmd | Lipg | Pgs1 | Pim1 | Spic | Tarm1 |
| H2-T10 | Lrch1 | Phldb1 | Plek | Tgm2 | Trim34b |
| H2-T22 | Mcc | Pnp2 | Ppp1r15a | Tnfrsf1b | Ttc39b |
| Hck | Minpp1 | Ppp6r1 | Ptger4 | Wnk2 | Uba7 |
| Herc6 | Mmp25 | Prpf38a | Rasgef1b | Zswim4 | |
| I830012O16Rik | Mndal | Ptgs2 | Rcsd1 | | |
| Ifi204 | Naaa | Rab11fip1 | Rel | | |
| Ifi205 | Noc4l | Rap2c | Rgs1 | | |
| Ifi35 | Otud1 | Relb | Rnd3 | | |
| Ifi44 | Phf6 | Rhbdf2 | Sbds | | |
| Ifit1 | Phip | Ripk2 | Sdc4 | | |
| Ifit2 | Pik3r6 | Rnf135 | Sept11 | | |
| Ifit3 | Plcb3 | Rnf19b | Slc12a4 | | |
| Igf2bp2 | Plekha2 | Samsn1 | Slc2a6 | | |
| Igtp | Plekha4 | Serpine1 | Slc35b2 | | |
| Iigp1 | Plod3 | Skil | Socs7 | | |
| Ikbke | Pnpt1 | Slamf7 | Srgn | | |
| Il15 | Pou2f2 | Slc31a2 | Stat5a | | |
| Il15ra | Ppa1 | Slc44a1 | Swap70 | | |
| Il18 | Ppargc1b | Socs3 | Tfec | | |
| Il27 | Ppfibp1 | Sod2 | Tlr2 | | |
| Il4ra | Ppm1k | Sowahc | Tnf | | |
| Irf2 | Prdm9 | Spred1 | Tnfaip2 | | |
| Irf7 | Prnp | Stx6 | Tnfaip3 | | |
| Irgm1 | Prpf4 | Tagap | Tnfsf9 | | |
| Irgm2 | Psme1 | Tax1bp1 | Tnip3 | | |

| | | | | | |
|---|---|---|---|---|---|
| Isg15 | Rassf4 | Tiparp | Ube2f | | |
| Isg20 | Rcl1 | Tra2a | Zfp36 | | |
| Jak2 | Rcn1 | Traf1 | Zyx | | |
| Kcna3 | Rgs14 | Trex1 | | | |
| Klf3 | Rnd1 | Ube2l6 | | | |
| Lass6 | Sbno2 | Vasp | | | |
| Lgals9 | Sco1 | Vcam1 | | | |
| Lpar1 | Sema4c | Wdr59 | | | |
| Lztfl1 | Serpina3g | Zfp800 | | | |
| Man2a1 | Sertad3 | | | | |
| Map2k4 | Setdb2 | | | | |
| Mapkbp1 | Sgk3 | | | | |
| Mcmbp | Slc30a4 | | | | |
| Mfsd7a | Sntb1 | | | | |
| Mier3 | Sos1 | | | | |
| Mlkl | Spata13 | | | | |
| Ms4a4b | Spsb1 | | | | |
| Ms4a6d | Steap4 | | | | |
| Msr1 | Tbc1d13 | | | | |
| Mtdh | Tmem86b | | | | |
| Mthfr | Tmprss2 | | | | |
| Mtus1 | Tnfsf10 | | | | |
| Mx1 | Tnfsf15 | | | | |
| Mx2 | Trim36 | | | | |
| Mxd1 | Upp1 | | | | |
| Myo10 | Usp12 | | | | |
| N4bp1 | Usp42 | | | | |
| Naa25 | Vezt | | | | |
| Ncrna00085 | Ythdf1 | | | | |
| Nlrc5 | | | | | |
| Nod1 | | | | | |
| Notch1 | | | | | |
| Nr3c1 | | | | | |
| Nt5c3 | | | | | |
| Nxf1 | | | | | |
| Oas1b | | | | | |
| Oas1g | | | | | |
| Oas3 | | | | | |
| Oasl1 | | | | | |
| Oasl2 | | | | | |
| P4ha1 | | | | | |
| Papd7 | | | | | |
| Parp10 | | | | | |
| Parp11 | | | | | |
| Parp12 | | | | | |
| Parp14 | | | | | |

