Persistent Nuclear Factor-κB Activation in Ucp2−/− Mice Leads to Enhanced Nitric Oxide and Inflammatory Cytokine Production*

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One of the phenotypes of mice with targeted disruption of the uncoupling protein-2 gene (Ucp2−/−) is greater macrophage phagocytic activity and free radical production, resulting in a striking resistance to infectious microorganisms. In this study, the molecular mechanisms of this enhanced immune response were investigated. We found that levels of nitric oxide measured in either plasma or isolated macrophages from Ucp2−/− mice are significantly elevated in response to bacterial lipopolysaccharide challenge compared with similarly treated Ucp2+/+ mice. Likewise, expression of inducible nitric-oxide synthase and inflammatory cytokines is higher in Ucp2−/− mice in vivo and in vitro. Key steps in the activation cascade of nuclear factor (NF)-κB, including IkB kinase and nuclear translocation of NF-κB subunits, are all remarkably enhanced in Ucp2−/− mice, most notably even under basal conditions. The elevated basal activity of IkB kinase in macrophages from Ucp2−/− mice can be blocked by cell-permeable inhibitors of superoxide and hydrogen peroxide generation, but not by a specific inhibitor for inducible nitric-oxide synthase. Isolated mitochondria from Ucp2−/− cells produced more superoxide/hydrogen peroxide. We conclude that mitochondrial-derived reactive oxygen from Ucp2−/− cells constitutively activates NF-κB, resulting in a "primed" state to both potentiate and amplify the inflammatory response upon subsequent stimulation.

Uncoupling protein (UCP)1−2 is a mitochondrial inner membrane carrier protein that was discovered through its homology to the brown fat UCP1 (1). Whereas UCP1 has been clearly established as the molecular mediator of non-shivering thermogenesis (reviewed in Ref. 2), the function of UCP2 remains somewhat of an enigma. Several features of UCP2 led us to initially propose that it was a bona fide uncoupling protein involved in the dissipation of excess metabolic fuel as heat. These aspects of UCP2 included its structural resemblance to UCP1, its ability to uncouple respiration in model assay systems, and a chromosomal location to a region with genetic linkage to obesity and hyperinsulinemia (1, 3). However, although Ucp2 mRNA is expressed in a broad array of tissues in humans and rodent models (3, 4), including metabolically important organs, it exists in minute amounts compared with the level of UCP1 in brown fat. Moreover, it is present in cell types such as pancreatic β-cells, lymphocytes, and neurons that are not typically associated with thermogenesis (5–7). These and other features of UCP2 (8), together with the critical finding that targeted disruption of the Ucp2 gene did not result in obesity, cold sensitivity, or demonstrable differences in coupling efficiency in isolated mitochondria (9–11), strongly suggested a functionally distinct role for this protein.

One of the phenotypes of Ucp2−/− mice was a striking resistance to infectious microorganisms associated with greater macrophage phagocytic activity and free radical production (9). We showed that macrophages from Ucp2−/− mice produced reactive oxygen species (ROS) using the nitro blue tetrazolium reduction assay and hypothesized that UCP2 plays a role in host immune defense by regulating the production of ROS. A broad definition of ROS can include reactive oxygen as well as nitrogen species, and macrophages are known to generate reactive nitrogen species (RNS) such as nitric oxide (NO) in addition to superoxide. However, the nitro blue tetrazolium assay cannot distinguish between the ROS and RNS generated (12) or the mechanisms responsible for it. In the present study we explored the molecular basis of this amplified immune response in Ucp2−/− mice using lipopolysaccharide (LPS), a membrane glycolipid of Gram-negative bacteria that is a well-established activator of both the “reactive oxygen” and “reactive nitrogen” generating pathways (13, 14). We show that in response to LPS challenge, Ucp2−/− mice produce large amounts of nitric oxide and cytokines both in vivo and from isolated macrophages in vitro as compared with wild-type mice.

