Activated Ets2 Is Required for Persistent Inflammatory Responses in the Motheaten Viable Model

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Activated Ets2 Is Required for Persistent Inflammatory Responses in the Motheaten Viable Model


The Ets2 transcription factor is constitutively phosphorylated on residue Thr^72 in macrophages derived from mice homozygous for the motheaten viable (me^-v) allele of the hemopoietic cell phosphatase (Hcph) gene. To genetically test the importance of signaling through residue Thr^72 of Ets2 during inflammation, the Ets2^A72 mutant allele, which cannot be phosphorylated on Thr^72, was combined with the Hcph^me^-v allele in mice. Ets2^A72/A72 moderated the inflammation-related pathology of Hcph^me^-v/me^-v mice, as demonstrated by the increased life span and the decreased macrophage infiltration in skin and lungs of these mice. Macrophage apoptosis induced by cytokine withdrawal was also increased in the double-mutant mice. Importantly, the Ets2^A72 allele resulted in decreased expression of inflammatory response genes, including TNF-α, CCL3, matrix metalloprotease 9, integrin αM, and Bcl-X in alveolar macrophage. Ets2 phosphorylation was required for persistent activation of TNF-α following LPS stimulation of bone marrow-derived macrophages. The phosphorylation of Ets2 functions in the severe inflammatory phenotype of the me^-v model by mediating both macrophage survival and inflammatory gene expression.


The ETS family, consisting of 28 members in the human genome, encodes for sequence-specific DNA-binding proteins that are transcriptional activators and repressors (1). The family is defined by a highly conserved DNA binding domain referred to as the ETS domain. However, the DNA-binding properties of these factors are similar and cannot entirely account for the specificity required for the precise activation of target genes that occurs during the diverse biological processes mediated by individual family members. Modification of discrete family members by signal transduction pathways provides an additional mechanism to determine specificity (1).

For example, phosphorylation of the ETS family members Ets1 and Ets2 by ras-dependent pathways leads to persistent expression of target genes, including extracellular proteases such as urokinase plasminogen activator (uPA) and stromelysin/matrix metalloprotease-3 (MMP-3) (2, 3). Ets1 and Ets2 are phosphorylated at a conserved residue (Thr^44 and Thr^72, respectively) by the well-characterized ras-effector pathway, the Raf/MAPK pathway (4–6). The key phosphorylation event occurs within a region of Ets1 and Ets2 that is conserved through evolution with the Drosophila P2 protein, and has been termed the pointed domain (7, 8). This region of homology plays a role in protein-protein interactions, both homophilic and with other proteins (1, 9). Changing the codon for Thr^72 in the Pointed domain of Ets2 to alanine (Ets2^T72A) results in a gene product with lowered trans activation potential especially evident in the presence of ras signaling (2–6). When the Ets2^A72 mutation is introduced into the mouse germline, it behaves as a hypomorphic allele that is haploinsufficient for embryonic development, but homozygous viable (10).

To define the biological role of Ets2 phosphorylation, we have previously studied Ets2 activation in macrophages, particularly in the context of the motheaten viable (hemopoietic cell phosphatase (Hcph^me^-v/me^-v)) mouse mutation (11). We demonstrated that Ets2 was constitutively phosphorylated on residue Thr^72 by a P38Kjun N-terminal kinase pathway in the Hcph^me^-v/me^-v model of acute inflammation, in contrast to wild-type cells, in which Ets2 phosphorylation is dependent on cytokine action, for example CSF-1. Furthermore, constitutive Ets2 activation correlated with expression of target genes such as Bcl-X and uPA and with the increased survival of Hcph^me^-v/me^-v macrophages. To directly test the role of Ets2 phosphorylation in the pathology of Hcph^me^-v/me^-v mice, we combined the Ets2^A72/A72 allele with Hcph^me^-v/me^-v genes in mice. This genetic analysis demonstrates the importance of MAPK activation of Ets2 in the chronic inflammatory responses characteristic of Hcph^me^-v/me^-v mice such as TNF-α induction, and confirms a key role for Ets2 in mediating CSF-1 survival signaling in macrophages.