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| Parp4 | | | | | | |
| Parp8 | | | | | | |
| Pcgf5 | | | | | | |
| Peli1 | | | | | | |
| Phf11 | | | | | | |
| Plagl2 | | | | | | |
| Pml | | | | | | |
| Pnp | | | | | | |
| Ppp1r15b | | | | | | |
| Psmb10 | | | | | | |
| Psmb8 | | | | | | |
| Psmb9 | | | | | | |
| Pstpip2 | | | | | | |
| Pyhin1 | | | | | | |
| Rapgef2 | | | | | | |
| Rbl1 | | | | | | |
| Rgl1 | | | | | | |
| Rin2 | | | | | | |
| Rnf31 | | | | | | |
| Rsad2 | | | | | | |
| Samhd1 | | | | | | |
| Sec24b | | | | | | |
| Sh3bp4 | | | | | | |
| Slc25a12 | | | | | | |
| Slc4a7 | | | | | | |
| Slco3a1 | | | | | | |
| Slfn3 | | | | | | |
| Slfn8 | | | | | | |
| Slfn9 | | | | | | |
| Snx10 | | | | | | |
| Socs1 | | | | | | |
| Sp100 | | | | | | |
| Sp140 | | | | | | |
| Src | | | | | | |
| St7 | | | | | | |
| Stat2 | | | | | | |
| Tap1 | | | | | | |
| Tap2 | | | | | | |
| Tapbp | | | | | | |
| Tifa | | | | | | |
| Tlr3 | | | | | | |
| Tmem170b | | | | | | |
| Tmem2 | | | | | | |
| Tmem67 | | | | | | |
| Tnfrsf14 | | | | | | |
| Tor3a | | | | | | |

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|---|---|---|---|---|---|
| Tpst1 | | | | | |
| Traf2 | | | | | |
| Trim21 | | | | | |
| Trim26 | | | | | |
| Trim30a | | | | | |
| Trim30b | | | | | |
| Trim30d | | | | | |
| Tspo | | | | | |
| Txn1 | | | | | |
| Ubash3b | | | | | |
| Ubr4 | | | | | |
| Usp18 | | | | | |
| Usp25 | | | | | |
| Vcan | | | | | |
| Vps54 | | | | | |
| Whamm | | | | | |
| Xaf1 | | | | | |
| Xrn1 | | | | | |
| Zbtb5 | | | | | |
| Zcchc2 | | | | | |
| Zufsp | | | | | |