As one of the major transcription factors responsible for increasing the expression of iNOS and inflammatory cytokines, we find that in spleen or isolated macrophages of Ucp2−/− mice, the NF-κB system is activated at several steps in its pathway (importantly, even under basal conditions). NF-κB is most frequently composed of two DNA-binding subunits, p50 and p65, which exist as an inactive form in the cytoplasm bound to an inhibitory protein (IκB). Almost all of the signals...
that lead to activation of NF-κB converge on IκB kinase (IKK). Activation of IKK leads to the phosphorylation of IκBα, which targets them for ubiquitination and degradation, thus allowing the NF-κB dimer to translocate to the nucleus to regulate target gene expression (15). In the present study we show that IKK activity is elevated in spleen as well as isolated macrophages from Ucp2−/− mice, even under basal conditions, and isolated mitochondria from Ucp2−/− spleen produce significantly more hydrogen peroxide as compared with wild-type mice. Together with the finding that cell-permeable preparations of the ROS inhibitors superoxide dismutase and catalase could block the basal elevation of IKK activity in isolated macrophages, our results support the idea that deficiency of UCP2 results in greater O2^−/H2O2 release from mitochondria (16), leading in turn to persistent activation of NF-κB and exacerbation of subsequent inflammatory challenges.

**EXPERIMENTAL PROCEDURES**

**Animals and Sample Collection—**Generation of Ucp2−/− mice was described previously (9). Twelve-week-old male mice were used that had been backcrossed from the mixed C57Bl/6J-129Sv/J strain back- ground to C57Bl/6J for 10 generations. All animal experiments were approved by the Institutional Animal Care and Use Committees of Duke University and CIIT Centers for Health Research. LPS (Sigma) was administered to mice at a dose of 4 μg/g or 15 μg/g body weight (bw) (17) in 100 μl of PBS by intraperitoneal injection or given PBS vehicle. Blood samples were collected 6 or 16 h later under pentobarbital anesthesia. Plasma or macrophage culture supernatants was detected as nitrite/nitrate (18). Plasma NO levels and cytokines were measured as described below. For isolation of resident peritoneal macrophages, mice were anesthetized with pentobarbital, and the peritoneal cavity was gently flushed with chilled Dulbecco’s modified Eagle’s medium supplemented with 1% fetal calf serum (Highveld). Lavage fluid was pooled and centrifuged three times with chilled Dulbecco’s modified Eagle’s medium supplemented with pentobarbital, and the peritoneal cavity was gently flushed with chilled Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum supplemented with 1% fetal calf serum (Highveld). The membrane was pre-hybridized with Expresshyb (Clontech) for 60 min at 68 °C, followed by hybridization with probe overnight at 68 °C. The arrays were washed four times in 2 × SSC/0.1% SDS (30 min each) and two times in 0.1 × SSC/0.1% SDS. The arrays were then sealed in plastic wrap and exposed to an Amersham Biosciences PhosphoImage screen or to radiographic film. The images were analyzed densitometrically using ImageJ software (NIH). The gene expression intensities were normalized with a total of nine housekeeping genes present on the array. Comparisons between sample groups were made by Student’s t test. p < 0.05 was considered statistically significant.

**Electrophoretic Mobility Shift Assays (EMSAs)—**Nuclear extracts were prepared as described above and either used immediately or stored at −70 °C. Single-stranded oligonucleotides (Integrated DNA Technologies, Inc.) were end-labeled with γ-[32P]ATP labeled (Boehringer-Mannheim) and purified by reversed-phase HPLC. The consensus oligonucleotides containing the underlined NF-κB-binding site (5′-ATATGAGGGGACCTTCCCAGG-3′) and 5′-ATACTCCGAGAGTTGCTC-CTCA-3′ and two other oligonucleotides containing the underlined iNOS promoter-specific NF-κB-binding site (NFkB1D, 5′-ACTGGGAGACT- CCTTTGGG-3′ and 5′-TCCCAAAGGGAGATGCCCTC-CTCA-3′) were used for EMSA.

**Western Blot Analysis—**Total RNA was isolated from spleen with Tri Reagent (Molecular Research Center, Inc.). A total of 10−15 μg of total RNA was fractionated by electrophoresis in 1.2% agarose gels, transferred to nylon membranes, and hybridized to 32P-labeled probes as previously described (25). Images were visualized and quantified in a PhosphorImager. All measurements were normalized to the internal standard cyclophilin or 18S RNA.

**Cytochrome Measurements by ELISA—**Plasma samples for ELISA were collected as described under “Animals and Sample Collection.” Detection of IFN-γ and TNF-α was performed using sandwich ELISA reagents from Chemicon (Temecula, CA).