Materials and Methods

Mice

The Ets2^A72A mutation (Ets2^A72A/v in the work as Ets2^A72) was generated by knockin gene targeting, as described (10), and a congenic FVB/N line carrying this mutation was created by nine generations of backcross. Ets2^A72A/me^-v mice are viable and fertile, but limit the growth of mammary tumors (10). The Hcph^me^-v mutation was originally obtained in the C57BL/6J background from The Jackson Laboratory (Bar Harbor, ME) and a congenic FVB/N line established after nine generations of backcrosses. The phenotype of Hcph^me^-v mice in the FVB/N background was identical with that reported for the mutation in the C57BL/6J background (12). All mice were genotyped by PCR. Primers and conditions used are available upon request. Use and care of mice in this study were approved

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2 Address correspondence and reprint requests to Dr. Michael C. Ostrowski, Department of Molecular Genetics, Ohio State University, Riverside Building, Room 834, 406 West 12th Avenue, Columbus, OH 43210. E-mail address: ostrowski.48@osu.edu
3 Abbreviations used in this paper: uPA, urokinase plasminogen activator; BMM, bone marrow-derived macrophage; Hcph, hemopoietic cell phosphatase; me^-v, motheaten viable; MMP, matrix metalloprotease.
by the Ohio State University Institutional Animal Care and Use Committee.

**Cell culture**

Bone marrow-derived macrophages were obtained, as described (11). The collection of thioglycolate-elicited peritoneal macrophages was described previously (13). The lavage fluid was centrifuged, and the cells were plated in six-well dishes (non-tissue culture treated). Thirty minutes later, the unattached cells were removed and total RNA was extracted from attached macrophages. The collection of murine alveolar macrophage was conducted, as described (14). The lung alveolar cells were lavaged, centrifuged, and resuspended in 1 ml of RPMI 1640 medium with 0.1% heat-inactivated calf serum, and transferred to a well of a 24-well dish (non-tissue culture treated). Six hours later, nonadherent cells were removed and RNA was prepared from the adherent macrophages.

**Cell survival assays**

The viability assay was conducted using the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes, Eugene, OR), using procedures described by the manufacturer. DNA fragmentation assay was performed, as described (15). The fragmented DNA from apoptotic cells was mixed with SyBr Green (Molecular Probes), and analyzed in 1.2% agarose gels. The gel was visualized under UV light in a gel imager (Bio-Rad, Hercules, CA). The caspase 3 assay was performed exactly as described (16).

**Quantitative real-time PCR**

Total RNA was extracted from cells and tissues by TRIzol (Invitrogen Life Technologies, Carlsbad, CA). DNA was degraded by DNase I (Roche, Basel, Switzerland) digestion, and RNA was purified by RNaseasy mini kit (Qiagen, Valencia, CA). For lung alveolar macrophage, RNA was extracted with RNaseasy mini kit column (Qiagen), and DNA was removed by on-column DNase I (Qiagen) digestion. Total RNA was reverse transcribed by Superscript III reverse transcriptase (Invitrogen Life Technologies) with random hexamer primers. Primers used for real-time PCR were picked by Primer 3 software (17), and sequences used are available upon request. The real-time PCR was conducted using SyBr Green super mix (Bio-Rad) in an iCycler iQ Real-Time Detection system (Bio-Rad). The PCR threshold was determined by iCycler PCR baseline subtracted curve fit method. The threshold for the gene being studied was adjusted by that of a reference gene (either ribosomal protein L4 gene or lysosome, as indicated in figure legends). The raw mRNA copy number was calculated as: 

\[ \text{Relative copy number} = \frac{\text{fluorescence}_{	ext{gene}}}{\text{fluorescence}_{	ext{reference}}} \]

The relative copy number was obtained by dividing each raw mRNA copy number with the reference sample raw mRNA copy number.