| CLUSTER 7 | CLUSTER 8 | CLUSTER 9 | CLUSTER 10 | CLUSTER 11 | CLUSTER 12 |
|---|---|---|---|---|---|
| 6030422M02Rik | 3010026O09Rik | 1200009I06Rik | 5031414D18Rik | Akt3 | 4933437F05Rik |
| A430084P05Rik | 9130230L23Rik | Abcc1 | AB124611 | Ankib1 | Ccr3 |
| Adamts6 | A3galt2 | Akap13 | Abhd16a | Arid5a | Cdk5r1 |
| Adora3 | Apol8 | Akap2 | Alpk1 | Atf3 | Chaf1b |
| Aldh1b1 | Arl5b | Akna | Atp13a1 | Ccl12 | Clcf1 |
| Angpt1 | BC031781 | Arl5c | B630005N14Rik | Ccl7 | F3 |
| Arhgef37 | Bcl2l11 | Atp2b4 | Brwd3 | Cd86 | Frmd6 |
| Arl4a | Btg2 | Bcor | Cmtm6 | Chd7 | Htr2a |
| Asap3 | C920009B18Rik | Bcorl1 | Csrp1 | Dennd1a | Htra4 |
| Bco2 | Cpm | Cacnb3 | D1Ertd622e | Dock10 | Ifnb1 |
| C1ra | Cxcl1 | Cdc42ep2 | Dgka | Eif2ak2 | Iglon5 |
| Casp12 | Cxcl2 | Clec4e | Dr1 | Etv6 | Il12b |
| Ccdc101 | Dhx40 | Dennd4a | Etnk1 | Fam53c | Irf4 |
| Cd200 | Dnajb4 | F11r | Etv3 | Fndc3a | Lrrc63 |
| Crim1 | Dusp4 | Fam20c | Golga3 | H3f3b | Map3k5 |
| Dpy19l1 | Dusp5 | Fscn1 | Gpr108 | Hivep2 | Mybbp1a |
| Fap | Dusp8 | Gja1 | Gramd1a | Il1rn | Olr1 |
| Fbn1 | Egr1 | Gm8909 | Hmgn3 | Marcks | Prkab2 |
| Fcrl5 | Egr2 | H2-Q4 | Hn1 | Mnda | Pvr |
| Fcrlb | Egr3 | H2-Q8 | Hsd17b11 | Mt2 | Rhof |
| Flt1 | Ets2 | Hivep3 | Iqsec2 | Nfil3 | Sele |
| Gfi1 | Fam71a | Hspa1a | Irf8 | Ralgapa2 | Sh3bgrl2 |
| Gm13315 | Fos | Hspa1b | Itpr1 | Ranbp2 | Tmem200b |
| Gm15987 | Fosb | Ift57 | Ktn1 | Rictor | Zbtb10 |
| Gm9895 | Gdf15 | Jdp2 | Mertk | Sik3 | |
| Ifitm7 | Gem | Kctd12 | Nlgn2 | Slfn2 | |
| Il12rb1 | Glp2r | Lphn2 | Nono | Slfn5 | |
| Il2ra | Gm4980 | Myo1g | Prkx | Stx11 | |
| Itgb1bp2 | Gtf2ird2 | Nfkbid | Rhoc | Tet2 | |
| Itgb8 | Ier2 | Nle1 | Rps6ka3 | Tlk2 | |
| Klrk1 | Ier3 | Nr4a3 | Slc12a9 | Tmod3 | |
| Mfsd6l | Ifngr1 | Orai2 | Slc25a22 | Zcchc6 | |
| Nox1 | Ifrd1 | Plagl1 | Stard7 | | |
| Ovol1 | Irgq | Plaur | Tgs1 | | |
| Pdk3 | Irs2 | Prdm1 | Tle3 | | |
| Pecam1 | Jun | Ptch1 | Tmem131 | | |
| Pla2g16 | Kcnj2 | Slc25a37 | | | |
| Pla2r1 | Kdm6b | Sphk1 | | | |
| Plod2 | Mmp12 | Sqstm1 | | | |
| Pou3f1 | Mybpc3 | St3gal1 | | | |
| Rab3ip | Myc | Stim2 | | | |
| Rac3 | Nlrp3 | Tnfrsf11a | | | |
| Rpgrip1l | Nr4a1 | Tpbg | | | |
| Rtn1 | Odc1 | Trpm4 | | | |
| Sectm1a | Olfr1444 | Tshz1 | | | |

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|---|---|---|---|---|---|---|
| Sell | Olfr920 | Ttc39c | | | | |
| Slamf1 | Osgin2 | Vash1 | | | | |
| Slc16a1 | Osm | Zc3h12c | | | | |
| Tex14 | P2ry2 | Zhx2 | | | | |
| Tmem176a | Phlda1 | | | | | |
| Tmtc2 | Plau | | | | | |
| Trip6 | Plk2 | | | | | |
| Tuba8 | Pmaip1 | | | | | |
| Uaca | Rcvrn | | | | | |
| Xkr8 | Rgs2 | | | | | |
| Zeb1 | Trib1 | | | | | |
| Zfp811 | | | | | | |

# 14 References


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