**Microarray Hybridization—**Atlas Mouse Inflammatory/Stress cdNA expression microarray analyses were performed according to the manufacturer’s instructions (Clontech). Ucp2+/+ and Ucp2−/− mice for this experiment were treated with 15 μg LPS/g bw or left untreated. All hybridizations were carried out in quadruplicate. Before labeling, RNA samples from two biological replicates were pooled in equal amounts. Briefly, total RNA was converted to [α-32P]ATP-labeled cdNA probe using Moloney murine leukemia virus reverse transcriptase and the Atlas Mouse Inflammatory/Stress CDS primer mix. The [32P]-labeled cdNA probe was purified using a NucTrap column (Clontech) and hybridized to the membrane. The membrane was washed three times with chilled Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Highveld). The membranes were washed three times with wash buffer (PBS/0.1% Tween 20) for 30 min at 4 °C and probed with primary antibodies overnight at dilutions recommended by the suppliers. Membranes were washed three times with wash buffer (15 min/wash) before incubation with second antibodies (1 h at room temperature). Blots were scanned and visualized with a PhosphorImager (Typhoon; Amersham Biosciences) and quantified by densitometry (Quantity One).
Northern blotting data for RNA are shown as the fold change relative to Fig. 2, expression were more robust in the cells from molation was linear through 30 min, and the data are expressed as conducted, equal amounts of mitochondria (350 μg/100 μl) from two mice of each genotype were incubated at room temperature in assay medium under state 4 conditions in the presence of the Amplex® Red reagent mixture. Mitochondrial production of H$_2$O$_2$ was measured at 10-min intervals over a 30-min period using a SPECFRMax M2 spectrofluorometer (Molecular Devices). Calibration curves for H$_2$O$_2$ concentrations were generated as previously described (27) in the presence of NO (nitrite and nitrate) in plasma from Ucp2−/− mice. This difference between the genotypes illustrates that upon LPS treatment, total NO production (Fig. 2A) and Western blotting (Fig. 1C). In both genotypes, iNOS expression was not detectable under basal conditions by these methods (data not shown). In addition, macrophages isolated from the two genotypes and stimulated by LPS in vitro gave identical responses to those observed in spleen. As shown in Fig. 2, A and B, generation of NO and the induction of iNOS expression were more robust in the cells from Ucp2−/− mice compared with those from Ucp2+/+ mice. Pre-treatment of the cells with the specific iNOS inhibitor 1400W completely prevented NO production (Fig. 2A), confirming that iNOS is the sole source of this NO.

Because cytokines released from macrophages and other immune cells are important facilitators of the host defense response and augment the induction of iNOS, we next measured the expression of several inflammatory cytokine genes by Northern blotting using total RNA from spleen of wild-type or Ucp2-null mice treated or not treated with LPS. As shown in Fig. 3A, LPS-induced expression of IFN-γ, TNF-α, interleukin-1β, and interleukin-6 was significantly higher in Ucp2−/− mice than in their wild-type littermates following LPS treatment. When we examined plasma levels of two of these key inflammatory cytokines, IFN-γ and TNF-α, both were substantially greater in Ucp2−/− mice than in Ucp2+/+ mice (Fig. 3, B and C). Similar results of elevated cytokine gene expression were obtained from microarray analyses of spleen RNA from Ucp2+/+ and −/− mice (Fig. 4). There was also a greater representation of a subset of tissue-degrading matrix metalloproteinases and anti-apoptotic gene products in samples from LPS-treated Ucp2−/− mice than from wild-type animals.

Table I

| Genotypes          | NADPH oxidase (protein) | COX2 (mRNA) |
|--------------------|------------------------|------------|
|                    | Membrane | Cytosol | Membrane | Cytosol | Membrane | Cytosol |
| LPS (4 μg/g bw, 6 h) |          |      |          |      |          |      |
| Ucp2+/+            | 1.00 ± 0.02 | 1.78 ± 0.02 | 1.00 ± 0.08 | 2.28 ± 0.14 | 1.00 ± 0.05 | 1.95 ± 0.03 | 1.00 ± 0.01 | 1.42 ± 0.02 |
| Ucp2−/−            | 1.00 ± 0.01 | 1.49 ± 0.02 | 1.08 ± 0.08 | 1.98 ± 0.15 | 1.01 ± 0.01 | 2.09 ± 0.03 | 1.04 ± 0.01 | 1.75 ± 0.02 |

