Type 2 Immune Response Plays A Critical Role in Trained Immunity

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Abstract

Trained immunity, or innate immune memory, is vital in inducing heterologous protection against infection. Type 2 cytokines such as IL-4 and IL-13 induce M2 macrophage differentiation, characterized by anti-inflammatory phenotype permitting inflammation resolution and wound healing. Here we report that peritoneal macrophages isolated from OVA-allergen sensitized mice possessed both M2 signatures and a pro-inflammatory phenotype. Moreover, mice were more resistant to bacterial infection in the OVA-induced allergy model. Mechanistically, we found that IL-4 induced trained immunity in a STAT6-independent and IRS-dependent manner. Furthermore, IL-4 induces epigenetic memory in the pro-inflammatory gene enhancer regions, ensuring a higher transcription activity upon stimulation. In addition, IL-4 trained macrophages are metabolically more glycolytic upon LPS stimulation. To conclude, this unrecognized role of IL-4 in trained immunity broadens our understanding of IL-4 mediated physiological changes and provides immediate impacts on how type 2 immune response could re-direct disease progression in response to pathogen infection.

Introduction

Innate immunity as the sentinels of host immunity elicits rapid response towards intruding pathogens. Unlike adaptive immunity, the self-nonself discrimination of innate immunity is antigen non-specific and acts via germline-encoded pattern recognition receptors (PRRs). Therefore, immunological memory is regarded as an exclusive characteristic of adaptive immunity that could form antigen-specific memory against the previously encountered antigen. However, in the past decade, innate immune cells such as natural killer cells[1], monocytes[2], macrophages[3] and neutrophils[4] were reported to possess immunological memory in a limited-antigen specific or antigen non-specific manner. This innate immunity memory is termed "trained immunity" , which is mediated via metabolic rewiring and epigenetic reprogramming[5,6].

Based on the effector functions and pathophysiological effects, the innate and adaptive effector immunity could be distinguished into type1, type 2, and type 3 immunity, respectively[7]. Type 1 and 3 immunity are critical in host defense against viral, bacterial and fungal infections. The dysregulated type 1 and 3 immunity also regulates inflammatory and autoimmune diastases. Type 2 immunity is essential for protection against helminth and venoms and is responsible for allergic and asthmatic diseases. IL-4 is one of the hallmark cytokines in Type 2 immune response. The canonical role of IL-4 in inducing alternative activation in macrophages (AAMφ), also known as M2[8,9], is extensively investigated. M2 macrophages are traditionally regarded as anti-inflammatory macrophages, secreting Arginase-1, IL-10, TGF-β, and other anti-inflammatory cytokines, promoting the resolution of inflammation and wound healing and favoring tumor development and progression in the context of tumor-associated macrophages (TAMs). Upon binding to IL-4Rα, IL-4 induces the phosphorylation of STAT6, which in turn dimerizes and translocates into the nucleus for promoting transcription of M2 signature genes.

Asthmatic patients had a decreased risk for hospital mortality, septicemia, sepsis, and septic shock across all infections[10]. In a retrospective study, patients who survive *S. aureus* bacteremia have higher numbers of Th2 and fewer Th17 lymphocytes in the blood[11], suggesting that type 2 immune response might have a beneficial bactericidal effect in humans. Furthermore, the accumulation of mast cells, eosinophils, and innate lymphoid cells 2 (ILC2) have been suggested to engage in the bactericidal effect in the acute type 2 immune response[11]. Notably, trained immunity in macrophages has been demonstrated to be important in host anti-bacterial response. We speculate that type 2 immunity might induce trained immunity in macrophages aiding in the anti-bacterial effect. Here, we demonstrated that type 2 immune response primed macrophages to produce more pro-inflammatory cytokines upon stimulation and rendered mice more resistant to bacterial infection. We further uncovered a non-canonical feature of IL-4 in inducing "trained immunity". We delineated that different sets of epigenetic reprogramming controlled the canonical M2- and non-canonical trained immunity-inducing effects of IL-4. Therefore, we propose that IL-4 induces non-canonical trained immunity in macrophages via epigenetic memory besides its well-characterized canonical anti-inflammatory role. This previously neglected

aspect of IL-4 biology might open up a new vista to broaden our understanding of the unresolved association between asthma and other inflammatory diseases such as sepsis[10] and obesity[12,13].

Materials and Methods

Animals

Wild-type C57BL/6 mice were purchased from Xiamen University Laboratory Animal Center. All mice were maintained under specific pathogen-free conditions at the Xiamen University Laboratory Animal Center. These mouse experiments were approved by the Institutional Animal Care and Use Committee. In addition, they were in strict accordance with good animal practice as defined by the Xiamen University Laboratory Animal Center.

Reagents

Recombinant M-CSF (CB34), IL-4 (CK74), IL-5 (CW73), IL-13 (CX57), and IFNγ (CM41) were purchased from Novoprotein, Shanghai, China. E. coli LPS (S11060), NT157 (S80314) were purchased from Shyuanye, Shanghai, China. Syk inhibitor BAY 61-3606 (1615197-10-8) and Tunicamycin (11445) were purchased from Cayman Chemical. BPTES, GSK-J4 (HY-15648B), Histone methyltransferase inhibitor MTA (HY-16938), AS1517499 (HY-100614), and Etomoxir (HY-50202) were purchased from MedChemExpress. Wortmannin (9545-26-7) was purchased from Adooq bioscience. GW5074 (R026615) was purchased from Rhawn, Shanghai, China. SYBR Green Premix Pro Taq HS qPCR Kit (AG11701), Evo M-MLV reverse transcriptase (AG11605), Recombinant RNase inhibitor (AG11608) were purchased from Accurate biology. Amplex Red (119171-73-2) was purchased from Alfachem. N-(1-Naphthyl) ethylenediamine dihydrochloride (N9125) and Sulfanilamide (S9251) were purchased from Sigma-Aldrich.

Bone-Marrow-Derived Macrophage Culture and Differentiation

Bone marrow cells were harvested from femurs and tibia of 6-8 week-old C56BL6/J mice and differentiated in DMEM supplemented with 10 % FBS and 40 ng/ml of recombinant mouse M-CSF. Half volume of medium containing fresh 40 ng/ml of M-CSF was added to the cell culture at day 3. Differentiated BMDMs were detached, counted, and reseeded to the cell culture plate for subsequent experiments.

For macrophage polarization experiments, 20 ng/ml LPS and 100 ng/ml IFNγ were added to BMDMs to induce M1 macrophage polarization. 20 ng/ml IL-4 were used to induce M2 polarization. To assess the short-term effect of type II cytokines, BMDMs were cultured with 20 ng/ml of IL-4, IL-5, or IL-13. After 24 hours, BMDMs were washed with PBS once and stimulated with 100 ng/ml LPS to assess the cytokine production capacity. To assess IL-4 induced long-termed trained immunity, BMDMs were first cultured with 20 ng/ml IL-4 for 24 hours and then washed with PBS once and refreshed with DMEM for additional 5 days. BMDMs were then harvested for subsequent functional assessment. To assess the signaling pathways involved in IL-4 induced classic and non-classic effects, BMDMs were pre-incubated with various inhibitors for 30 min before being stimulated with IL-4. BMDMs were washed with PBS 24 hours post-IL-4 stimulation and harvested for subsequent functional assessment.

Quantitative Real-Time PCR

For quantitative RT-PCR, total mRNA was extracted with oligo-dT magnetic beads. In short, RNA was extracted by magnetic beads conjugated with Oligo-dT18. Isolated RNA was reverse transcribed into cDNA using dNTP (Beyotime, D7366) /oligodT mix, RNA transcriptase (Accurate Biology, AG11605), and RNase inhibitor (Accurate Biology, AG11608). qPCR was performed using the SYBR Green method (Accurate Biology, AG11701). Relative expression levels were calculated using theΔCT method and normalized to the expression of the β2M housekeeping gene. The primer sequences we used are listed in Table S1.

ELISA and Lactate Measurement

Cytokine levels in culture supernatant or mice serum were determined by TNF-α (Invitrogen; 88-7324-88) and IL-6 (Invitrogen; 88-7064-88) ELISA kits following the instructions of the manufacturer. As for lactate measurements, the cellular supernatant was diluted by PBS. Then the diluent is mixed with the mixture containing Amplex Red, HRP, and lactate oxidase for 10 min by one-to-one ratio. The fluorescence was detected at excitation wavelength at 528 nm and emission wavelength at 590 nm.