**Results**

**Increased viability and reduced pathology in Hcph<sup>me-v/me-v</sup> mice homozygous for the Ets2<sup>A72</sup> allele**

The mouse mutant <i>me-v</i> (Hcph<sup>me-v/me-v</sup>) results from a point mutation that affects splicing of transcripts encoded by the hemopoietic cell phosphatase gene (Hcph, also termed <i>src homology 2 tyrosine phosphatase-1</i>), and leads to expression of a protein with 5–10% of wild-type tyrosine phosphatase activity (18). These mutant mice accumulate massive numbers of macrophages and neutrophils in the peripheral tissues, including skin, spleen, and lung, and subsequently succumb to an interstitial pneumonia (12). Thus, Hcph apparently plays a central role in cell signaling events that regulate macrophage-dependent inflammatory responses. In previous work, we demonstrated that the transcription factor Ets2 was constitutively phosphorylated and activated in macrophages isolated from Hcph<sup>me-v/me-v</sup> mice, and that Ets2 activation correlated with expression of target genes such as Bcl-X and increased cell survival following growth factor withdrawal (11). To directly test the role of Ets2 activation in the pathology of Hcph<sup>me-v/me-v</sup> mice, we used a recently created Ets2<sup>A72</sup> knockin allele that produces a protein product that cannot be phosphorylated by ras-dependent signaling and is consequently a weak <i>trans</i> activator in studies performed in cell culture (2–6), and acts as a hypomorphic allele in vivo (10).

Mice homozygous for both Ets2<sup>A72</sup> allele and Hcph<sup>me-v</sup> allele (Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup>) showed increased survival at 100 days compared with Hcph<sup>me-v/me-v</sup> mice with wild-type Ets2 (Ets2<sup>+/+</sup>; Hcph<sup>me-v/me-v</sup>), with 75% of the former group surviving (<i>n</i> = 37), compared with 12% of the latter (<i>n</i> = 35; Fig. 1A). The body weight of the Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup> mice was approximately twice that of Ets2<sup>+/+</sup>; Hcph<sup>me-v/me-v</sup> mice, and indistinguishable from wild-type mice of the same age and genetic background (Fig. 1B). The Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup> mice, both males and females, were also fertile, in contrast to Ets2<sup>+/+</sup>; Hcph<sup>me-v/me-v</sup> mice, which were never fertile, as reported during the original characterization of these mice (12).

One major immunopathological abnormality in Hcph<sup>me-v/me-v</sup> mice is the accumulation of macrophages and granulocytes in the dermis with inflammatory cells penetrating as far as the panniculus pigment, giving the mice their characteristic motheaten appearance. Additionally, inflammation in the limbs and joints is associated with an arthritis-like condition in the joints (19). This type of inflammation is especially severe in both the feet and digits of the Journal of Immunology

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\textbf{FIGURE 1.} Increased viability and reduced inflammation in Hcph<sup>me-v/me-v</sup> mice homozygous for the Ets2<sup>A72</sup> allele. A, Cumulative survival of Hcph<sup>me-v/me-v</sup> mice with wild-type or mutant Ets2<sup>A72</sup>. Data were based on <i>n</i> = 37 Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup> mice and <i>n</i> = 35 Ets2<sup>+/+</sup>; Hcph<sup>me-v/me-v</sup> mice. B, Body weight of Hcph<sup>me-v/me-v</sup> mice. The data reflect the average weight of <i>n</i> = 6 mice from each genotype at 3 mo of age. Error bars indicate SD. C and D, Image of front paws from representative Ets2<sup>+/+</sup>; Hcph<sup>me-v/me-v</sup> mice (C) or Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup> mice (D). E-H, H&E-stained sections from front paws of Ets2<sup>+/+</sup>; Hcph<sup>me-v/me-v</sup> mice (E and G) or the Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup> mice (F and H).
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Ets2+/+; Hcph<sup>me-v/me-v</sup> (Fig. 1C). The Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice were observed to lack severe skin and joint inflammation (Fig. 1D).