Fig. 1. Nitric oxide production is elevated in Ucp2−/− mice upon stimulation by LPS. A, NO in plasma. Mice were treated with 4 μg/g bw LPS or PBS. NO levels in plasma were measured and are shown as the mean ± S.E. of three independent experiments (n = 8). B and C, iNOS expression in spleen. Following treatment of mice with 4 μg/g bw LPS, spleen cytoplasmic proteins (16 h) and total RNA (6 h) were prepared. mRNA levels of iNOS were measured by Northern blotting (B), and protein levels of iNOS and β-actin were detected by Western blotting (C). Data are representative of two independent experiments (n = 4–6). *, p < 0.05; **, p < 0.01 for comparison between genotypes and relative wild types.
Gene network analysis (29) illustrates that these molecules regulate the transcription factor NF-κB or are regulated by it. Activation of NF-κB Cascade in Ucp2−/− Mice, Even Under Basal Conditions—In the next series of experiments, we attempted to determine the mechanisms responsible for the exacerbated levels of expression of iNOS and cytokines in Ucp2−/− mice. Because NF-κB is a central regulator of these inflammatory mediators, we examined its relative abundance, subcellular distribution, and functional activity as assessed by its binding to NF-κB DNA response elements in nuclear extracts using electrophoretic mobility shift assays (Fig. 5). Under LPS treatment conditions, binding to oligonucleotides containing the two separate NF-κB response elements from the iNOS gene promoter, NF-κB(d) and NF-κB(u) (26), was elevated in Ucp2−/− mice (Fig. 5A), supporting the idea that the greater increase of iNOS expression in Ucp2−/− mice is NF-κB-dependent. Using a consensus NF-κB response element, we obtained essentially identical results (Fig. 5B). The specificity of these interactions was confirmed by the ability of antisera against the p50 and p65 subunits of NF-κB to retard the mobility (“supershift”) of these binding species (Fig. 5C). Surprisingly, we also observed that even under basal

**Fig. 2.** Nitric oxide production is elevated in resident macrophages from Ucp2−/− mice upon stimulation by LPS. A, NO levels in supernatant of resident peritoneal macrophages. Supernatants of macrophage cultures were collected following treatment with 1 μg/ml LPS in the absence or presence of 50 μM 1400W for NO measurement. Results are shown as the mean ± S.E. of two independent experiments, each representing pooled macrophages from three to four mice. B, iNOS protein levels in peritoneal macrophages. Macrophages were treated with LPS as described in A. Protein levels of iNOS and β-actin were measured by Western blotting. Data are representative of two independent experiments (n = 4). #, p < 0.05 for comparison within genotype with respect to non-inhibitory treatment. *, p < 0.05 for comparison between genotypes and relative wild types.

**Fig. 3.** Induction of inflammatory cytokines by LPS is increased in Ucp2−/− mice. A, Northern blotting. Spleen total RNA was isolated from mice treated with 4 μg/g bw LPS or PBS. Levels of indicated cytokine mRNAs, Ucp2, and cyclophilin (Cyclo) were detected by Northern blotting. Data are shown as the fold change of Ucp2−/− mice versus Ucp2+/+ mice (n = 4). #, p < 0.05. B and C, ELISA for IFN-γ and TNF-α, respectively. Mice were treated or not treated with 4 μg/g bw LPS for 6 h, and plasma was collected for ELISA measurement. Each result shown is the mean ± S.E. of plasma sample from four individual mice. #, *, and ** are as defined in the Fig. 1 legend.
NF-κB added for the supershift assays (from Ucp2-extracted after LPS treatment and used in EMSA (5). In certain pathological conditions (30–32), as shown in Fig. 6A, the relative levels of the p50 and p65 subunits of NF-κB in cytoplasmic and nuclear fractions from spleen of both genotypes under basal and stimulated conditions. As shown in Fig. 6B, the location and activation of the NF-κB subunits are regulated by the inhibitor proteins IκBa and IκBβ, which are in turn regulated by IKK activity. Cytoplasmic levels of IκBa and IκBβ were mildly decreased following LPS treatment in both genotypes, but IκBα levels declined more significantly in Ucp2–/– mice under both basal and LPS-treated conditions (data not shown). Although the functions of the two IκB subtypes are similar, IκBa is noted to primarily be regulated by acute cytokine stimulation, whereas IκBβ is mainly involved in the sustained activation of NF-κB by other pathways (33, 34). Together, these results are consistent with a pattern of low-level persistent activation.