NO Measurement

The cellular supernatant was mixed with solution A (ethylenediamine dihydrochloride) and solution B (sulfanilamide) in a one-to-one ratio. Then the absorbance was measured at 540 nm.

Western Blot

Cells were lysed in RIPA buffer (1 mM Tris-HCl, 0.3 M NaCl, 0.01% SDS, 1.5% NP40, 120 mM deoxycholate, 1 M $MgCl_2$) containing protease inhibitors. Proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Roche, # 3010040001). The membranes were blocked for 1 h in blocking buffer (5% BSA and 0.1% Tween 20 in TBS) at room temperature and then incubated with respective primary antibodies in 5% BSAat 4℃ overnight. After being rinsed with TBST for three times, HRP coupled secondary antibodies in TBST were used to blot for 1 h at room temperature. Antibody against β-Actin (21338) was purchased from SAB. Antibodies against HK1 (A10886), HK3 (A8428), Glut1 (A11727), Histone H3(A2348), TriMethyl-Histone H3K4 (A2357), Acetyl-Histone H3K27 (A7253) were purchased from ABclonal. Antibodies against Mono-Methyl-Histone H3K4 (Clone D1A9, 5326S) were purchased from CST.

Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq)

ATAC-seq was carried out mainly according to TruePrep DNA Library Prep Kit V2 for Illumina (Vazyme, TD501) with minor modification. Briefly, we spun 30,000 cells at 500 g for 5 min. Cells were lysed for 10 min at 4 ℃ by using pre-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl2 and 0.1% IGEPAL CA-630). Nuclei were harvested for transposition reaction. After tagmentation, DNA was purified using the Agencourt AMPure XP kit (Beckman Coulter, A63880). To reduce GC and size bias, we determined the final PCR cycles using qPCR to allow library amplification to be stopped before saturation. We performed initial amplifications for five cycles, after which we took an aliquot of the PCR reaction and added 10 μl of the PCR

cocktail with SYBR Green at a final concentration of 0.3×. We ran this reaction for 25 cycles to calculate the additional cycles required for the remaining 45 μL reaction. We amplified library fragments for 12-13 cycles. The libraries performed double size selection for 300-500bp DNA fragments. Fragment distribution of libraries was assessed with Agilent 4200. The library preparations were sequenced on an Illumina Hiseq platform, and 150 bp paired-end reads were generated.

Cut&Tag-sequencing

We adapted the Cleavage Under Target & Tagmentation (CUT&Tag) assay to perform chromatin-immunoprecipitation sequencing. All procedures were carried out by following the manufacturer's instructions described in NovoNGSR CUT&Tag 3.0 (Novoprotein, N259-YH01). Briefly, fresh cells were harvested and counted. 10 μl concanavalin A-coated beads slurry were added to 100,000 cells to allow cell capture on an end-over-end rotator for 10 min. Captured cells were separated by putting the tubes on a magnet stand. Primary antibodies against H3K4me1 and H3K27ac were added to the cells at RT for 2 hr with gentle vortex. Secondary antibody was added at RT for additional 1hr. Then mix pA/G-Tn5 adapter complex to a final concentration of 1:100 with 100 μL sample at RT for 1hr. After binding Tn5-adapter mixture, add 50 μL tagmentation buffer at 37 ℃ for 1 hr. 10% SDS were added to stop tagmentation and solubilize DNA fragments at 55 ℃ for 10 min. Add 2 volume Tagment DNA Extract beads to the sample after termination. Beads were washed with fresh 80% ethyl alcohol twice. Universal or barcoded i5/i7 primers were used for library amplification. Post-PCR DNA library was purified with DNA Clean Beads followed by library quality inspection and Pair-end Illumina sequencing.

RNA Isolation and Sequence

RNA from cells was isolated using RNAprep Pure Cell/Bacteria Kit (TIANGEN) per instruction of the manufacturer. RNA samples were quantified by Qubit and an Agilent Bioanalyzer for the RNA integrity assessment. All samples had an RNA integrity number (RIN) of about 10. Following the manufacturer's recommendations, sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA), and index codes were added to attribute sequences to each sample. The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library was sequenced on an Illumina Novaseq platform, and 150 bp paired-end reads were generated.

Metabolomics

Bone marrow-derived macrophages (2×10^6) were seed in a 6-well plate and treated as above. Especially for metabolites tracing experiment, DMEM was replaced with glucose-free medium supplemented with 12.5 mM U-[^{13}C]-glucose (Cambridge Isotope Laboratories) with or without 4 mM glutamine (Gibco). For metabolites extraction, cells were washed three times with pre-cold PBS. After completely aspirating the liquid, the plates were put on ice, 1 ml of extraction buffer (volume ratio 4:1 methanol/water) was added and scraped on ice.

The lysate was transferred to a 2 ml tube and vortex for 30 seconds, followed by additional 10 min sonication in the ice bath, and then immediately frozen in a liquid nitrogen tank. The mixtures were centrifuged at 13,000 rpm for 15 min at 4 ℃. Finally, supernatants were transferred to a new tube and lyophilized.

For metabolites analysis, the liquid chromatography with SCIEX Exion LC AD was prepared, and all chromatographic separations were performed with a Millipore ZIC-pHILIC column (5 μm, 2.1× 100 mm internal dimensions, PN: 1.50462.0001). Column was maintained at 40°C and the injection volume of all samples was 2 μL. The mobile phase A composed by 15 mM ammonium acetate and 3 ml/L Ammonium Hydroxide (> 28 %) in LC-MS grade water. The mobile phase B is composed by LC−MS grade 90 % (v/v) acetonitrile in HPLC water. The mobile phase runs at a flow rate of 0.2 mL/min. The column was eluted with the following gradient program: 95% B held for 2 min, increased to 45 % B in 13 min, held for 3 min, and the post time was set 4 min. The QTRAP mass spectrometer used a Turbo V ion source. The ion source was run in negative mode with a spray voltage of -4,500 V, Gas1 40 psi and Gas2 50 psi and Curtain gas 35 psi. Metabolites were measured using the multiple reactions monitoring mode (MRM). The relative amounts of metabolites were analyzed by MultiQuant Software Software (AB SCIEX).

IL4c administration and LPS induced sepsis

For the administration of IL-4 complex (IL-4c), 5 μg of recombinant mouse IL-4 was complexed to 25 μg of anti-mouse IL-4 antibody (BioXCell,11B11), diluted in 200 μl of PBS and administered intraperitoneally. 24 hr post-IL-4c administration, mice were injected with 20 mg/kg LPS to induce sepsis. Serum was harvested from the caudal tail 2 hr post-LPS injection. ELISA determined the IL-6 and TNF-α levels in serum according to the manufacturer's instructions. Mice survival after LPS injection was monitored for 6 days.

OVA-induced Allergy model

C57BL/6 mice were intraperitoneally injected with 100 μg OVA (Sigma, A5253) absorbed to 10 % Alum (Sigma, A6435) with the final volume ratio of OVA to Alum as 1:1 on days 0 and 14. Mice were intraperitoneally injected with 0.1 mg OVA in 200 μl PBS from days 21 to 25. Mice were intraperitoneally injected with *Staphylococcus aureus* at 5×10^3 CFU / 200 μL PBS per mouse. On day 26, whole blood from the caudal tail was collected for calculating the CFU.

RNA-seq data analysis

Paired-end sequence reads were aligned to mouse genome reference (mm10) with HISAT2 and option as defaults. These reads mapping to each gene were named raw-count through feature-count. FPKM of each gene is based on the length of the gene and its raw count. The DESeq2 was used for differential gene expression analysis. Differentially expressed genes were selected by fold change and significance relative based on two biological replications. The significant genes show fold change above 2 with pvalues below 0.05. ClusterProfiler (R) was used in pathway analysis and the resulting padj (pvalue adjusted using Benjamini and

Hochberg's) cut off 0.05. For clustering of M2 signature genes, the STEM program was used. RNAseq data have been deposited in the GEO database with accession number GSE184811.

GEO data analysis

Data from GSE110465 were extracted by SRA-Toolkits. The reads were aligned to the mouse reference genome (mm10) by bowtie2 program. Then, the peaks were called by MACS2 callpeak with option p-value cutoff 0.01 and model fold 5-50.

All heatmap are created with ComplexHeatmap in R.

Data Resource

The accession number for the sequencing data reported in this paper is NCBI GEO: GSE184811(RNA-seq) and GSE196099 (Cut&tag ATAC sequence).

Statistics

Differences were analyzed using the two-tailed Student's t-test. Analyses were performed using Prism (GraphPad Software). Significant difference was label with star sign (p-value < 0.05). Data is shown as means ±SD.