Histological analysis of sections made from the feet and digits confirmed that inflammation was attenuated in the Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (Fig. 1, E–H). In Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice, the epidermis was markedly thickened, and large areas of the dermis were heavily infiltrated by mixed inflammatory cells (see region indicated by arrowhead; Fig. 1E). There were numerous intraepidermal pustules in the epidermis, some of which had ruptured to the skin surface. In many areas, suppuration extended from the epidermis deep into the dermis. In addition, there was evidence of extensive remodeling of the cortices of the long bones in the digits (arrow in Fig. 1G). This remodeling was due both to bone resorption and new bone formation and resulted in skeletal deformity. In marked contrast, the epidermis of the Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice was only two to three cell layers thick, identical with the thickness for normal mice, and the dermis did not contain large numbers of inflammatory cells (area indicated by arrow in Fig. 1F). Cortical surfaces of long bones were smooth, and cortices were of uniform thickness (arrow in Fig. 1H). The skin, bones, and joints of the Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice were indistinguishable from those of normal mice.

The accumulation of macrophages and neutrophils in lungs results in a fatal pneumonitis in Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (12). At the histological level, this correlated with a massive accumulation of macrophages and neutrophils in the alveolar spaces in lungs of Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (Fig. 2, C and D; arrowheads indicate mononuclear cells within the alveolar space) compared with wild-type littermates (Fig. 2, A and B). However, there was little evidence of macrophage accumulation in the alveolar spaces of lungs from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (Fig. 2, E and F). The lung sections from the double mutant looked similar to those of wild-type mice. The results shown are representative of results obtained from nine Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice examined at age 90 days or older, while all Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (>10 examined) had similar lung pathology beginning at 50–60 days of age.

To quantify the accumulation of macrophages in lung, RNA was extracted from whole lungs from 90-day-old animals. Real-time quantitative PCR was used to measure the expression of two macrophage marker genes, c-fms and lysosome (Fig. 2G). This analysis revealed that Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice had 3-fold higher levels of expression of c-fms and lysosome compared with either wild-type or Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice, results consistent with the histology results.

**Increased apoptosis following CSF-1 withdrawal in macrophage derived from Hcph<sup>me−/me−</sup> mice homozygous for the Ets2<sup>+/+</sup> allele**

Our previous work demonstrated a correlation between Ets2 phosphorylation at Thr<sup>72</sup> and increased survival of bone marrow-derived macrophages (BMMs) from Hcph<sup>me−/me−</sup> mice upon CSF-1 withdrawal (11). To directly test the role of Ets2 phosphorylation in the increased survival of BMMs from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup>, we used three different assays to study apoptosis of BMMs following CSF-1 withdrawal: calcein acetoxyethyl/ethidium bromide staining (Fig. 3A), DNA fragmentation assays (Fig. 3B), and caspase 3 assays (Fig. 3C). All three assays confirmed our previous results showing that the BMMs from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice are refractory to apoptosis induced by CSF-1 withdrawal when compared with wild type. The calcein AM/ethidium bromide assay and DNA fragmentation assay also demonstrated that BMMs prepared from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice had a significant 2- to 3-fold increase in apoptosis (Fig. 3, A and B). These cells had a 30-fold increase in caspase 3 activation following CSF-1 withdrawal compared with either wild-type or Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (Fig. 3C). However, the BMM from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice still had 2- to 3-fold less apoptosis and 3-fold lower caspase 3 activation when compared with wild-type mice.

The proliferation of BMMs from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice was also compared with that of cells from Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice, but no significant difference was observed between the two genotypes (Fig. 3D), indicating that Ets2 was not necessary for cell proliferation.