**Increased IKK Activity in Ucp2–/– Mice in Both Basal and Stimulated Conditions Can Be Suppressed by O$_2$/$H_2$O$_2$ Inhibitors**—The IKK activity in spleen and in isolated macrophages was measured directly by immune complex kinase assay (21). The results shown in Fig. 7A indicate that IKK activity is higher in Ucp2–/– mice both under basal conditions and 2 h after LPS stimulation. Because the antibody used for immune-precipitation recognizes both IKKα and IKKβ, we cannot distinguish whether one isoform is differentially activated over the other. In any event, together with the previous data, these results show that the increase in IKK activity and the decrease of IκBβ level trigger the translocation and activation of NF-κB in Ucp2–/– mice under basal conditions. Although it is commonly observed that IKK can be activated in response to various stimuli including oxidative stress, cytokines, or ultraviolet radiation, the mechanism by which this occurs remains unclear (35). However, we can rule out any contribution from the classical mitogen-activated protein kinase pathways such as extra-
increased IKK activity could be the result of the interaction of multiple cell types and signals within the spleen as opposed to a “cell autonomous” event dependent solely on the lack of UCP2. However, as shown in the left panel of Fig. 7B, we confirmed that under basal conditions, IKK activity is increased in isolated macrophages from Ucp2−/− mice. To distinguish which of these reactive species might be produced to trigger basal activation of IKK, we tested the effect of specific inhibitors for O2− and H2O2 (cell-permeable polyethylene glycol-conjugated superoxide dismutase and catalase) or iNOS (1400W) (we used a combination of superoxide dismutase and catalase in order to completely remove O2− and its immediate breakdown product, H2O2). As shown in the left two panels of Fig. 7B, the O2− and H2O2 inhibitors completely blocked the enhanced IKK activity in isolated primary macrophages of Ucp2−/− mice. The iNOS inhibitor 1400W did not suppress the increased IKK activity and actually tended to increase it somewhat. Because NO can react with O2− to form peroxynitrite, inhibition of NO production might be expected to lead to a net increase in O2− and an elevated cellular response (37). These findings suggest that, under basal conditions, there is a source of superoxide and/or by-products thereof within macrophages of Ucp2-null mice that serves to activate IKK and the NF-kB cascade independent of other circulating factors in vivo.

Increased H2O2 Generation from Isolated Spleen Mitochondria of Ucp2−/− Mice—If superoxide is being generated in macrophages from Ucp2−/− cells to an extent that is able to increase IKK activity, the most likely source is from mitochondria for the following reasons. First, as shown in Table I, there were no differences between genotypes in subcellular distribution of the NADPH oxidase subunits under basal or stimulated conditions. There were also no differences in their relative expression levels as measured by reverse transcription-PCR (data not shown). Second, under physiological resting conditions, mitochondria are presumed to be the major source of ROS (38), and UCP2 is a mitochondria inner membrane protein. To test this idea, mitochondria were isolated from spleen of Ucp2+/+ and Ucp2−/− mice, and H2O2 (as the relatively stable immediate by-product of superoxide) was measured using the fluorometric substrate Amplex® Red. As shown in Fig. 7C, twice as much H2O2 was produced from the mitochondria of Ucp2−/− mice as compared with the mitochondria of wild-type mice.