Results

Type 2 immune response induced trained immunity

To examine whether type 2 immune response renders mice protection advantage against infection, we first sensitized mice with OVA followed by *S. aureus* (SA) challenge (Fig. 1A). Mice experiencing acute OVA-primed systemic allergy had delayed death upon lethal SA infection (Fig. 1B). The bacterial CFU in the blood was significantly lower in the OVA-primed group, suggesting that acute allergy response induced enhanced bactericidal effect (fig. S1A). Arg-1 and Rentla were explicitly upregulated in peritoneal macrophages isolated from the OVA-primed group (Fig. 1C), confirming that acute allergic response triggered type 2 response and induced M2 differentation. Moreover, pro-inflammatory genes such as IL-6, IL-1β, and NOS2 were upregulated (Fig. 1D). Furthermore, OVA-primed mice were still more resistant to SA infection even 7 days after the final OVA challenge (fig. S1B), suggesting acute allergy induced a long-lasting anti-bactericidal effect. IL-5 induced eosinophilia has been shown to mediate bactericidal function in type 2 immune response[11]. Therefore, to exclude the confounding effect induced by IL-5 induced eosinophilia in OVA-allergy model, we adopted another well-established IL-4c injection model to examine the role of macrophages[14]. To assess whether IL-4c treatment also conditions M2 macrophages with higher pro-inflammatory potential, LPS were injected into the peritoneal cavity one-day post-IL-4c injection. Serum (Fig. 1E) and peritoneal fluid (fig. S1C) IL-6 and TNF-α levels were greatly enhanced upon LPS stimulation. In addition, IL-4c treated mice were more susceptible to LPS sepsis (Fig. 1F), presumably due to the increased pro-inflammatory cytokine production. Furthermore, IL-4c pre-treated mice were more resistant to SA infection (Fig. 1G), suggesting the acute IL-4 treatment protected mice from bacterial infection, similar to the protective effect observed in β-glucan induced

trained immunity[15]. In summary, these data suggest that type 2 immune response induces trained immunity, such as producing more pro-inflammatory cytokines and more resistance to bacterial infection, in different animal models.

IL-4 induces trained immunity in macrophages

Based on the animal data, we speculated that IL-4 might have dual function on macrophages. On the one hand, IL-4 induced canonical M2 macrophages differentiation, while at the same time, IL-4 might train macrophages to produce more pro-inflammatory cytokines upon stimulation. To test this hypothesis, we assessed whether IL-4 could induce trained immunity in mouse bone marrow derived macrophages (BMDMs) as depicted in Fig. 2A. As a result, IL-6, IL-12, and IL-1β gene expression were upregulated (Fig. 2B), and the IL-6 protein level and NO production were upregulated (Fig. 2C), suggesting the long-lasting effect of IL-4 treatment, phenocopying the effect of the well-documented β-glucan induced trained immunity[16]. Noteworthily, the IL-10 expression was downregulated (fig. S2A). In addition, BMDMs received activation signaling constitutively from IL-4 for six days produced higher pro-inflammatory cytokines than the short-exposure group, suggesting a prolonged type 2 stimulation could induce a heightened pro-inflammatory effect (fig. S2B).

We further performed RNAseq to acquire a panoramic view of the transcriptomic change of IL-4 induced short-term (24 hours) and long-term (6 days) trained immunity. Gene expression profiles could be subdivided into 9 clusters (Fig. 2D). Cluster 1-3 are enriched with genes downregulated by IL-4 at both time points. Cluster 6, 7, and 9 are genes upregulated at 24 hours post-IL-4 but declined or returned to basal at day 6. Cluster 5 and 8 are enriched with genes upregulated at day six but not significantly altered at 24 hours. Cluster 7 contains M2 signature genes such as Arg1, Chil3, and Retnla, upregulated at 24 hours but returned to basal at day 6. Gene Ontology enrichment analysis of different clusters suggests cytokine production biological process was significant to downregulate in Cluster 1 and 3 (fig. S3A), and KEGG analysis suggesting that response against multiple pathogens were also enriched in Cluster 3 (fig. S3B), reinforcing the role of IL-4 in the induction of canonical anti-inflammatory response at basal state.

We further compared the gene expression profile upon LPS focusing on LPS upregulated genes. We subdivided the differential expressed genes into 3 clusters: trained, unaffected and tolerant cluster by comparing the expression level between control and IL-4 trained macrophages (Fig. 2E and fig. S4A). Pro-inflammatory genes, such as IL-6, IL-12a, and IL-1b, were encompassed in the trained cluster at both 24 hours and day 6, in line with the qPCR results (Fig. 2B). The GO enrichment analysis of the trained geneset also revealed the enrichment of cytokine/chemokine activity (Fig. 2F), supporting the idea that the non-canonical feature of IL-4 in promoting pro-inflammatory response.

Intrigued by the fact that IL-4 induces a more robust pro-inflammatory response in macrophages upon stimulation, we wonder whether other type 2 cytokines could also induce trained immunity. We found out that IL-13, but not IL-5, induced trained immunity in BMDMs as well (Fig. 2G & fig. S5), suggesting the signaling

transduction via IL-4Rα is crucial in inducing trained immunity. Surprisingly, IFNγ failed to induce a long-term pro-inflammatory phenotype, while stimulation with IFNγ and IL-4 simultaneously induced a similar response as IL-4 single stimulation (fig. S5). These results imply that type 2 cytokine IL-4 and IL-13 induce non-canonical trained immunity in BMDMs.

IL-4 induces non-canonical pro-inflammatory feature in a Gln/α-KG/jmjd3 axis- and STAT6-independent but IRS-dependent manner

Glutamine/α-ketoglutarate/jmjd3 axis is critical for IL-4 induced M2 polarization[17,18]. Therefore, to investigate whether this axis is also responsible for the non-canonical feature of IL-4, BMDMs were stimulated with IL-4 in the presence or absence of glutamine. In line with the previous report, M2 signature genes such as Arg1 and Chil3 were downregulated in glutamine-free conditions (Fig. 3A). In contrast, the pro-inflammatory gene (Fig. 3B&D) and protein expression (Fig. 3C&E) were not influenced both for short-term and long-term IL-4 stimulation. Similarly, inhibition with glutaminolysis or jmjd3 (Jumonji C domain containing 3) activity by BPTES or GSK-J4, respectively, impaired M2 signature gene expression (fig. S6A&B), while upregulation of IL-6 and downregulation of IL-10 were not altered (fig. S6C&D).

The STAT6 pathway is critical for IL-4 induced M2 polarization. We validated that STAT6 inhibition downregulated M2 gene expression (Fig. S6E) and STAT6 phosphorylation (fig. S6F). However, pro-inflammatory gene expression (Fig. 3F&G) and IL-6 protein and NO secretion (fig. S6G&H) were not influenced by STAT6 inhibitor both at 24 hours or 6 days. These results suggest IL-4 induced non-canonical trained immunity in a STAT6-independent manner.

Insulin receptor substrate (IRS) has been reported to transduce signals upon IL-4 stimulation[19]. Therefore, we examine whether inhibition of IRS by NT-157 impacts IL-4 induced non-canonical effect. Pretreatment of BMDMs with NT-157 significantly inhibited IL-4 induced pro-inflammatory gene expression (Fig. 3H). Meanwhile, M2 gene expression was also impaired by NT-157 treatment (fig. S6I), suggesting the IRS signal regulates IL-4 mediated canonical M2 differentiation and non-canonical pro-inflammatory feature. Furthermore, western blot analysis indicates that NT-157 blocks IL-4 induced STAT6 phosphorylation (fig. S6J), which partially explains why NT-157 inhibits M2 differentiation.

IL-4 induces distinct epigenetic remodeling in M2 signature and trained gene sets

Epigenetic modification and metabolic rewiring have been reported to be the two pillars underlying trained immunity. Therefore, we first assessed IL-4 stimulation-induced epigenetic reprogramming by re-examining the ATAC-seq data from the GEO database (GSE110465) to compare the chromatin accessibility of genes regulated by IL-4 in BMDMs. As expected, M2 signature genes Arg1, Egr2, Retnla, and Ccl24 had constitutively accessible chromatin regions, and higher gene expression in IL-4 stimulated macrophages (fig. S7A). In addition, the chromatin accessibility around the genes identified in the "trained" cluster, such as IL-6 and IL-12a, is also increased by IL-4 (fig. S7B). Noteworthily, IL-4 increases chromatin accessibility at distal

regulatory elements for genes in the trained cluster, while more relatively at promoter-proximal regions in the M2 set (Fig. 4A).

