Temporal gene expression profiles of target-ablated olfactory epithelium in mice with disrupted expression of scavenger receptor A: impact on macrophages


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Getchell, M. L., H. Li, R. A. Vaishnav, A. S. Borders, J. Witta, N. Subhedar, W. de Villiers, A. J. Stromberg, and T. V. Getchell. Temporal gene expression profiles of target-ablated olfactory epithelium in mice with disrupted expression of scavenger receptor A: impact on macrophages. Physiol Genomics 27: 245–263, 2006. First published August 1, 2006; doi:10.1152/physiolgenomics.00261.2005.—Target ablation [removal of the olfactory bulb (OBX)] induces apoptotic death of olfactory sensory neurons (OSNs) and an immune response in which activation and recruitment of macrophages (mφs) into the olfactory epithelium (OE) occupy a central role. Mφs phagocytose apoptotic neurons and secrete cytokines/growth factors that regulate subsequent progenitor cell proliferation and neurogenesis. Scavenger receptor A (SR-A) is a pattern recognition receptor that mediates binding of mφs to apoptotic cells and other relevant immune response functions. The aim of this study was to determine the impact of the absence of SR-A on the immune response to OBX. The immune response to OBX was evaluated in mice in which functional expression of scavenger receptor (MSR) was eliminated by gene disruption (MSR−/−) and wild-type (wt) mice of the same genetic background. OBX induced significant apoptotic death of mature OSNs in the two strains. However, subsequent mφ infiltration and activation and progenitor cell proliferation were significantly reduced in MSR−/− vs. wt mice. Gene expression profiling at short intervals after OBX demonstrated significant differences in temporal patterns of expression of several gene categories, including immune response genes. Many immune response genes that showed different temporal patterns of expression are related to mφ function, including cytokine and chemokine secretion, phagocytosis, and mφ maturation and activation. These studies suggest that impairment of the immune response to OBX in the OE of MSR−/− mice most likely resulted from decreased mφ adhesion and subsequent reduced infiltration and activation, with a resultant decrease in neurogenesis.

neurogenesis; microarray; phagocytosis; chemokines; cytokines; dendritic cells

APOPTOSIS OF MATURE olfactory sensory neurons (OSNs) in the olfactory epithelium (OE) and their replacement by neurogenesis occur throughout the lifespan of vertebrates. To study the regulation of apoptosis and neurogenesis in the OE, investigators have utilized a model in which the olfactory bulb, which contains the synaptic targets of OSNs, is surgically removed [olfactory bulbectomy (OBX)], severing the OSN axons. This results in the synchronous apoptosis of mature OSNs (e.g., 12); activation, infiltration, and recruitment of macrophages (mφs) into the OE (e.g., 27); phagocytosis of apoptotic OSNs (e.g., 74); proliferation of OSN progenitors (e.g., 9) leading to neurogenesis; and epithelial remodeling (e.g., 25). Our laboratory and others have utilized OBX to investigate the multiple roles played by mφs in maintaining epithelial homeostasis, including phagocytosis of apoptotic OSNs (74), production of chemokines that regulate recruitment of monocytes mφs to the OE (26, 27, 50), and the secretion of cytokines and growth factors that stimulate neurogenesis and epithelial remodeling (25, 61).

Scavenger receptors are pattern-recognition receptors that contribute to tissue homeostasis and immune response (29, 75). Scavenger receptor A (SR-A, CD204), encoded by the murine gene Msr1 (macrophage scavenger receptor 1), is expressed primarily by mature mφs (but not monocytes) and dendritic cells (DCs), as well as some endothelial and vascular smooth muscle cells (13, 64). SR-A has been implicated in multiple processes, including the uptake of modified low-density lipoprotein, bacteria (e.g., 59, 64), and, of particular relevance to this study, apoptotic cells (64, 76); adhesion to cells such as activated B lymphocytes (81) and to extracellular matrix (e.g., 20, 30, 47, 65); and major histocompatibility complex (MHC) class I-restricted antigen presentation (58, 62). Its expression has been detected in models of neuronal degeneration (5, 60) and in microglia associated with β-amyloid plaques in the brain (7, 11).

Phagocytosis, a key process in the immune response to OBX, is a highly complex, multistep, coordinated activity (e.g., 15, 51, 55, 67, 72). Apoptotic cells initiate phagocytosis by releasing “find-me” signals, which attract and activate mφs (e.g., 32, 52). Activated resident mφs secrete chemokines and cytokines that perpetuate and amplify the response. Chemokines are potent chemoattractants that are responsible for the recruitment of monocytes into tissues (e.g., 43), their subsequent activation and maturation (56), and the activation of microvascular endothelial cells to facilitate phagocyte recruitment (37). Cytokines also regulate monocyte maturation and mφ activation into specific phenotypes (56) and stimulate local endothelial cell activation (45). Following infiltration into the tissue, activated phagocytes identify apoptotic cells through their display of “eat-me” or engulfment signals that bind to engulfment receptors on the phagocyte, sometimes in association with bridging molecules that link the two cells (e.g., Refs. 14, 17). This triggers signaling that results in the formation of the phagosome. The phagosome encloses the apoptotic cell and/or its fragments and ultimately processes them for disposal (22) or antigen presentation (33). Phagocytosis culminates in the release from mφs of anti-inflammatory mediators that
suppress the development of tissue-destructive immune processes and of endothelial and epithelial growth and survival factors that promote neurogenesis and epithelial remodeling (16, 28).

The availability of a murine model in which exon 4 of the gene encoding SR-A was disrupted, blocking trimerization of the gene product into functional integral membrane receptors of both type I and type II SR-A (73), made it possible to investigate the potential involvement of SR-A in the immune response of the OE to OBX. MSR^+/− mice had normal blood levels of plasma cholesterol, triglycerides, and lipoproteins when maintained on a standard diet (73). Additionally, during their embryonic development, there was no impairment in the clearance of apoptotic cells due to the participation of and compensation by other cell types and receptors (46).

The aims of this study were 1) to determine if there were differences in the cellular responses to OBX, particularly mφ infiltration into the OE and progenitor cell proliferation, between wild-type (wt) C57BL/6 and MSR^+/− mice, 2) to identify categories of genes in which significantly different temporal patterns of expression in the OE occurred in response to OBX in the two strains, and 3) to describe in detail the temporal pattern of expression of genes related to the innate immune response whose expression in the OE was significantly up- or downregulated by OBX in wt mice and to compare their expression patterns in the two strains. By comparing the cellular and molecular responses to OBX in the OE of wt and MSR^+/− mice, we identified key genes and pathways involved in the innate immune response to OBX and identified a role for SR-A in OE homeostasis.