**FIGURE 2.** Reduced lung inflammation of Hcph<sup>me−/me−</sup> mice homozygous for the Ets2<sup>+/+</sup> allele. Representative lung sections from wild-type (A and B), Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> (C and D), and Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> (E and F) mice. Arrowheads show examples of mononuclear cells within the alveolar space (C), or the absence of these cells in Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> mice (E). B, D, and F, Originally ×100 magnification, and A, C, and E, originally ×400 magnification, respectively. G, Relative mRNA levels of macrophage markers of lungs from wild-type control (WT), Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> (M), and Ets2<sup>+/+</sup>; Hcph<sup>me−/me−</sup> (AM) mice. Total RNA was isolated from freshly dissected lungs, reverse transcribed, and then subjected to real-time PCR analysis. The expression levels of each gene were standardized to the level of ribosomal protein L4 mRNA. The data represent the average obtained from two individual mice of each genotype, with each experiment performed in duplicate. Error bars indicate SD of measurements.
Inflammatory response gene expression is decreased in lungs and macrophage isolated from Hcphme-v/mice homozygous for the Ets2A72 allele

To further define the molecular basis underlying the attenuation of the inflammatory phenotype of Ets2^A72/A72; Hcph^me-v/me-v mice, quantitative real-time PCR was used to study gene expression in lungs obtained from mice of the three different genotypes used for these studies (Fig. 4). For this analysis, a set of well-defined target genes of Ets2, including extracellular proteases MMP1, MMP3, MMP9, uPA, and the antiapoptotic gene Bcl-X (2-4, 11, 20), was studied (Fig. 4A), as well as genes involved in macrophage-inflammatory responses, such as genes encoding chemokines (Fig. 4B), chemokines (Fig. 4C), and adhesion molecules (Fig. 4D).

In this analysis, a number of genes were identified that were more highly expressed in lungs obtained from Ets2^+/+; Hcph^me-v/me-v vs wild-type mice. Most of these genes were also down-regulated in Ets2^A72/A72; Hcph^me-v/me-v mice. In particular, defined Ets2 targets such as MMP1, MMP9, uPA, IL-12, and TNF-α (21) were all genes that were down-regulated in the mice homozygous for the Ets2^A72 allele. Additionally, genes known to have ets sites in their promoters, but with no evidence that they are direct targets for Ets2 such as cytokine gene IL-1α, chemokine genes CCL3 and CCL4 (MIP-1α and MIP-1β), and integrin genes αM and β2 (20), were all expressed at higher levels in Ets2^+/+; Hcph^me-v/me-v than in wild-type or Ets2^A72/A72; Hcph^me-v/me-v mice. Expression of other genes, such as MMP3 (stromelysin), integrin β1, CCL5 (RANTES), and in particular Bcl-X, was not different between the three genotypes studied (Fig. 4A).

To determine whether expression differences in lung tissue reflected differences in gene expression in macrophages specifically, alveolar macrophages were prepared and expression of key genes was identified in the previous analysis determined (Fig. 5A). With a few exceptions, the same genes were found to be differentially expressed in alveolar macrophages. In particular, cytokine genes TNF-α and IL-1α, chemokine genes CCL2 and CCL3, integrin genes α4, and extracellular protease genes MMP9 and uPA were all expressed at significantly higher levels in cells isolated from Ets2^+/+; Hcph^me-v/me-v than from either wild-type or Ets2^A72/A72; Hcph^me-v/me-v mice (Fig. 5A). In addition, Bcl-X was expressed at 4-fold higher levels in alveolar macrophage obtained from Ets2^+/+; Hcph^me-v/me-v than from Ets2^A72/A72; Hcph^me-v/me-v.