H3K4me1 marked poised enhancer has been proposed to serve as a fingerprint of epigenetic memory regulating the gene expression during embryonic cell development[20]. We speculate that IL-4 induced trained immunity might cause H3K4me1 marked poised enhancer around the "trained" genes, thus facilitating the higher transcription activity upon subsequent stimulation. As H3K4me3 and H3K4me1 have previously been reported to be the epigenetic landmark of β-glucan induced trained immunity in macrophages, we further examined the epigenetic profiles of IL-4 trained macrophages by CUT & tag approach. The distal enhancers of proinflammatory genes such as IL-6 and IL-12a were indeed marked with poised enhancer signatures with enriched H3K4me1 peak but lack of ATAC and H3K27ac peaks (Fig 4B). Similar to the ATACseq result, H3K4me1 peaks were more enriched at the distal region in the trained geneset (fig. S7C). The poised enhancer bears epigenetic memory as the subsequent LPS stimulation resulted in higher proinflammatory gene expression in IL-4 trained group (Fig 4C).

WDR5 forms a complex with MLL and Set H3K4 histone methyltransferase family. Knocking down of WDR5 partially inhibits IL-4 induced augmentation of IL-6 and IL-1β expression (Fig. 4C & fig. S7D), suggesting a potential role of H3K4 methylation in IL-4 induced trained immunity. These results imply that WDR5 mediated H3K4me1 methylation might induce poised enhancers at the "trained" genes and results in the IL-4 induced non-canonical trained immunity in macrophages. Furthermore, inhibition of histone methylation by MTA (pan-methyltransferase inhibitor) impaired IL-4 induced IL-6 and IL-1β gene expression (Fig. 4D) and the production of IL-6 and NO (Fig. 4E), while MTA did not influence IL-4 induced M2 differentiation as evidenced by the expression of Arg-1 and Rentla (fig. S7E). Putting together with the data from the glutamine deficiency experiment, we speculate that IL-4, on the one hand, could induce canonical M2 gene expression via Gln/α-/jmjd3/H3K27me3 axis[17,18]. On the other hand, IL-4 induces non-canonical pro-inflammatory trained immunity via H3K4me1 marked poised enhancer at the proinflammatory genes.

IL-4 primed macrophages are more energetic and switch toward glycolysis upon LPS stimulation

Upon stimulation, metabolic rewiring from oxidative phosphorylation to aerobic glycolysis is essential for mounting efficient pro-inflammatory responses in macrophages. In addition, we previously reported that β-glucan trained macrophages exhibit higher aerobic glycolysis metabolic status enabling a prompt response upon stimulation [21]. We found that lactate production was significantly increased in IL-4 trained macrophages upon LPS stimulation but not at the basal state (Fig. 5A). In line with the elevated lactate level, essential glycolysis proteins such as HK3 and GLUT1 were also increased (Fig. 5B), indicating the elevated pro-inflammatory feature of IL-4 trained macrophages possibly came from the robust shift towards glycolysis upon LPS stimulation. However, 24 hours short-term IL-4 treatment does not upregulate HK3 and GLUT1 protein levels, while IL-4 pre-treated macrophages still produce more lactate upon LPS stimulations (fig. S8A&B).

We further assess the effect of IL-4 on the energetic status both at acute (24 hours) or trained (6 days) states. Acute IL-4 treatment increases macrophages' glycolysis and glycolytic capacity at basal and upon LPS stimulation (Fig. 5C). Basal respiration, ATP production, and maximal respiration capacity are also increased by acute IL-4 treatment (Fig. 5D). In line with the increased lactate secretion, IL-4 trained macrophages have similar basal glycolysis compared to control macrophages. In contrast, trained macrophages upregulate glycolysis upon LPS stimulation and have a higher glycolytic capacity (Fig. 5E). The OCR and mitochondrial capacity of IL-4 trained macrophages had a similar pattern (Fig. 5F). Overall, IL-4 trained macrophages are more energetic at the basal and upon stimulation (fig. S8C). We further performed stable isotope tracing in IL-4 stimulated macrophages with the tracer [U-$^{13}C_6$]-Glucose. In agreement with the seahorse result (Fig. 5C), ^{13}C-labeled glycolytic intermediates were significantly increased in IL-4 stimulated macrophages (Fig. 5G). The proportion of ^{13}C-labeled TCA cycle intermediates, such as citrate, α-ketoglutarate, succinate, and fumarate, was also primarily upregulated in IL-4 stimulated macrophages (fig. S8D), implying with higher OXPHOS capacity and in line with the OCR result (Fig. 5D). Noteworthily, the elevated enrichment of ^{13}C-labeled α-ketoglutarate, succinate, and fumarate were blunted when macrophages were cultured in a glutamine-free medium (fig. S8D). In agreement with the observed metabolic rewiring, the glycolysis, TCA cycle, and glutamine metabolism genes were also upregulated in IL-4 stimulated macrophages (Fig. 5H). We further performed metabolomics analysis for IL-4 stimulated BMDMs. Principle component analysis (PCA) suggests that LPS stimulation induced a significant metabolic change at PC1, while LPS stimulated IL-4 day 6 macrophages further separated from the rest of the LPS stimulated group (fig. S9A). The TCA cycle and glycolytic intermediates were increased in IL-4 trained macrophages at both time points (fig. S9B). Noticeably, the one-carbon metabolism intermediates such as SAM and SAH are notably upregulated (Fig. S9C). The elevated one-carbon metabolism might provide a methyl donor for IL-4 induced histone methylation.

Discussion

Type 2 immunity is critical for host defense against helminth infection and plays an essential role in allergic and asthmatic diseases. IL-4 is one of the central cytokines upregulated in type 2 immune response. The canonical function of IL-4 in inducing M2 macrophage differentiation and mediating anti-inflammatory responses is well documented. The signaling transduction and pathway involved in IL-4 induced canonical features are well characterized. However, an unrecognized non-canonical feature of IL-4 induction of trained immunity has never been described. We report that type 2 immunity enhanced host bactericidal capacity via a trained immunity mechanism. Pre-conditioning with IL-4 in acute type 2 immune response induced a non-canonical, more robust responsiveness to microbial stimuli, similar to β-glucan-induced trained immunity, upon stimulation in macrophages.

Metabolic rewiring and epigenetic control have been reported to play critical roles in the canonical M2 macrophage polarization. Jha et al. demonstrated that M2 polarization activates glutamine catabolism and

UDP-GlcNAc-associated modules[22]. When macrophages were polarized towards M2 in glutamine deprived medium or with N-glycosylation inhibitor tunicamycin, M2 polarization and production of CCL22 were significantly reduced. In addition, jmjd3, an H3K27me3 demethylase, has been reported to be upregulated by IL-4 during M2 polarization, and knockdown of jmjd3 also impairs M2 polarization *in vitro*[23]. Furthermore, helminth-induced M2 polarization is significantly reduced in Jmjd3 -/- mice[17]. α-ketoglutarate has recently been demonstrated to serve as a central hub bridging glutamine metabolism and jmjd3-mediated epigenetic programming in M2 macrophages[18]. In addition to glutaminolysis, α-ketoglutarate is also derived as a by-product of the de novo serine synthesis pathway via the upregulation of phosphoglycerate dehydrogenase (Phgdh) activity induced by IL-4[24]. Although the Gln/α-KG/jmjd3 axis has been demonstrated to be crucial for IL-4 mediated M2 differentiation via downregulating H3K4me3, this axis is dispensable for the non-canonical trained immunity induced by IL-4 in macrophages. Our data suggest that IL-4 treatment, on the one hand, induces M2 gene expression via STAT6- and Gln/α-KG/jmjd3-axis, which opens up the promoter region of M2 signature genes by downregulation of the H3K27me3[17,18]. On the other hand, IL-4 induces trained immunity through an IRS-dependent manner and via recruiting WDR5 interacting H3K4 methyltransferase, upregulating H3K4me1 around the enhancer region of the "trained" genes, including pro-inflammatory and glycolysis genes. The H3K4me1 poised enhancer serves as epigenetic memory, facilitating a higher transcription activity upon stimulation resulting in a more robust pro-inflammatory phenotype (Fig. 6).