MATERIALS AND METHODS

Animals and tissue preparation. We obtained 36 6-wk-old male mice of the wt C57BL/6 strain from the Jackson Laboratory (Bar Harbor, ME). We obtained 36 6-wk-old male mice of the MSR^+/− strain, in which exon 4 of the Msr1 gene was disrupted (73), that were backcrossed onto a C57BL/6 background (2) from a breeding colony maintained at University of Kentucky. MSR^+/− mice were genotyped according to the protocol of Babaev et al. (2) to confirm the absence of SR-A. All mice were maintained in the facilities of the Department of Laboratory Animal Research on a 12:12-h light/dark cycle and were given food and water ad libitum. All protocols were implemented in accordance with National Institutes of Health guidelines and approved by the University of Kentucky Institutional Animal Care and Use Committee.

We used 21 mice of each strain for cellular studies to determine their olfactory phenotype and to investigate their response to bilateral OBX. Fifteen mice of each strain underwent bilateral OBX; three of the remaining six mice of each strain underwent sham bilateral OBX (0 h sham OBX), and three of each strain were euthanized without any surgical manipulation. The latter six mice were perfused, and tissues were removed and processed as described below; the tissues from these mice were used for histological and immunohistochemical procedures. No differences in the cellular parameters described in RESULTS were noted between the latter two groups; only the data from the 0-h sham OBX mice are reported. Both bilateral sham OBX and bilateral OBX were performed according to this laboratory’s protocols as previously described (26, 61). All mice whose tissues were used for histological and immunohistochemical studies were injected with 5-bromo-2’-deoxyuridine (BrdU, 100 mg/kg ip; Sigma Chemical, St. Louis, MO) in sterile saline 2 h before perfusion. For tissue collection, three mice of each strain with no surgical manipulation and three mice of each strain per time point were euthanized by CO2 asphyxiation at 0 (sham), 2, 8, 16, or 48 h or 3 days post-OBX. These mice were perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 3% paraformaldehyde. Tissues were postfixed for 2 h in 3% paraformaldehyde, cryoprotected, embedded, frozen, and sectioned as previously described (26, 61).

We used 15 mice of each strain at 0 h (sham) and 2, 8, 16, and 48 h post-OBX, 3/time point, for microarray analysis and real-time RT-PCR studies. They were euthanized by CO2 asphyxiation, and olfactory-nasal mucosa and liver were rapidly removed, weighed, frozen in liquid N2, and stored at −80°C.

All reagents used in animal preparation, tissue collection, and in situ hybridization protocols were molecular biology grade. Surgical instruments and disposables were RNase-free, and surgery and tissue collection and handling were performed with RNase-free techniques.

Histology and immunohistochemistry. Cresyl violet staining was performed on defatted, rehydrated frozen sections with 1% aqueous cresyl violet (Sigma). DNA staining was performed on rehydrated frozen sections with a 1:105 dilution of bisbenzimide (Hoechst 33258, Sigma) in PBS.

A standard indirect immunofluorescence technique was used as previously described (61) to visualize immunoreactivity for the mature OSN marker olfactory marker protein (OMP; a generous gift of Dr. Frank Margolis) and macrophages (CD68 and F4/80). The height of the OE on the nasal septum was measured from the basement membrane to the top of the visible ciliary layer as previously described (61). The numbers of CD68+ and F4/80+ macrophages and BrdU+ proliferative basal cells were counted in the same region along 6–9 lengths of OE basement membrane, each measuring 200 μm in length as previously described (61). Statistical analysis was performed using two-tailed unpaired t-tests (GraphPad Instat v. 3.06; GraphPad Software, San Diego, CA).

RNA isolation. Frozen tissue was pulverized in TRI reagent (Sigma-Aldrich) and processed through a QIAshredder column (Qiagen, Valencia, CA), and total RNA was purified using the Qiagen RNEasy Mini-Kit according to the manufacturer’s protocol. Total RNA yield and purity were assessed spectrophotometrically and with the model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA); all samples had A260/A280 ratios of 1.9–2.2 and showed two sharp peaks corresponding to 18S and 28S RNA on Bioanalyzer electropherograms.

High-density oligonucleotide arrays. Affymetrix Murine Genome U74Av2 GeneChips (Affymetrix, Santa Clara, CA) were processed at the University of Kentucky Microarray Core Facility. Thirty GeneChips were used. Hybridization of unpoled RNA, normalization, and identification of present genes were performed as previously described (50). The microarray data set was deposited into the Gene Expression Omnibus database; the series accession number is GSE3455.

Statistical analyses of microarray data. Mean hybridization signals were analyzed using a 2 × 5 ANOVA (overall P < 0.05), with strain as one factor and time post-OBX [0, 2, 8, 16, or 48 hours post-OBX (hpO)] as the other. The false discovery rate (FDR) correction (6, 66) at 1% was applied, reducing the effective P value to <3.2E-03. Identification of genes whose temporal pattern of expression differed significantly in the two strains was determined by using αnew = 3.2E-03, determined by the previous FDR procedure as the cutoff point. The protected Fisher’s least significant difference test procedure at the level of αnew was adopted to control the family-wise error rate in identifying the subsequent interaction effect between strain and time, main effects, and/or simple effects. Data were analyzed using Excel (Microsoft, Redmond, WA), SAS (SAS Institute, Cary, NC), and SigmaPlot (SPSS, Chicago, IL). To determine if functional categories as defined by the Gene Ontology (http://www.geneontology.org) were overrepresented among genes with highly significant differences (P < 3.2E-03) in expression levels in the two strains at Physiol Genomics • VOL 27 • www.physiolgenomics.org
RESULTS