FIGURE 3. Phosphorylation of Ets2 is critical for macrophage survival. Present are the effects of CSF-1 withdrawal from BMMs of the three genotypes studied: wild-type control (Wt); Ets2^+/+; Hcph^me-v/me-v (M), and Ets2^A72/A72; Hcph^me-v/me-v (AM) mice, respectively. See Materials and Methods for details of these assays. A, Cell viability assays using calcein AM/ethidium bromide (live/dead) staining. Shown are the percentage of cells surviving as a function of time following CSF-1 withdrawal. B, DNA fragmentation assays. DNA was extracted 24 h after CSF-1 withdrawal and analyzed using agarose gel electrophoresis of extracted DNA. MK is the lane that contained DNA size markers. C, Caspase 3 assays. Assays were performed on cell extracts 24 h after CSF-1 withdrawal. D, Cell proliferation assays, determined by counting cells for the indicated times following initial CSF-1 treatment. The data presented for the viability assays (A), caspase 3 assays (C), and proliferation assays (D) represent the average results for cells obtained from three individual mice of each genotype. Error bars in each of the panels indicate the SD of the measurements. A result representative of three independent DNA fragmentation experiments is shown in B.

FIGURE 4. Reduced expression of inflammatory genes in lungs of Hcphme-v mice homozygous for the Ets2^A72 allele. Total RNA was isolated from freshly dissected lungs from wild-type control (Wt), Ets2^+/+; Hcph^me-v/me-v (M), and Ets2^A72/A72; Hcph^me-v/me-v (AM) mice, respectively, then reverse transcribed, and subjected to real-time PCR analysis. The genes and genotypes studied are as indicated in the figure panels. The expression levels of genes encoding extracellular proteases and Bcl-X (A), cytokines (B), chemokines (C), and integrins (D) were standardized to the level of lysozyme mRNA. The data presented are the average of two experiments performed in duplicate with lungs from two independent mice from each genotype. Error bars indicate the SD.
The differential expression of genes between cells derived from mice with the three different genotypes (Fig. 5B), as previously demonstrated (11, 22), but not in all of the many other cell types present in lung tissue.

These data indicate that Bcl-X was a target of Ets2 in macrophages, as previously demonstrated (11, 22), but not in all of the many other cell types present in lung tissue.

To test whether the changes in gene expression observed were limited to lung macrophages or reflected a general change in tissue macrophage gene expression, expression of these genes was compared in peritoneal macrophages prepared from mice with the three different genotypes (Fig. 5B). The differential expression of genes between cells derived from Ets2+/+; Hcph<sup>me-v/me-v</sup> and Ets2<sup>A72/A72</sup>; Hcph<sup>me-v/me-v</sup> mice, including for Bcl-X, was similar in the two macrophage populations (Fig. 5B). However, there were differences in the relative levels of expression of several of these genes in peritoneal compared with alveolar macrophages. For example, MMP9 was expressed at higher relative levels in alveolar cells, while CCL2 was expressed at higher relative levels in peritoneal cells. In addition, IL-12 and MMP1 expression were more dramatically different between the genotypes in peritoneal cells than alveolar (Fig. 5B). This might reflect inherent differences in absolute levels of gene expression between the macrophage populations.

BMMs do not express high levels of inflammatory response genes, but LPS treatment can stimulate such responses, for example, the expression of TNF-α. We compared LPS induction of TNF-α in BMMs derived from wild-type, Ets2<sup>+/+;</sup> Hcph<sup>me-v/me-v</sup>, and Ets2<sup>A72/A72;</sup> Hcph<sup>me-v/me-v</sup> mice (Fig. 5C). This analysis demonstrated that early induction of TNF-α expression was indistinguishable in cells of the three different genotypes: after 30 min of LPS treatment, TNF-α mRNA levels were uniformly induced ∼150-fold compared with untreated cells. However, after 18 h of LPS treatment, TNF-α mRNA expression remained 3- to 4-fold higher in Ets2<sup>+/+;</sup> Hcph<sup>me-v/me-v</sup> than in either wild-type or Ets2<sup>A72/A72;</sup> Hcph<sup>me-v/me-v</sup> cells, in which the level of expression had returned to near baseline activity. Therefore, persistent activation of TNF-α in Hcph<sup>me-v/me-v</sup> cells depended on phosphorylation of Ets2.