The memory feature of innate immune memory or trained immunity differs from the antigen-specific memory induced by adaptive immunity[5]. Instead, epigenetic reprogramming and metabolic rewiring are responsible for the non-specific antigen augmentation of immune response responding to subsequent same or different antigens in trained immunity[25]. In the well-defined β-glucan trained immunity, enhanced glutaminolysis and accumulation of fumarate induced by β-glucan simulation are responsible for the H3K4me3 and H3K27ac in the trained macrophages[26]. In addition, β-glucan upregulated Set7 methyltransferase and induced the H3K4me1, thus increasing the MDH2 and SDHB and oxidative phosphorylation capacity of trained macrophages[27]. Moreover, the rewired glycolysis capacity has been suggested to be critical for β-glucan induced trained immunity both *in vitro* and *in vivo*[16]. The epigenetic memory could further be explained by the existence of latent enhancers induced by external stimulation, which have a persistent effect mediating a faster and more robust response upon stimulation[28]. This latent enhancer-mediated epigenetic memory could be the underlying mechanism of IL-4 induced non-canonical trained immunity, with the genes in the "trained" cluster being robustly induced upon stimulation but remaining quiescent at the basal state.

Previous report suggested that type 2 immune response might render host a beneficial bactericidal effect (*10*, *11*). Although the accumulation of eosinophils and ILC2 has been suggested to play a role in the bactericidal effect in the acute type 2 immune response, here we uncovered the role of macrophages in the enhanced bactericidal capacity. Acute IL-4c injection has been demonstrated to induce peritoneal macrophages

differentiation toward M2-biased phenotype[29]. However, *ex vivo* stimulation of peritoneal macrophages with IL-4 enhanced IL-6 and TNF-α production upon nematode *Neisseria meningitidis* infection[30] which is in accordance with the non-canonical feature of IL-4 observed here. In addition to IL-4, another type 2 cytokine IL-13, but not IL-5, could also induce trained immunity in macrophages *in vitro*. This prompts us to wonder whether type 2 response could have an acute and chronic effect on macrophages *in vivo*. Mice injected with IL-4c were more susceptible to LPS induced sepsis and secreted more IL-6 and TNF-α, suggesting acute IL-4 priming could enhance the pro-inflammatory response *in vivo*. This result is in line with a previous report demonstrating that pretreatment with peritoneal macrophage with IL-4 augments the production of cytokines and chemokines[31]. As trained immunity has been reported to possess a higher bactericidal effect in several infection models[16], we also found that IL-4 pretreatment mice were more resistant to an *S. aureus* infection. In addition, type 2 immune response induced by OVA also renders mice better protection against the subsequent bacterial infection. Moreover, the peritoneal macrophages from OVA-challenged mice have upregulated IL-6 production compared to control mice, suggesting that macrophages could acquire a pro-inflammatory phenotype in the acute type 2 immune response. Therefore, our data suggest an acute type 2 immune response could induce trained immunity *in vivo*. It would be worthwhile to examine whether circulating monocytes from patient with acute allergic or post-allergic response bears trained immunity phenotype for the translational purpose.

In contrast to our finding, Czimmerer et al. reported that IL-4 stimulated macrophages suppressed NLRP3 inflammasome activation and IL-1β secretion upon LPS stimulation in a STAT6 dependent manner [32]. This seemingly contradictory result could be due, at least partly, to the way of BMDMs differentiation. Czimmerer et al. used a conditioned medium from L929 cells while we used recombinant M-CSF for BMDM differentiation. As L929 conditioned medium contains M-CSF and other soluble factors, such as macrophage migration inhibitory factor (MIF), osteopontin, Ccl2, and Ccl7, derived from L929, L929 conditioned medium differentiated macrophages were functionally different from recombinant M-CSF derived macrophages[33]. In addition, Czimmerer et al. used *Helgmosomoides polygyrus* infection model to induce a type 2 immune response in mice. However, instead of assessing the role of IL-4 on peritoneal macrophage by bacterial infection, they examined the peritoneal macrophages isolated from *H. polygyrus* infected mice *ex vivo* and showed that peritoneal macrophages from *H. polygyrus* infected group have lower Nlrp3 and Il1b expression upon LPS stimulation. In contrast, we adopted a different model to assess whether a prior type 2 response could induce the non-canonical effect of IL-4 *in vivo,* and the collective results point to the same effect that type 2 response causes a more robust pro-inflammatory response *in vivo* and renders a better protection capacity upon bacterial infection.

Metabolically, IL-4 stimulated macrophages are more energetic with higher basal respiration rate and elevated glycolysis at basal while switching toward robust glycolysis upon stimulation and have higher glycolytic

capacity. Metabolomics data reveal that IL-4 trained macrophages had elevated one-carbon metabolism, TCA cycle, and glycolysis. The elevated one-carbon metabolism may provide SAM as a methyl donor for epigenetic modification. Succinate accumulation might be responsible for the enhanced IL-1β expression[34] and subsequent H3K4me1 accumulation[27]. Fumarate accumulation has been suggested to be involved in β-glucan induced H3K4me3 accumulation[26]. Therefore, the elevated intermediate TCA cycle metabolites accumulation in IL-4 stimulated macrophages might impact IL-4 induced trained immunity via epigenetic modification.

To sum up, we propose that IL-4 induced both canonical M2 and non-canonical trained immunity via different sets of epigenetic modifications. The non-canonical trained immunity induced by IL-4 is characterized by a poised enhancer, enabling the trained macrophages to produce more pro-inflammatory cytokines upon stimulation. Due to the association between asthma and chronic inflammatory diseases, such as obesity, or asthma and acute inflammatory response, such as sepsis, we believe a further characterization of IL-4 induced trained immunity will fill the gap of the Th2-biased disease asthma and chronic and acute inflammatory diseases. Thus, our data here provide a new vista considering the well-characterized anti-inflammatory cytokine IL-4 and offer a new horizon to cope with Th2 disease-associated inflammatory diseases.

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Writing – review & editing: DB, JZ, NX, W-SL, KM, SL, JH, SC-CH, P-CH, S-CC

Conflict of interest

Authors declare that they have no conflict of interest.

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Figures Legends

Figure. 1. Type II immune response induced trained immunity *in vivo*. (**A**) Schematic presentation of mice OVA-allergy model. (**B**) Survival curve of control or OVA-primed mice after *S. aureus* infection. (**C**) qPCR analysis of the M2 marker genes from peritoneal macrophages isolated at day 25 from the OVA challenged or PBS control group. (**D**) Peritoneal macrophages from the OVA challenged or PBS control group were stimulated with LPS for 4 hours *ex vivo*. The expression of cytokine genes was determined by qPCR. *P < 0.05, unpaired, two-tailed Student's t-test. Data are representative of 2 independent experiments with 10 mice per group (mean ± SD). (**E**)IL-6 and TNF-α were determined from serum taken 3 hours post LPS injection in control and IL-4c treated mice. Survival of wild-type C57BL/6J pre-treated with PBS or IL-4c for 24 hours followed by (**F**) LPS i.p. injection or (**G**) *S. aureus* infection. Data are representative of 2 independent experiments with 10 mice per group.

Figure 2. IL-4 induced non-canonical trained immunity in macrophages. (A) Schematic presentation of the experimental setup for assessing short-term or long-term IL-4 non-canonical effect in macrophages. (B) Short-term (Day 2) or long-term (Day 6) IL-4 stimulated BMDMs were stimulated with LPS for 4 hours, and mRNA was harvested for subsequent qPCR analysis. (C) IL-6 and NO production were determined from the culture supernatant 24 hours post LPS stimulation. (D) Differential gene expression analysis between the control, IL-4 24 hours stimulated, and IL-4 trained BMDMs at day 6 was presented as a heatmap. (E) Control and IL-4 24 hours stimulated BMDMs were stimulated with LPS or left untreated for 4 hours. LPS upregulated genes were filtered out, and the expression level was ranked normalized according to the Log2FC value between the control-LPS group and IL-4-LPS group. Genes were further divided into three clusters: trained (Log2FC ≥0.5), unaffected (0.5 ≥ Log2Fc ≥ -0.5), and tolerant (Log2FC ≤ -0.5). (F) Gene ontogeny analysis of genes in the trained clusters. (G) IL-13 stimulated BMDMs were stimulated with LPS for 4 hours, and mRNA was harvested for subsequent qPCR analysis for the expression of IL-6 and IL-12. IL-6 and NO production were determined from the culture supernatant 24 hours post LPS stimulation. *P < 0.05, unpaired, two-tailed Student's t-test. Data are representative of 3 independent experiments with 3 to 4 samples per group (mean ± SD).