Cellular changes following target ablation. The cellular organization of the OE of MSR−/− mice (Fig. 1A) appeared indistinguishable from that of wt mice (e.g., 50, 61). OSNs, identified by immunohistochemistry with OMP (Fig. 1B), comprised the lower 3/4 of the OE, with a thin two- to three-cell high band of progenitor cells at the base of the epithelium. As in wt mice (61), small numbers of resident mgs expressing the pan-mg marker CD68 occurred primarily in the lamina propria beneath the OE; very rare intraepithelial resident mgs were also observed in both wt (Fig. 1C) and MSR−/− mice. OE height was comparable in sham wt and MSR−/− mice (Fig. 2A). At 3 days after OBX, OE height was reduced by 57% in wt mice and 68% in MSR−/− mice (Fig. 2A). The numbers of infiltrating mgs increased significantly in both strains compared with their respective shams (Figs. 1D, 2A), due primarily to the loss of most of the OMP-immunoreactive OSNs (Fig. 1E); the height of the OE was 26% less in MSR−/− than in wt mice following OBX (P < 1.0E-03). The numbers of infiltrating mgs increased significantly in both strains, particularly in the OE (Fig. 1F), but there were notable differences. While the numbers of CD68+ mgs were initially comparable and increased significantly (P < 1.0E-03) following OBX in both wt and MSR−/− mice, significantly fewer (P = 0.012) infiltrated the OE in MSR−/− mice (2.9-fold increase) vs. wt mice (4.8-fold in-

Fig. 1. Effects of olfactory bulbectomy (OBX) on olfactory epithelium (OE) of macrophage scavenger receptor (MSR)−/− mice. A and D: cresyl violet staining; B and E: olfactory marker protein (OMP) immunohistochemistry (IHC); C and F: CD68 IHC. A, D: in OE of 0-h sham MSR−/− mice (A), pattern of organization was qualitatively the same as in wild-type (wt) mice; lightly stained sustentacular cell nuclei lay just below epithelial surface, and a thick layer of darkly stained nuclei of olfactory sensory neurons (OSNs) occupied most of the OE height; duct of Bowman’s gland (d) penetrated basement membrane. In OE of MSR−/− mice at 3 days post-OBX (D), most OSN nuclei were gone, resulting in large decrease in OE height; apoptotic nucleus (arrow) was present in OSN layer. Sustentacular cell nuclear layer remained intact. B, E: in 0-h sham MSR−/− mice (B), closely packed OMP+ OSN cell bodies (arrows) occupied most of the lower 3/4 of OE, with dendrites projecting to OE surface and a thin band of unstained progenitor cells at base. In MSR−/− mice at 3 days post-OBX (E), a large reduction in the number of OMP+ cells (arrows) resulted in decreased OE height. C, F: in OE of 0-h sham MSR−/− mice (C), an occasional intraepithelial CD68+ macrophage (arrow) was observed. In OE of MSR−/− mice at 3 days post-OBX (F), intraepithelial CD68+ macrophages (arrows) were more numerous. Scale bar (in D), 15 μm for A–F.
increase, Fig. 2B); thus there were 48% fewer macrophages in the OE of MSR<sup>−/−</sup> vs. wt mice after OBX. The numbers of activated m<sub>0</sub>s, identified by immunoreactivity for F4/80, increased significantly in wt mice (P = 0.023, 3.3-fold), but there was no increase in their numbers in MSR<sup>−/−</sup> mice (Fig. 2C). The number of proliferative BrdU<sup>+</sup> progenitor cells was significantly higher initially in MSR<sup>−/−</sup> sham vs. wt sham mice (P = 2.0E-03, 5.1 fold); following OBX, the number increased significantly in both wt and MSR<sup>−/−</sup> mice but was significantly lower in MSR<sup>−/−</sup> vs. wt mice (P < 1.0E-03, 1.9-fold). Another difference between the two strains was the apparent persistence of apoptotic bodies in the OE of MSR<sup>−/−</sup> vs. wt mice (Fig. 3, A and B) as indicated by nuclear staining with bisbenzimide. There appeared to be more individual large apoptotic bodies and more numerous clusters of small apoptotic bodies, particularly in the turbinates, in MSR<sup>−/−</sup> vs. wt mice at 3 days post-OBX. These results demonstrated substantial differences in OBX-induced cellular responses, including decreased numbers of recruited and activated m<sub>0</sub>s, reduced progenitor cell proliferation, and an apparent defect in the clearance of apoptotic OSNs in the OE of MSR<sup>−/−</sup> vs. wt mice.

*Global and interactive gene analyses.* The flow chart (Fig. 4) outlines the analyses applied to the hybridization signals on the GeneChips to identify genes in the OE that had significantly different patterns of expression at at least one time point following OBX in wt and MSR<sup>−/−</sup> mice. Among the probe sets for known genes, an Absolute Call analysis identified 6,458 (70%) as present on at least one chip. A (mouse strains) × 5 (0-h sham OBX and 2, 8, 16, and 48 hPO time points) ANOVA performed on the Present probe sets identified 4,006 significantly (P < 0.05) regulated genes. The FDR correction set to a stringent 1% was performed to evaluate the approximate number of type I errors represented as false positives, which reduced the number of significantly regulated probe sets by ~52% to 2,092 genes with P values of <3.2E-03.

A major aim of this study was to identify genes regulated by OBX as a result of OSN apoptosis and the subsequent innate immune response, neurogenesis, and epithelial remodeling that exhibited different temporal patterns of expression in MSR<sup>−/−</sup> and wt mice. We separated the significantly regulated genes into two groups based on their expression levels in the 0-h sham OBX mice of each strain. For ~52% of the genes, there was no significant difference (NSD, P > 0.05) between the magnitudes of the mean hybridization signals at 0 h in the two strains; ~48% exhibited significant differences (P < 0.05) between the 0-h shams (Fig. 4). Among the 1,095 genes with NSD at 0 h, 293 genes (27%) had a highly significant differ-

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**Fig. 2.** Comparison of effects of OBX in wt and MSR<sup>−/−</sup> mice. A: OE height, which was equivalent in 0-h shams of strains (open bars), was significantly reduced compared with respective shams at 3 days post-OBX (striped bars) in both strains (P < 0.001). OE height in MSR<sup>−/−</sup> mice was significantly less than in wt mice at 3 days post-OBX (P < 0.001). B: numbers of CD68<sup>+</sup> macrophages recruited to the OE at 3 days post-OBX increased significantly in wt and MSR<sup>−/−</sup> mice (P < 0.001), but significantly fewer were recruited in MSR<sup>−/−</sup> mice (P < 0.01), but there was no change in their numbers in MSR<sup>−/−</sup> mice (P < 0.05), but there was no change in their numbers in MSR<sup>−/−</sup> mice. C: the numbers of F4/80<sup>+</sup> activated macrophages recruited to the OE at 3 days post-OBX increased significantly in wt mice (P < 0.001), but there was no change in their numbers in MSR<sup>−/−</sup> mice (P < 0.05), but there was no change in their numbers in MSR<sup>−/−</sup> mice. D: ~5 times more 5-bromo-2′-deoxyuridine (BrdU)<sup>+</sup> progenitor cells occurred in OE of 0-h sham MSR<sup>−/−</sup> vs. wt mice (P < 0.05). Numbers of BrdU<sup>+</sup> cells increased significantly in both strains (P < 0.001) at 3 days post-OBX, but increase in proliferating progenitor cells was significantly less (P < 0.001) in MSR<sup>−/−</sup> mice. *P < 0.05, **P < 0.01, ***P < 0.001.