**Discussion**

The results presented in this work provide genetic evidence demonstrating that Ets2 is necessary for the pathology of Hcph<sup>me-v/me-v</sup> mutant mice, extending previous correlations between Ets2 and Hcph action surmised by biochemical and cell biological approaches (11). As depicted in Fig. 6, Hcph acts as a negative regulator upstream of MAPK pathways that can lead to phosphorylation and activation of Ets2 in macrophages. The Hcph<sup>me-v</sup> allele encodes a product no longer capable of down-regulating signals that lead to MAPK activation, including activation of jun N-terminal kinase in macrophages (11), thus leading to constitutive activation of Ets2. Activation of Ets2 leads to increased expression of genes encoding cytokines, chemokines, extracellular proteases, and adhesion molecules, all involved in various aspects of inflammation. Ets2 also directly regulates Bcl-X (11, 22), and thus decreases apoptosis of activated macrophages.

![FIGURE 6. Model for Ets2 action in macrophage inflammation. Mutation of the Hcph gene results in constitutive activation of MAPK signaling pathways and constitutive activation of Ets2. This leads to activation of gene targets, both direct Ets2 targets and indirect targets, that contribute to various aspects of cell inflammation. When the hypomorphic Ets2<sup>A72</sup> allele is present, expression of these target genes is reduced, and pathology due to inflammation is reduced.](http://www.jimmunol.org/)

For the calculation of the SD of the measurements, the expression of each target gene was determined in duplicate. Error bars indicate the SD of the measurements.
As predicted by this model, a mutation in the negative regulator Hcph is masked by the hypomorphic Ets24/– allele, resulting in decreased expression of these inflammatory response genes, increased apoptosis, and a subsequent attenuation of acute inflammation. Several of the affected genes are well-defined target genes of Ets2, including MMP1, MMP9, uPA, TNF-α, and IL-12 (2–4, 11, 20, 21). Other affected genes, including CCL3, CCLA, and integrin genes αM and β2, contain ets sites in their cis-regulatory regions, although there is no direct evidence that Ets2 directly regulates them (20). Overall, these data suggest that Ets2, either directly or indirectly, regulates a set of genes that are necessary for the severe inflammatory phenotype in the me-v model.

TNF-α plays a critical role in the pathology of me-v mice, as injection of an anti-TNF Ab into mutant mice results in a decrease in lung pathology (23). Chromatin immunoprecipitation assays indicate that Ets2 directly binds to the TNF-α gene in macrophage cell lines (21, 24), an observation extended by the in vivo results presented in this study. Consistent with our model (Fig. 6), TNF-α is not constitutively expressed in BMMs obtained from me-v mice until after LPS stimulation, and phosphorylation of Ets2 is important for this persistent expression pattern. These results all suggest that the persistent regulation of TNF-α expression may provide the mechanism by which Ets2 contributes to the pathology of the me-v model.

Studying the me-v inflammatory model has revealed an important role for MAPK pathway signaling through a single amino acid in Ets2 that was not deduced from previous genetic studies with Ets2 null or hypomorphic mutations (10, 25). Although the genetically determined pathology of the me-v mice may be an extreme condition, our results suggest that the activation of Ets2 might also be important in other naturally occurring inflammatory processes, for example in tumor progression (10). When the immune system of rescued Ets2-deficient animals is not acutely challenged, the functions of Ets2 may be sufficiently complemented by Ets1, the most closely related Ets family member (M. Ostrowski and G. Wei, unpublished observations), or by other signaling pathways.

Genes such as Bcl-X and TNF-α identified as Ets2 targets are also targets for other transcription factors known to play important roles in inflammatory responses, API and NF-κB (21, 22, 26). Interestingly, both API and NF-κB can directly interact with Ets2 or the highly related gene product Ets1 to activate target genes in many cell types, including in the immune system (21, 22, 26). Studying genetic interactions between Ets2 and these other transcription factors and signaling pathways will provide a more detailed understanding of transcriptional regulatory networks controlling terminal differentiation states in monocytes and macrophages, and may suggest new combinatorial strategies to limit inflammation pathology involving these key pathways.

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