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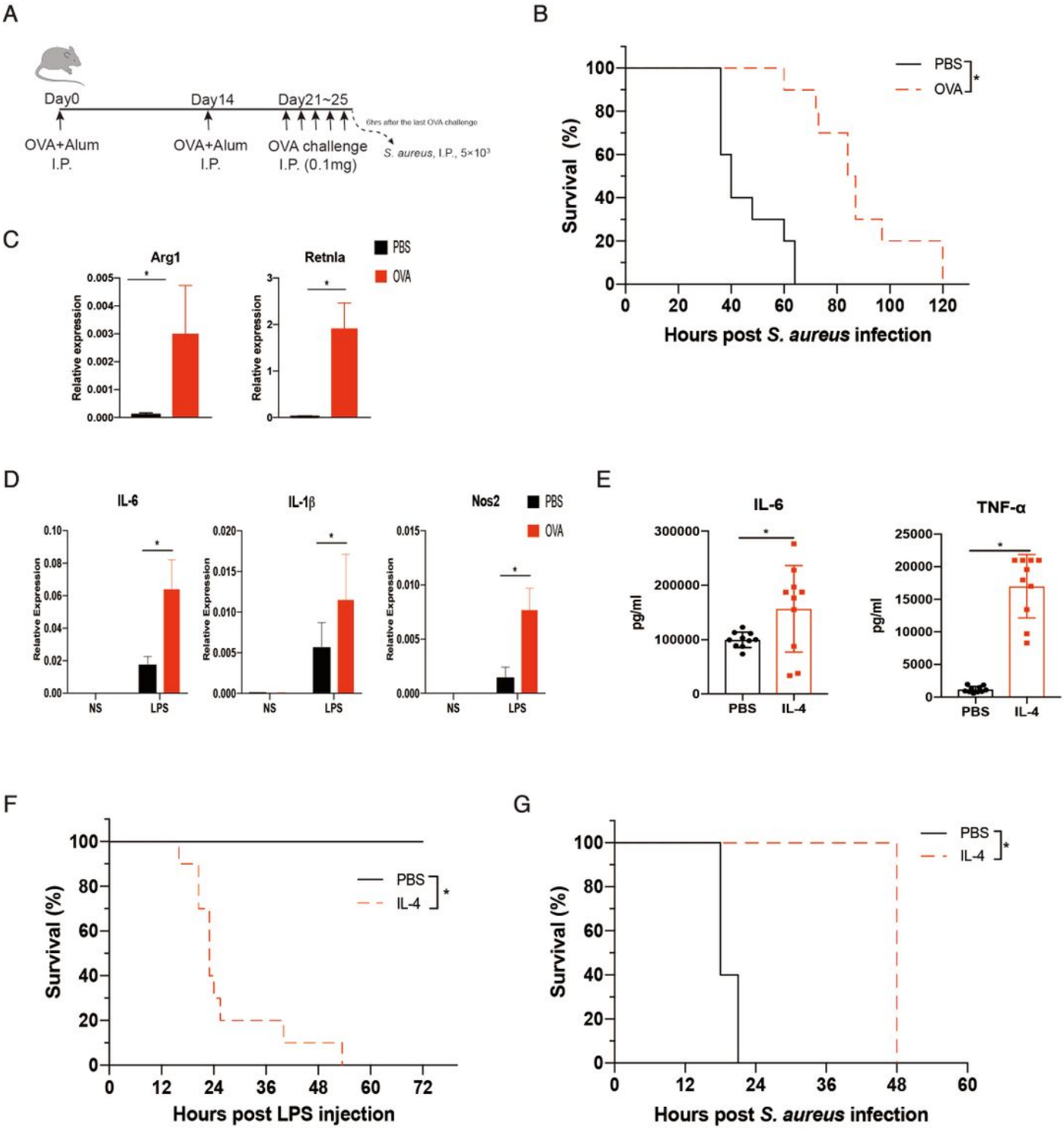
Figure 5. Metabolic characterization of IL-4 induced trained immunity in macrophages. (**A**) IL-4 trained BMDMs at day 6 were stimulated with LPS. The supernatant was harvested 24 hours post LPS, and lactate level was determined. (**B**) Western blot from cell lysate harvested from control or IL-4 trained BMDMs at day 6 or 3 hours post LPS stimulation. Antibodies specific for endogenous GLUT1, HK3 and actin were used to blot the target protein. Representative blots of three independent experiments are shown. Seahorse analysis of control or IL-4 induced trained BMDMs at both acute (24h) or trained (6 days) at basal or 3 hours post LPS stimulation. ECAR and glycolysis capacity (**C&E**) and OCR and mitochondrial respiration capacity (**D&F**) were determined from two independent experiments. (**G**) Representative metabolites derived from $^{13}C_6$-Glucose following the glycolysis pathway determined by LC-MS were shown. (**H**) Differential gene expression in glycolysis, TCA cycle, and glutamine metabolism between the control, IL-4 24 hours stimulated, and IL-4 trained BMDMs at day 6 was presented as a heatmap.

Fig. 6. Schematic model of IL-4 induced canonical and non-canonical responses.

Figures

Figure 1



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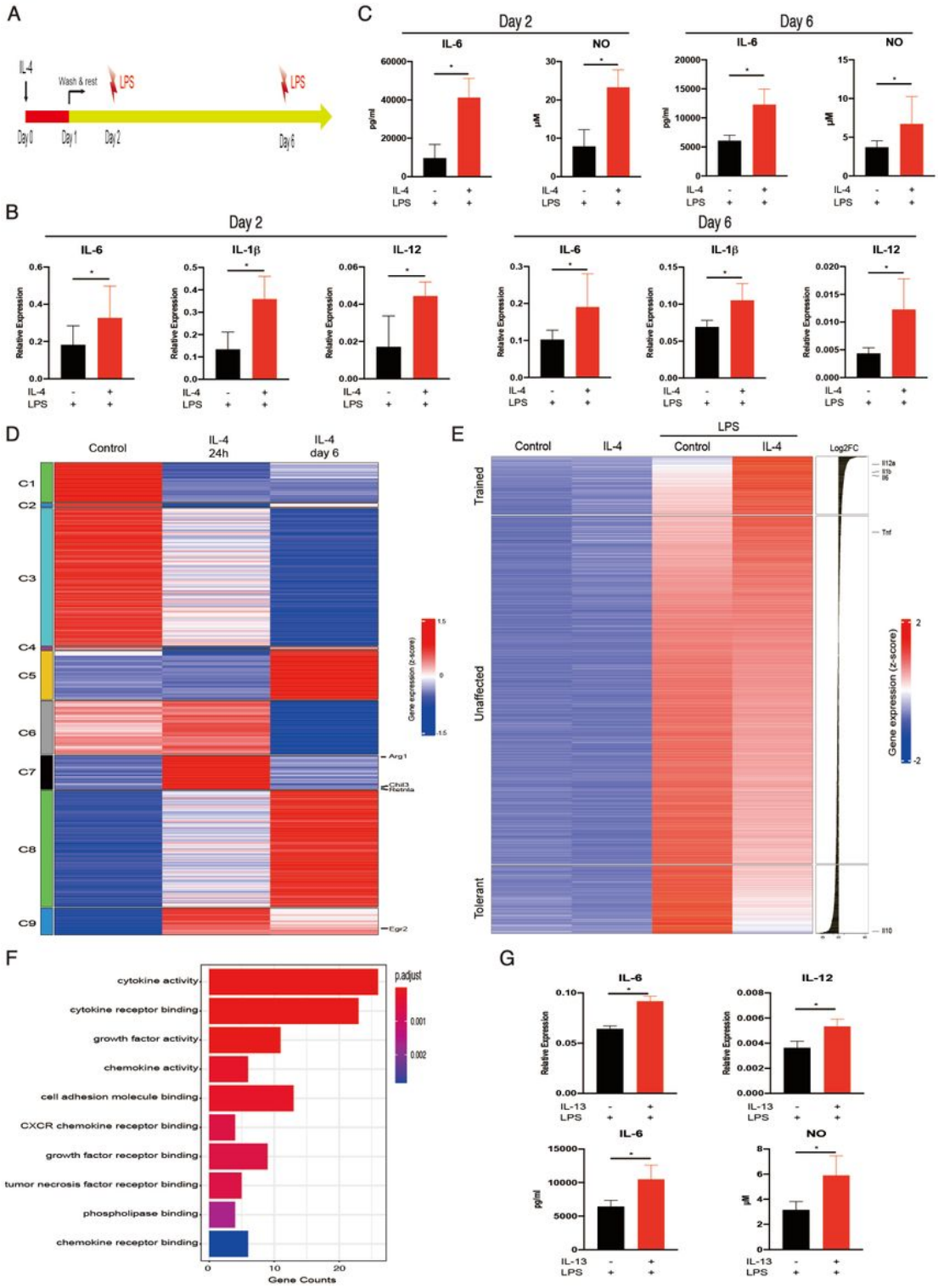