**Fig. 3.** Apoptotic bodies in the OE at 3 days post-OBX. DNA staining demonstrated apoptotic bodies (arrows) in the OE of both wt mice (A) and MSR<sup>−/−</sup> mice (B). In MSR<sup>−/−</sup> mice, single apoptotic bodies (arrows) appeared larger and more numerous, and clusters of small apoptotic bodies (brackets) were more numerous. Scale bar (in A), 15 μm for A and B.
ence (P values ranging from 7.8E-13 to 3.2E-3) between the magnitudes of their mean hybridization signals in the two strains at least one time point post-OBX; that is, these genes differed significantly in their temporal pattern of expression in the two strains following OBX. We further classified these genes by identifying the time point at which the first statistically significant difference in expression between the two strains occurred. Seventy-six genes showed their first highly significant difference in the two strains at 2 h, 25 genes at 8 h, 56 genes at 16 h, and 136 genes at 48 h following target ablation. For example, there was NSD at 0 h in the expression of \( \text{Cp} \) (ceruloplasmin, Fig. 5A), an IL-1 inducible gene expressed by masts and glia that is upregulated following retinal and brain injury (10, 44, 49), in the wt and MSR/−/− strains. In wt mice, \( \text{Cp} \) was significantly upregulated at 16 h (P = 3.4E-04, 1.5-fold) and at 48 h (P = 7.8E-05, 3.8-fold). The first statistically significant difference between the expression of \( \text{Cp} \) in the two strains occurred at 16 h (P = 1.0E-03) when the magnitude and direction of expression in the two strains diverged sharply and the gene was expressed at a 1.7-fold higher level in wt than in MSR/−/− mice. At 48 h, \( \text{Cp} \) was again expressed at a significantly higher level in wt than in MSR/−/− mice (P = 4.2E-03, 2.5-fold). Of the remaining genes with NSD at 0 h, 337 (31%) had a significant difference (P values ranging from 3.3E-3 to 0.049) between the magnitudes of their mean hybridization signals in the two strains at least one time point post-OBX. The remaining genes (465 genes, 42%) were main-effect genes; that is, their patterns of expression were parallel in the two strains, indicating that these genes responded to OBX with similar changes in their magnitude and direction of expression over time. For example, there was NSD in the expression of \( \text{Bmp6} \) (bone morphogenetic protein 6, Fig. 5B), a member of the transforming growth factor (TGF)-β superfamily of growth factors that is expressed by mature OSNs (63). In wt mice, \( \text{Bmp6} \) was significantly downregulated at both 16 h (P = 0.013, 1.5-fold) and at 48 h (P = 0.017, 2.0-fold), and there was a parallel downregulation of expression at each time point in both wt and MSR/−/− mice, showing the main effect of treatment at all post-OBX time points.

Among the 997 genes for which there were significant differences (P < 0.05) at 0 h between the two strains, 283 genes (28%) exhibited highly significant differences between strain and time (P values ranging from 2.2E-09 to 3.1E-3). One hundred eighty-two genes showed their first statistically significant difference in expression at 2 h, 15 genes at 8 h, 40 genes at 16 h, and 46 genes at 48 h following target ablation. Of the remaining genes with a significant difference at 0 h, 369 (37%) had a significant difference (P values ranging from 3.3E-3 to 0.049) between the magnitudes of their mean hybridization signals in the two strains at least one time point post-OBX. The remaining genes (345 genes, 35%) were main-effect genes.

In summary, 2,092 genes were differentially regulated (1% FDR, P = < 0.0032) in the two strains, of which about half

![Flow chart demonstrating statistical analysis of microarray data.](Fig. 4)
A Gene with Highly Significant Interaction between Strain and Time

![Graph showing hybridization signals for Cp (ceruloplasmin) gene]

**Cp** 92851_at

p = 1.9E-06

- Mean hybridization signal in wt and MSR -/- mice are shown by dashed lines and ●; time point(s) at which there was a significant difference (P < 0.05) with mean hybridization signal in 0-h sham mice. Mean hybridization signals ± SD for wt mice are shown by solid lines and ●, *time point(s) at which there was a significant difference (P < 0.05) with mean hybridization signal in 0-h sham mice. Mean hybridization signals ± SD for MSR -/- mice are shown by dashed lines and ●, •, time point at which first statistically significant difference in temporal pattern of expression between the 2 strains occurred; **time point(s) at which there was a significant difference (P < 0.05) with mean hybridization signal in wt mice. A: genes with significant interaction between strain and time. *Mean hybridization signals for Cp (ceruloplasmin) were significantly greater at 16 and 48 hPO than in 0-h sham mice. The temporal pattern of expression showed the first significant difference between wt and MSR -/- mice at 16 hPO (*). There was also a significant difference in expression at 48 hPO (**). B: main-effect gene. Mean hybridization signals for Bmp6 (bone morphogenetic protein 6) was significantly lower at 16 hPO and 48 hPO (*) than in 0-h sham mice. Pattern of expression of Bmp6 in the 2 strains changed in parallel over time in response to OBX; that is, there were no statistically significant differences between the magnitudes and/or direction of change of mean hybridization signals at any post-OBX time point.

B Main Effect Gene

![Graph showing hybridization signals for Bmp6]

**Bmp6** 161028_at

p = 0.990

- Mean hybridization signal in wt and MSR -/- mice are shown by dashed lines and ●; ●, time point at which first statistically significant difference in temporal pattern of expression between the 2 strains occurred; **time point(s) at which there was a significant difference (P < 0.05) with mean hybridization signal in wt mice. A: genes with significant interaction between strain and time. *Mean hybridization signals for Cp (ceruloplasmin) were significantly greater at 16 and 48 hPO than in 0-h sham mice. The temporal pattern of expression showed the first significant difference between wt and MSR -/- mice at 16 hPO (*). There was also a significant difference in expression at 48 hPO (**). B: main-effect gene. Mean hybridization signals for Bmp6 (bone morphogenetic protein 6) was significantly lower at 16 hPO and 48 hPO (*) than in 0-h sham mice. Pattern of expression of Bmp6 in the 2 strains changed in parallel over time in response to OBX; that is, there were no statistically significant differences between the magnitudes and/or direction of change of mean hybridization signals at any post-OBX time point.