**DISCUSSION**

When we first generated UCP2-deficient mice and observed that they were exceptionally resistant to chronic infection with *Toxoplasma* and had increased bacteriocidal activity (9), we concluded that the lack of UCP2 in macrophage mitochondria led to increased “ROS” in the broad sense of the term, including all reactive “oxygen” species such as superoxide, hydroxyl radical, nitric oxide, or their various by-products. However, at the same time, we noted that whereas UCP2 is a mitochondrial protein, we could not rule out a role for NAPD oxidase as a major source of this ROS or even determine which of these ROS was the predominant form produced. In the present study, we first investigated the production of reactive oxygen and nitrogen species from wild-type and Ucp2−/− mice following treatment with LPS. As a membrane glycolipid of Gram-negative bacteria with strong immunostimulating activity, it is a well-established activator of both the “reactive oxygen” and “reactive nitrogen” generating pathways (13, 14). From these experiments, we demonstrated that in response to LPS challenge, iNOS-derived NO production is markedly increased in Ucp2−/− mice in vivo as well as in macrophages isolated from Ucp2-null mice and cultured *in vitro*. A recent report by Kizaki et al. (39) of reduced NO production in a macrophage cell line engineered to overexpress UCP2 is consistent with our findings.
NF-κB phenotype of the and was further supported by our report on the microbicidal production was first put forth by Casteilla and co-workers (16) levels in the activation cascade as far back as IKK activity. Because IFN-γ is produced mainly by T lymphocytes and natural killer cells, rather than macrophages (36), it was reasonable to speculate that the ability of Ucp2−/− mice to generate more NO might be a consequence of a larger overall immune response involving many cell types as opposed to a cell autonomous mechanism of NO generation in macrophages alone. We believe that both processes are probably occurring. Macrophages isolated and cultured in vitro are capable of producing more NO and expressing iNOS to a higher degree than wild-type cells, but that response is probably amplified in vivo in view of the elevated IFN-γ. Therefore, an issue to investigate in the future is whether other cell types in the Ucp2−/− mouse elicit a more robust response to antigenic challenge.

As one of the key transcription factors responsible for increasing the expression of iNOS and various cytokines, we found that upon LPS treatment, the NF-κB pathway was activated in spleen to a greater extent in Ucp2−/− mice at all levels in the activation cascade as far back as IKK activity. Notably this was evident even under basal conditions and in isolated cultured macrophages from the Ucp2−/− animal. NF-κB also regulates the expression of a number of chemokines, matrix metalloproteinases, and anti-apoptotic genes either directly or indirectly (47, 48), and our preliminary microarray results found that these genes were also increased in spleen of Ucp2−/− mice. Based on cell fractionation studies, the p50 subunit of NF-κB, but not p65, appeared to be more active in the Ucp2−/− mouse under basal conditions. It also appears that under basal conditions there is a preferential degradation of IkBα in Ucp2−/− spleen. These two characteristics have both been associated in other studies with the persistent activation of NF-κB (30–34). In the LPS-stimulated situation, even though p50 is more robustly translocated to the nucleus in both Ucp2+/+ and the Ucp2−/− mice, we still observed a significantly greater amount of nuclear p50 in the Ucp2−/− mouse, although the relative difference between genotypes under these conditions is less than that seen under basal conditions. We also observed that, after LPS stimulation, p65 nuclear translocation was increased to a greater extent in Ucp2−/− mice than in wild-type animals. Together, we interpret this behavior of the two subunits, p50 and p65, as indicating a greater response by the Ucp2−/− mouse under both basal and stimulated conditions.

The concept that UCP2 may serve as a modulator of ROS production was first put forth by Casteilla and co-workers (16) and was further supported by our report on the microbicidal phenotype of the Ucp2−/− mouse (9). More recently, superoxide itself was proposed as an activator of UCP2 (49, 50), although it is still unclear exactly what might be “activated” because there are discrepancies in the literature regarding the ability of UCP2 (or UCP3 for that matter) to facilitate proton movement or transport of molecules across the inner membrane (5, 6, 11, 51–53). In any event, our findings here provide an additional pathway to explore how UCP2 controls cell function. Here we show that, at least in macrophages, the absence of UCP2 results in increased mitochondrially derived superoxide and/or hydrogen peroxide, which serves as a stimulus for the activation of IKK, even under basal conditions. As illustrated in Fig. 8, upon antigenic challenge the inflammatory response, particularly the generation of NO and cytokines, is amplified in the Ucp2−/− mouse. UCP2 has a relatively broad tissue distribution (1) and has been negatively associated with metabolic dysfunction in humans and animals (1, 10, 54–57). The increasing evidence that metabolic disorders such as obesity, diabetes, and atherosclerosis have an inflammatory component and oxidative stress in their etiology (52, 58–61) raises the possibility that our finding in macrophages may be relevant to the development of these other diseases.

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