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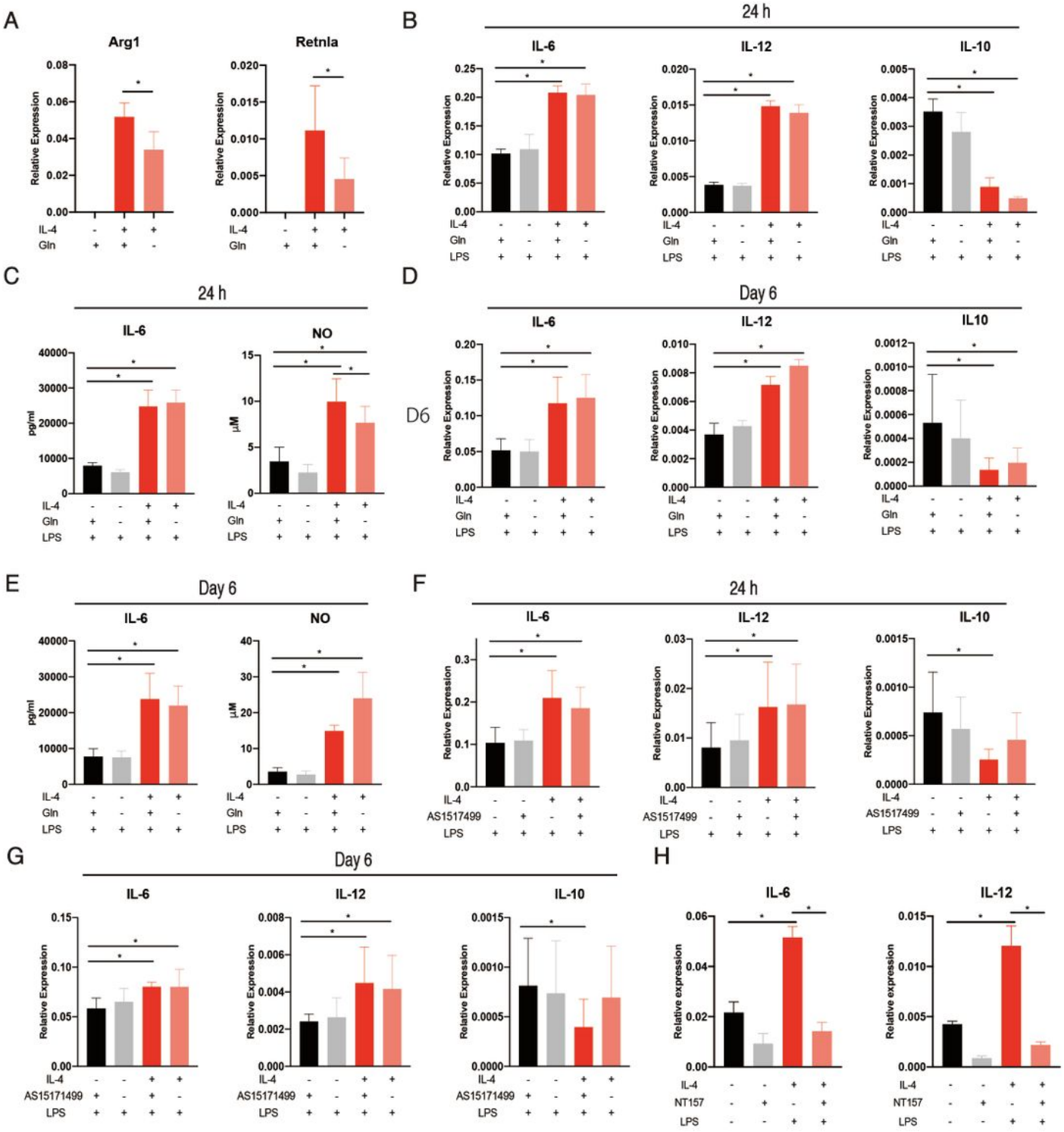

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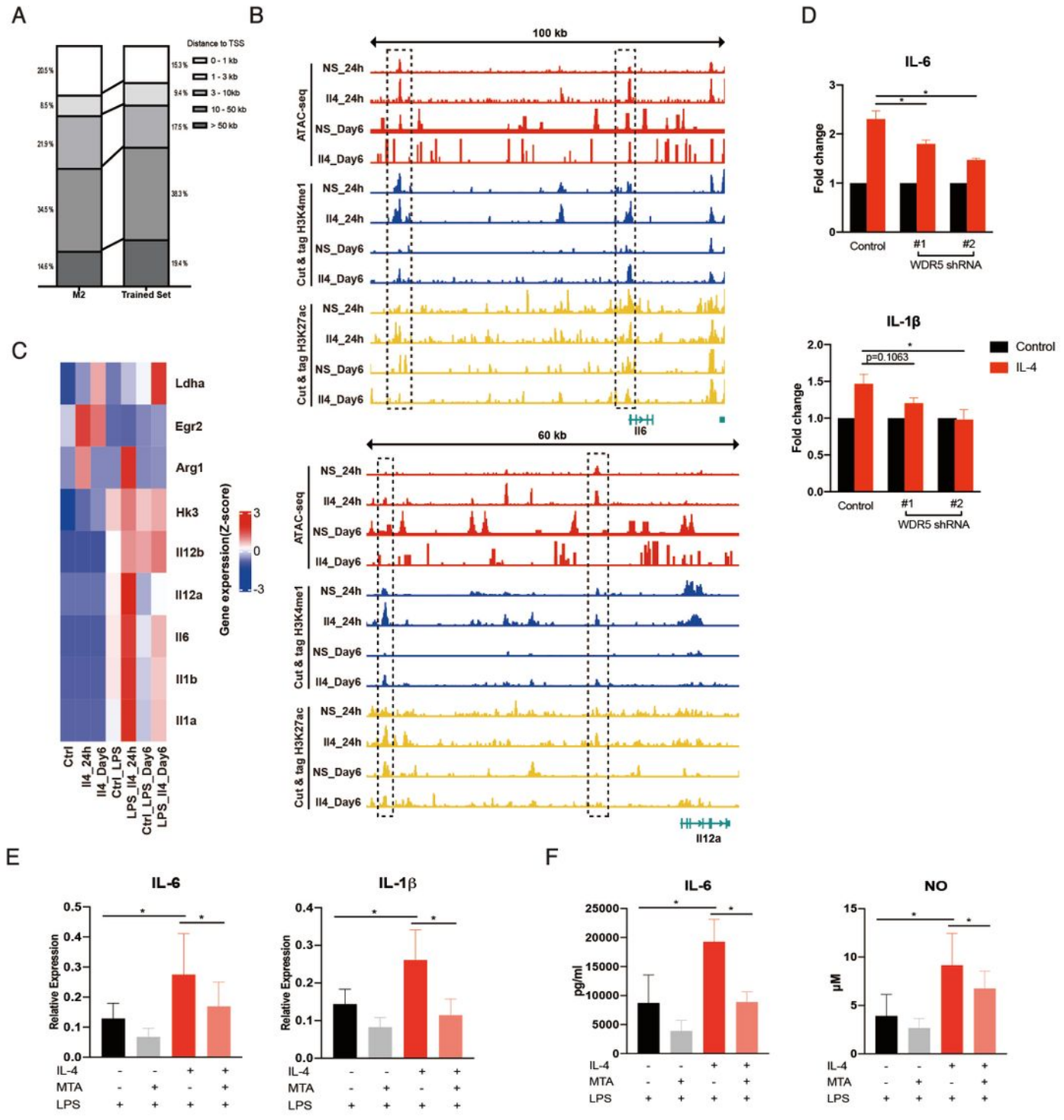

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BMDMs. (**B**) Representative ATACseq H3K4me1, H3K4ac CUT & tag screenshots of control or IL-4 24h and day 6 stimulated BMDMs in the gene region pro-inflammatory genes IL-6 and IL-12a. The dashed box pointed to the poised enhancer region. (C) The gene expression profile of selected genes at basal state or 4h-post LPS stimulation. (**D**) Control or WDR5 stable knockdown Raw264.7 cells were pre-treated with IL-4 for 24 hours and stimulated with LPS for 4 hours to assess the mRNA expression of IL-6 and IL-1β by qPCR. (**E**) qPCR mRNA expression of cytokine genes in BMDMs pre-treated with MTA for 30 minutes followed by 24 hours IL-4 stimulation. The gene expression was determined 4 hours post LPS stimulation. (**F**) IL-6 and NO production was determined from 24 hours supernatant post LPS stimulation. *P < 0.05, unpaired, two-tailed Student's t-test. Data are representative of 3 independent experiments with 3 samples per group (mean ± SD).