Fig. 5. Differences in patterns of expression of genes that are significantly regulated in response to OBX. The text in each graph shows gene symbol, probe set ID, and overall P value for the significance of the interaction between strain and time. Mean hybridization signals ± SD for wt mice are shown by solid lines and ●, *time point(s) at which there was a significant difference (P < 0.05) with mean hybridization signal in 0-h sham mice. Mean hybridization signals ± SD for MSR -/- mice are shown by dashed lines and ●, **time point(s) at which there was a significant difference (P < 0.05) with mean hybridization signal in wt mice. A: genes with significant interaction between strain and time. *Mean hybridization signals for Cp (ceruloplasmin) were significantly greater at 16 and 48 hPO than in 0-h sham mice. The temporal pattern of expression showed the first significant difference between wt and MSR -/- mice at 16 hPO (*). There was also a significant difference in expression at 48 hPO (**). B: main-effect gene. Mean hybridization signals for Bmp6 (bone morphogenetic protein 6) was significantly lower at 16 hPO and 48 hPO (*) than in 0-h sham mice. Pattern of expression of Bmp6 in the 2 strains changed in parallel over time in response to OBX; that is, there were no statistically significant differences between the magnitudes and/or direction of change of mean hybridization signals at any post-OBX time point.

exhibited either NSD or a significant difference in their initial level of expression in the two strains. About 28% of the differentially regulated genes displayed a temporal pattern of expression following OBX that showed highly significant differences (P < 3.2E-03) between wt and MSR -/- mice.

**Categorical gene analysis.** The genes used in Fig. 5 to illustrate the statistical categories of genes with a significant interaction between strain and time and main-effect genes suggest that these statistical categories cut across functional categories. Functional overrepresentation in the genes with NSD at 0 hPO and a significant difference at 0 hPO were analyzed separately using the EASE program and are listed in Supplemental Table 1. (The online version of this article contains supplemental data.) Genes with an SD at 0 hPO included intracellular genes related to ribosomes and mitochondrial electron transport. These categories are consistent with the biological and molecular categories of protein biosynthesis and RNA binding, and nucleotide triphosphate metabolism, hydrogen ion transporter activity, and oxidoreductase/antioxidant activity, respectively. The mitochondrial electron transport chain and hydrogen ion transporter activity/oxidoreductase activity categories contain many of the same genes, including those in the NADH dehydrogenase (ubiquinone), ubiquinol cytochrome c reductase, and cytochrome c oxidase families. In the hydrogen ion transporter activity category, 18/20 genes that are expressed exclusively in the mitochondrial electron transport chain were expressed at significantly higher levels in MSR -/- mice, and in the antioxidant category, 7/11 genes, including peroxiredoxins 1–4, were expressed at higher levels, indicating the occurrence of oxidative stress in the OE of MSR -/- mice. Another overrepresented category, regulation of apoptosis, contained primarily proapoptotic genes such as caspases 2, 3, and 9 that were expressed at significantly lower levels in MSR -/- mice; however, apoptotic nuclei were not detected in the OEIs of sham mice of either strain. At 2 hPO, genes with significant interactions in both groups (NSD or significant difference at 0 hPO) were primarily intracellular. Among genes with NSD at 0 hPO with a significant interaction at 2 hPO were genes associated with the endoplasmic reticulum in the secretory pathway (e.g., Rab6, Ssr1). The hydrolase activity and GTPase molecular function categories contained five of the same genes, including Rab6 as well as two genes of the Ras family and their related G proteins that are involved in cytoskeletal modulation. Among genes with a significant difference at 0 hPO and a significant interaction at 2 hPO there were several transcription factors in the ribonucleoprotein complex/nucleotide/nucleic acid metabolism categories and several mitochondrial complex I NADH dehydrogenase flavoproteins in the NADH dehydrogenase/electron transporter categories. These results indicate that OBX-induced gene transcription and energy-dependent processes were regulated differently in the two strains. Of particular relevance to this study was the overrepresentation of genes with a significant difference at 0 hPO and a significant difference in expression levels at 2 hPO that are associated with the regulation of apoptosis. Among the genes in this category are Cebpb (CCAAT/enhancer binding protein-β), Pdcd6 (programmed cell death 6), Rnf7 (ring finger protein 7), and Traf3 (TNF receptor-associated factor 3). Other apoptosis genes that were previously reported to be regulated by OBX in wt mice (25), such as Casp2 and Bax (see Apoptosis genes below), also show this pattern. No categories related to apoptosis were overrepresented at later time points, suggesting that the regulation of apoptosis genes by OBX was similar in the two strains by 16–48 hPO.

Other genes of particular relevance to this study also appear in the overrepresented categories. Il1rn (IL-1 receptor antagonist) is in the growth factor activity category, probably relating to its role in the regulation of insulin and lipase levels, among genes with NSD at 0 hPO and a significant interaction at 8 hPO. Among the genes with NSD at 0 hPO that had a significant interaction at 16 hPO, Grn (granulin) encodes a growth-

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promoting protein that interacts with the product of Slpi (secretory leukocyte protease inhibitor) to promote epithelial wound healing. Among the genes with NSD at 0 hpO that had a significant interaction at 48 hpO, genes related to the extra-cellular space were significantly overrepresented, most likely related to the overrepresentation of genes in the immune response, chemotaxis, and chemokine activity biological/molecular categories that also had significant EASE scores at this time point. Genes with related functions in other over-represented categories at 48 hpO included Il1r2 (IL-1 receptor, type II) and Il1rl1 (IL-1 receptor-like 1) in the growth factor binding category; and Slpi in the endopeptidase/protease inhibitor activity category.

Although EASE analysis provides insight into functional categories of genes that were regulated differently in the two strains, the overrepresentation of a particular functional category does not provide information on the relative magnitude of the expression levels or direction of these changes. Therefore, we examined the detailed temporal expression patterns of genes whose expression level in wt mice was significantly regulated (P < 0.05) at one or more time points after OBX and that were classified in the following categories: 1) genes expressed in the OE primarily by OSNs; 2) genes previously associated with apoptotic pathways involved in OSN cell death; and 3) because SR-A is expressed primarily by mØs and endothelial cells, genes associated with cytokine and chemokine secretion and response, diapedesis, and phagocytosis that are relevant to OE degeneration and remodeling.

**Olfactory-related genes.** As we previously reported (25), genes expressed only or primarily by OSNs showed a characteristic pattern of expression following OBX in wt mice, with significantly lower levels of gene expression beginning at 16 hpO for many genes (e.g., Bmp6, Fig. 5B; Omp, Fig. 6A) and for all by 48 hpO (e.g., Uchl1 (ubiquitin carboxy-terminal hydrolase L1) or PGP9.5, a neuronal marker; Fig. 6B); this pattern indicates the loss of OSNs by apoptosis. In general, in MSR−/+ mice, these genes exhibited a sharp decrease in mean hybridization signals at 2 hpO followed by an increase to near 0-h sham levels at 8 hpO; at 16 hpO, the level of expression was greater in MSR−/+ than in wt mice but not significantly greater than in 0 hpO sham MSR−/+ mice. At 48 hpO, there was no significant difference between the two strains in the levels of expression for most of these genes. This pattern occurred in 15/19 genes expressed primarily by OSNs that we previously identified as regulated by OBX. Nine of the 19 OSN genes (Supplemental Table 2) were main-effect genes.

**Apoptosis genes.** Because OBX causes apoptosis primarily of mature OSNs, we anticipated that relatively few apoptosis genes would exhibit significant differences in temporal patterns of expression in the two strains. Nine of the 17 apoptosis genes
(Supplemental Table 3) were main-effect genes, including all of the caspases that were regulated by OBX (25). For example, the proapoptotic mediator Casp3 (caspase 3) was a main-effect gene with a significant difference at 0 h, and a significant increase in expression at 8 hpO (P = 0.012, 1.3-fold), 16 hpO (P = 2.0E-04, 1.5-fold), and 48 hpO (P = 6.0E-04, 1.4-fold) in wt mice, with a highly similar pattern in MSR−/− mice. Another main-effect gene, proapoptotic Bid (BH3 interacting domain death agonist; Fig. 6), had NSD at 0 h and significant increases in expression at 16 hpO (P = 8.5E-04, 1.8-fold) and 48 hpO (8.0E-06, 2.4-fold) in wt mice, with a highly similar pattern of expression in MSR−/− mice. A downstream target of Bid, Ctsd (cathepsin D), which was significantly upregulated in wt mice at 48 hpO (P = 4.8E-03, 1.5-fold), was also a main-effect gene. Among apoptosis genes that showed different patterns of expression in the two strains was Bax (Bcl2-associated X protein, Fig. 6D), a proapoptotic regulator that significantly increased in expression in wt mice at 16 hpO (P = 0.012, 1.2-fold) and 48 hpO (P = 1.8E-03, 1.3-fold) and Casp2 (caspase 2), a negative regulator of apoptosis in neurons; notably, the levels of expression of these genes at 16 and 48 hpO was equivalent in the two strains. Apoptosis genes with highly significant differences in expression levels at 16 and/or 48 hpO were all expressed at lower levels in MSR−/− mice than in wt mice.

Cytokines. Our microarray results identified a potential candidate for an OSN-derived cytokine that may initiate the local immune response to OBX: IL-1β. At 2 hpO, there was a significant upregulation of Il1b (IL-1β, Fig. 7A) in both wt (P = 0.013, 2.1-fold,) and MSR−/− mice (P = 0.033, 2.5-fold). This was consistent with the upregulation of the IL-1β-inducible Ccl2 [chemokine (C-C motif) ligand 2, monocyte chemoattractant protein 1 (MCP-1)] mRNA by 8 hpO (see Chemokines below) and protein by 16 hpO (27). In contrast to the early parallel pattern of expression of Il1b in the two strains, there was a significant difference at 48 hpO (P = 9.8E-06, 8.5-fold), with an increase in expression in wt mice and a decrease in MSR−/− mice. This was also consistent with the pattern of regulation of Ccl2 (see Chemokines below). Il1r2 (IL-1 receptor antagonist) increased significantly at 48 hpO in wt mice; in MSR−/− mice, mean hybridization signals increase significantly over those of wt mice at 8 hpO but at 48 hpO did not occur.

**Fig. 7. Expression patterns in response to OBX for interleukin (IL)-1-related genes.** See Fig. 5 for symbols and text for details. A: mean hybridization signals for Il1b (IL-1β) increased significantly at 2 and 48 hpO in wt mice; in MSR−/− mice, mean hybridization signals increase at 2 hpO but not at 48 hpO. B: mean hybridization signals for Il1r2 (IL-1 receptor, type II) increased in parallel in the 2 strains at 2 hpO, but the significant increase in wt mice at 48 hpO did not occur in MSR−/− mice. C: the expression pattern of Il1a (IL-1α) was different from that of Il1b in that there was no significant increase in expression until 48 hpO in wt mice. In MSR−/− mice, mean hybridization signal increased but to a significantly lower level than in wt mice. D: mean hybridization signals for Il1rn (IL-1 receptor antagonist) increased significantly at 48 hpO in wt mice; in MSR−/− mice, mean hybridization signals increase significantly over those of wt mice at 8 hpO but at 48 hpO did not occur.
was no upregulation in either strain at 2 hPO, and, at 48 hPO, the level of \( \text{Il}1a \) expression increased significantly in both strains (wt mice, \( P = 6.3E-05, 4.3\)-fold; MSR\(^{-/-}\) mice, \( P = 0.013, 2.1\)-fold), although it was expressed at a significantly lower level than in wt mice (\( P = 3.6E-05, 2.0\)-fold). In wt mice, \( \text{Il}1\text{rn} \) (IL-1 receptor antagonist, Fig. 7D) was significantly upregulated at 48 hPO (\( P = 2.1E-04; 5.2\)-fold); in MSR\(^{-/-}\) mice, the gene was significantly upregulated at 8 hPO (\( P = 0.011, 3.4\)-fold), differing significantly from its expression level in wt mice (\( P = 4.2E-04, 3.2\)-fold). At 48 hPO, there was no upregulation of expression as seen in the wt mice, resulting in a significantly lower level of expression in the MSR\(^{-/-}\) mice (\( P = 4.5E-03, 14.3\)-fold). The two main-effect IL-1-related genes, \( \text{Tollip} \) (Toll interacting protein) and \( \text{Tom1} \) (target of myb1 homolog, Supplemental Table 4), which interact with each other and are negative regulators of IL-1 signaling, were significantly downregulated at 48 hPO in wt mice (\( \text{Tollip}, P = 0.023, 1.4\)-fold; \( \text{Tom1}, P = 0.015, 1.4\)-fold).