Figure 5

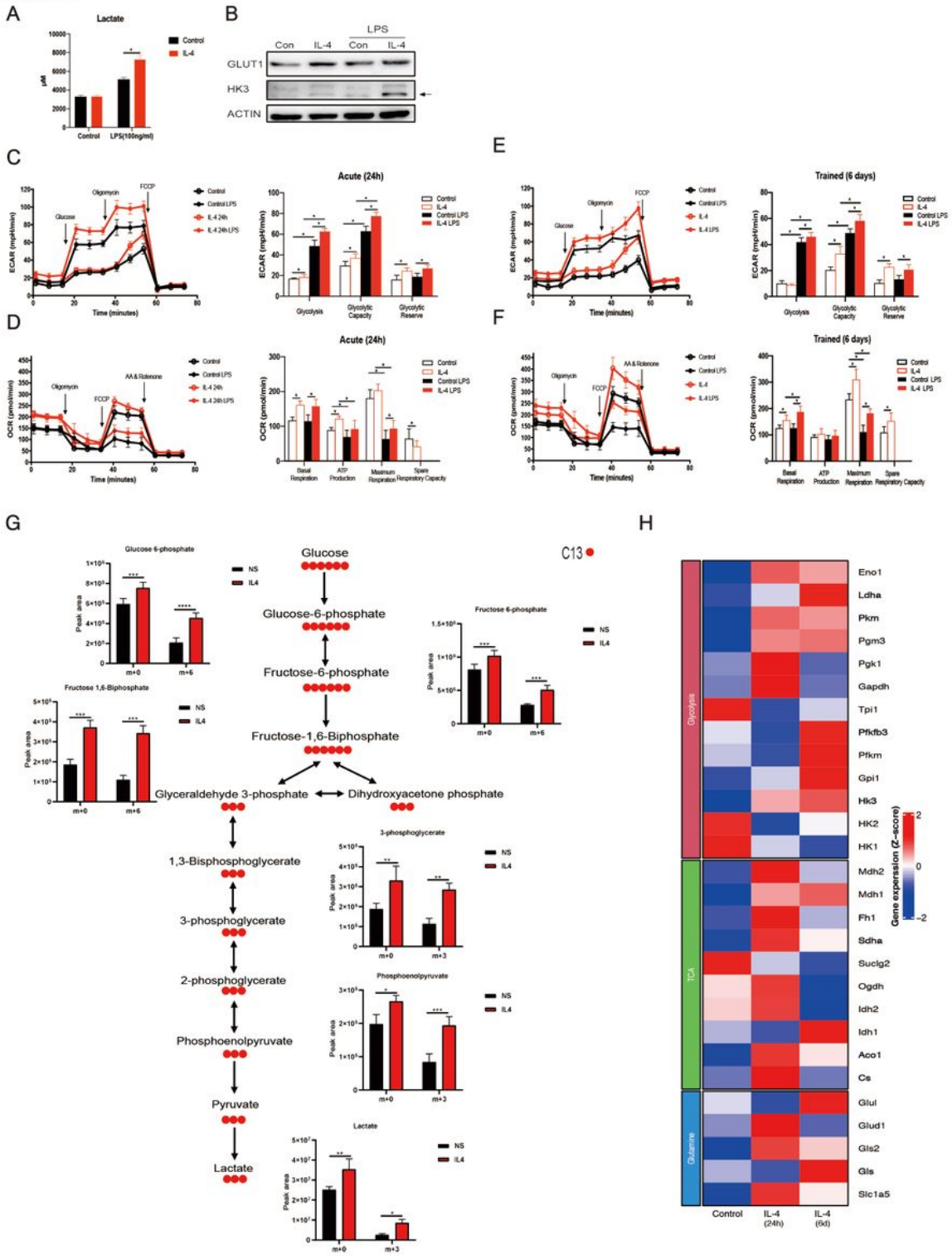

Figure 5

Metabolic characterization of IL-4 induced trained immunity in macrophages. (**A**) IL-4 trained BMDMs at day 6 were stimulated with LPS. The supernatant was harvested 24 hours post LPS, and lactate level was determined. (**B**) Western blot from cell lysate harvested from control or IL-4 trained BMDMs at day 6 or 3 hours post LPS stimulation. Antibodies specific for endogenous GLUT1, HK3 and actin were used to blot the target protein. Representative blots of three independent experiments are shown. Seahorse analysis

of control or IL-4 induced trained BMDMs at both acute (24h) or trained (6 days) at basal or 3 hours post LPS stimulation. ECAR and glycolysis capacity (**C&E**) and OCR and mitochondrial respiration capacity (**D&F**) were determined from two independent experiments. (**G**) Representative metabolites derived from $^{13}C_6$-Glucose following the glycolysis pathway determined by LC-MS were shown. (**H**) Differential gene expression in glycolysis, TCA cycle, and glutamine metabolism between the control, IL-4 24 hours stimulated, and IL-4 trained BMDMs at day 6 was presented as a heatmap.

Figure 6

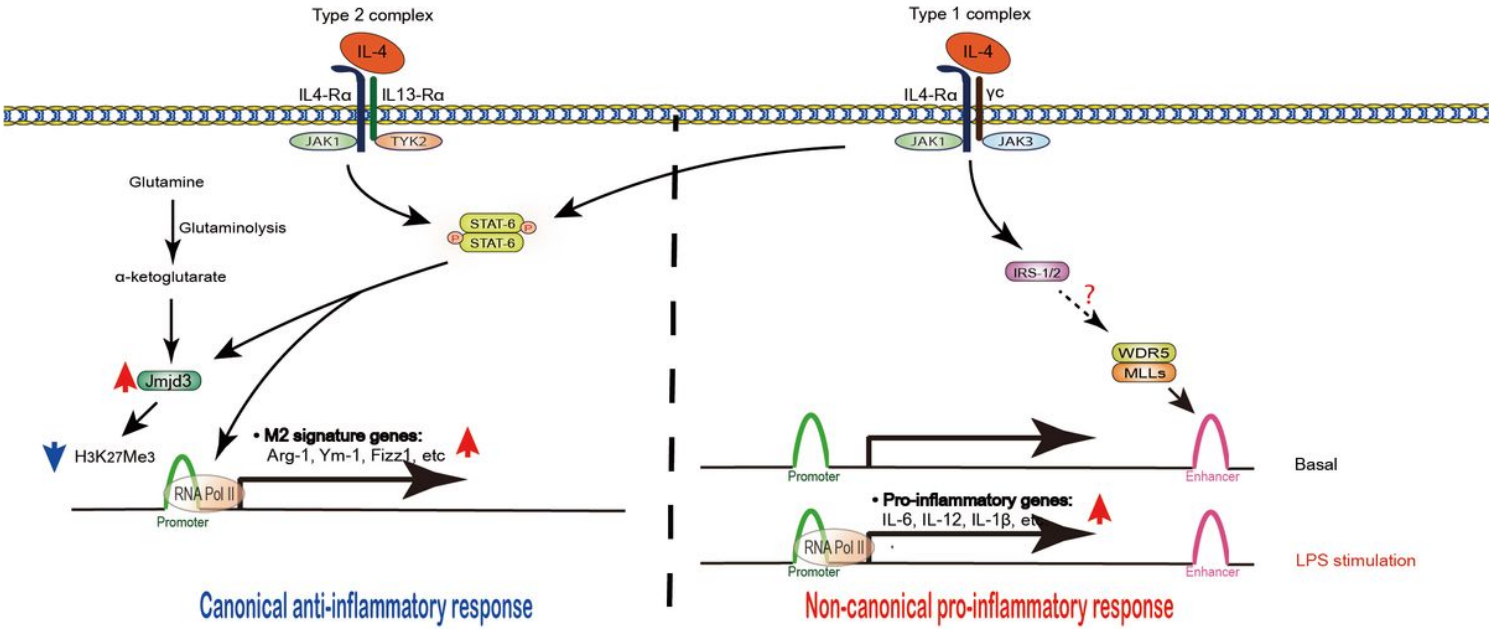

Figure 6

Schematic model of IL-4 induced canonical and non-canonical responses.

Supplementary Files

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- Supplementaryfigures.pdf