Other \( \text{mb} \)-derived cytokines whose gene expression patterns exhibited significant differences between strain and time included members of the neuropoietic cytokine, interferon-\( \alpha/\beta \), and complement families, and several related transcription factors and signaling molecules (Supplemental Table 4), and one TGF-\( \beta \)-related anti-inflammatory cytokine that is associated with phagocytosis (\( \text{Tgfb1} \), TGF-\( \beta \) induced; Supplemental Table 6). Of the 25 cytokine and cytokine-related genes with significant differences in expression patterns between the two strains at one or more time points post-OBX, most were significantly upregulated in wt mice at 16 hPO (6 genes) and 48 hPO (11 genes). In MSR\(^{-/-}\) mice, most of these genes were not upregulated at 48 hPO, with near-sham levels of expression of six of eight IL-1-related genes, five of eight neuropoietic cytokine-related genes, one of three complement-related genes, two of three signaling molecules, and the TGF-\( \beta \)-related gene.

Among genes in the neuropoietic or IL-6-type cytokine family with patterns of expression that showed significantly different patterns of expression in the two strains, \( \text{Lifr} \) (leukemia inhibitory factor receptor) increased significantly at 48 hPO in wt mice; in MSR\(^{-/-}\) mice, there was no significant increase in expression at 48 hPO. \( \text{Il}6 \) (interleukin 6) increased significantly at 48 hPO in wt mice; they first differed significantly in the 2 strains at 2 hPO when its expression was significantly upregulated in MSR\(^{-/-}\) but not wt mice, and at 48 hPO when its expression was significantly upregulated in wt but not MSR\(^{-/-}\) mice. C: expression pattern of \( \text{Osmr} \) (oncostatin M receptor) differed from \( \text{Lifr} \) and \( \text{Il}6 \), being upregulated (significantly in MSR\(^{-/-}\) mice) at 8 hPO in both strains, but at 48 hPO, mean hybridization signals were significantly upregulated in wt but not MSR\(^{-/-}\) mice. D: similarly, \( \text{Socs3} \) (suppressor of cytokine signaling 3) expression was significantly upregulated at 8 hPO in both strains, but only in wt mice at 48 hPO.
hpO (P = 1.2E-03, 3.0-fold), but in MSR−/− mice, it was expressed at 0-h sham levels that were significantly lower than in wt mice (P = 0.011, 26.7-fold). The expression pattern of OsMr (oncostatin M receptor, Fig. 8C) differed from that of both LIFr and IL6 in that it was upregulated (significantly in MSR−/− mice) at 8 hpO in both strains (wt, 2.8-fold; MSR−/−, P < 1.0E-04, 2.8-fold). The pattern of expression between the strains differed at 48 hpO, when in wt mice, OsMr expression was significantly upregulated (P = 0.012, 3.9-fold) to a level significantly higher than in MSR−/− mice (P = 1.5E-03, 2.4-fold). The receptors through which these cytokines signal share a common element, gp130, encoded by Il6st (IL-6 signal transducer), which was upregulated significantly at 48 hpO in wt mice (P = 3.4E-03, 1.2-fold) and was a main-effect gene. Soc3 (suppressor of cytokine signaling 3, Fig. 8D), a regulator of signaling through gp130-containing receptors, was expressed in the three strains in a pattern resembling that of OsMr. At 8 hpO, there was a significant increase in expression over 0-h sham levels in both wt mice (P = 7.4E-03, 1.6-fold) and MSR−/− mice (P = 0.011, 2.0-fold). At 48 hpO, there was a significant increase in expression in wt mice (P = 6.2E-03, 2.3-fold) that was significantly higher than in MSR−/− mice (P = 2.9E-05, 3.1-fold).

Chemokines. The expression patterns of four CC chemokines and three CXCL chemokines differed significantly in wt and MSR−/− mice (Supplemental Table 5). Ccl2 (Fig. 9A) showed a biphasic expression pattern in wt mice, with significant upregulation at 8 hpO (P = 1.6E-04, 14.7-fold), 16 hpO (P = 9.1E-03, 9.4-fold increase), and 48 hpO (P = 5.0E-04, 23.7-fold). In MSR−/− mice, the pattern of expression was highly similar at 8 and 16 hpO, but at 48 hpO there was a significant difference in expression levels between the 2 strains (P = 2.2E-05, 6.1-fold). In wt mice, the other CC chemokines with highly significant differences in expression patterns, Ccl3 [chemokine (C-X-C motif) ligand 3, macrophage inflammatory protein (MIP)-1α] and Ccl6 [chemokine (C-C motif) ligand 6, c10] showed no change in expression levels until significant upregulation at 48 hpO (Ccl3, P = 3.4E-03, 5.2-fold; Ccl6, P = 0.011, 1.4-fold). In MSR−/− mice, the expression of Ccl3 and Ccl6 remained at 0-h sham levels at all time points, resulting in significant differences in expression levels in the two strains (Ccl3, P = 8.1E-06, 3.8-fold; Ccl6, P = 0.018, 2.0-fold). The CXC chemokines showed similar patterns of expression to those of the CC chemokines. In wt mice, the expression levels of Cxcl1 [chemokine (C-X-C motif) ligand 1, Gr1 oncopogene] and Cxcl2 [chemokine (C-C motif) ligand 2, Gro2, Fig. 9C] were significantly upregulated at only 48 hpO (Cxcl1, P = 2.7E-03, 7.5-fold; Cxcl2, P = 2.4E-03, 51.5-fold). In MSR−/− mice, the level of expression of Cxcl1 at 2 hpO was significantly greater than that in wt mice (P = 0.031, 5.7-fold), and for both, there were significant differences at 48 hpO with the wt strain (Cxcl1, P = 6.6E-03, 16.0-fold; Cxcl2, P = 1.9E-07, 42.7-fold).

Diapedesis-related genes. Several genes encoding key adhesion molecules and their receptors as well as cytoskeletal components and associated signaling pathways associated with the diapedesis of recruited monocytes (10, 76) showed significantly different patterns of expression after OBX in wt and MSR−/− mice (Supplemental Table 5). For example, Selp (P-selectin, Fig. 9D) was significantly upregulated only in wt mice at 48 hpO, when there was a significant increase in expression (P = 0.011, 8.6-fold); the lack of upregulation in MSR−/− mice resulted in a significant difference in expression levels between the strains (P = 3.0E-05, 10.3-fold). Two genes encoding monocyte adhesion molecules were regulated by OBX, including Itgb2 (integrin-β2, LFA-1) whose significant upregulation at 48 hpO in wt mice (P = 4.6E-03, 1.7-fold) was previously reported (25), were main-effect genes. In wt mice, Vcam1 (vascular cell adhesion molecule 1, Fig. 9E), an endothelial adhesion molecule, was significantly upregulated at 48 hpO (P = 4.0E-04, 2.5-fold) as previously reported (25); in MSR−/− mice, its expression level remained at 0-h sham levels, resulting in a significant difference in expression between the two strains (P = 1.2E-04, 1.9-fold). The receptor for VCAM-1, Icam1 (intercellular adhesion molecule 1), was significantly upregulated at 48 hpO in wt mice (P = 1.1E-03, 3.1-fold); in MSR−/− mice, Icam1 was expressed at significantly lower levels at both 16 hpO (P = 0.024, 2.8-fold) and 48 hpO (P = 9.0E-04, 3.4-fold).

Genes related to cytoskeletal remodeling were also differentially expressed in the two strains, including numerous genes encoding components of the actin cytoskeleton and signaling molecules associated with its regulation (not shown, see discussion); these patterns were consistent with the lower expression levels of Vcam1 and Icam1, whose interactions with their integrin ligands regulate cytoskeletal remodeling, in MSR−/− vs. wt mice. The S100 calcium-binding proteins S100a8 (Fig. 9F) and S100a9, which regulate the interaction between microtubule and actin cytoskeletal remodeling pathways, were also differentially expressed in the two strains. In wt mice, both genes showed no significant changes in expression levels until 48 hpO, when both were significantly upregulated (S100a8, P = 2.1E-03, 4.0-fold; S100a9, P = 4.5E-03, 3.8-fold); both genes remained at 0-h sham levels in MSR−/− mice, significantly lower than in wt mice (S100a8, P = 9.7E-03, 3.8-fold; S100a9, P = 0.016, 4.3-fold).

Phagocytosis: engulfment of apoptotic cells by macrophages. Numerous phagocytosis-related genes are regulated by OBX (Supplemental Table 6). An enzyme that regulates the exposure of phosphatidylserine groups on the surface of apoptotic cells, Plscr1 (phospholipid scramblase 1, Fig. 10A), was significantly upregulated at 8 hpO (P = 1.3E-04, 2.0-fold) in wt mice, and its expression remained significantly elevated through 48 hpO (16 hpO, P = 8.7E-03, 1.6-fold; 48 hpO, P = 4.7E-04, 2.6-fold). In MSR−/− mice, Plscr1 was expressed at significantly higher levels at 0 h than in wt mice; its expression at 8 hpO was significantly upregulated (P = 9.4E-03, 1.6-fold) as in wt mice, but by 48 hpO, it was expressed at a significantly lower level than in wt mice (P = 4.9E-03, 1.9-fold). Another eat-me signal on apoptotic cells, Anxa1 (annexin A1), was expressed at comparable levels in the shams of both strains (Fig. 10B). In wt mice, Anxa1 was significantly upregulated at 8 hpO (P = 2.1E-03, 1.9-fold) and 16 hpO (P = 0.018, 1.6-fold), with a parallel pattern of expression in MSR−/− mice. However, at 48 hpO, it was expressed at a significantly lower level in MSR−/− mice (P = 2.1E-06, 2.0-fold).

Among molecules that bridge apoptotic cells to phagocytes, Mfge8 (milk fat globule-EGF factor 8 protein) was a main-effect gene that was significantly upregulated in both strains at 48 hpO (wt mice, P = 0.017, 1.3-fold; MSR−/− mice, P = 6.9E-03, 1.3-fold). The three bridging molecules regulated by OBX that had significantly different patterns of expression in
the two strains differed significantly at 48 hPO, consistent with expression patterns in eat-me signals.

Engulfment receptors on m/H9278s include several classes of scavenger receptors. In wt mice, Msr1 was upregulated at 48 hPO with marginal significance (P/110005 0.050, 1.6-fold); the upregulation was validated by real-time RT-PCR (P/11021 0.001, 25.0-fold; Fig. 12), indicating the participation of SR-A in the engulfment of apoptotic OSNs. Several other scavenger receptor genes were significantly upregulated in wt mice, including Cd36 (Cd36), a class B scavenger receptor, at 16 hPO, Lgals3bp (galectin-3 binding protein) and Stab1 (stabilin 1) at 16 and 48 hPO, and Scarb1 (scavenger receptor class B, member 1, Fig. 10C) and CD68 (Cd68, Fig. 10D), a class D scavenger receptor, at 48 hPO. Among the scavenger receptors
that were regulated by OBX in wt mice, the expression level of only Scarb1 (Fig. 10C) was significantly upregulated in MSR−/− compared with wt mice at 48 hpO (P = 0.022, 1.3-fold), suggesting that SR-B1 partially compensated for the absence of SR-A. We confirmed the upregulated expression of Scarb1 at 48 hpO with in situ hybridization (Fig. 13). Cd68 (Fig. 10D), which was significantly upregulated in both wt and MSR−/− mice at 48 hpO (wt mice, P = 5.9E-03, 1.8-fold; MSR−/− mice, P = 8.6E-03, 1.6-fold), was a main-effect gene.

The genes encoding numerous phagosomal proteins were regulated by OBX and many showed significant differences in their expression patterns in the two strains. Among these were several actin- and tubulin-associated genes associated with cytoskeletal rearrangement. For example, Capg (gelsolin-like actin filament capping protein, Fig. 11A), was significantly upregulated in wt mice at 16 hpO (P = 0.013, 1.8-fold) and 48 hpO (P = 2.1E-03, 2.8-fold). In MSR−/− mice, Capg was expressed at a significantly lower level at 48 hpO than in wt mice (P = 0.012, 2.3-fold). Similarly, Arhgdia (Rho GDP dissociation inhibitor−α) was also significantly upregulated in wt mice at 16 hpO (P = 7.4E-03, 1.3-fold) and 48 hpO (P < 1.0E-04, 1.6-fold); in MSR−/− mice, Arhgdia was expressed at significantly lower levels at both 16 and 48 hpO (16 hpO, P = 0.046, 1.2-fold; 48 hpO, P = 0.033, 1.3-fold). H2-D1 (histocompatibility 2, D region locus 1, Fig. 11B), an antigen of the major histocompatibility class I associated with phagosomes, showed similar patterns of expression. H2-D1 was significantly upregulated at 16 hpO (P = 7.4E-03, 2.0-fold) and 48 hpO (P = 0.012, 2.0-fold) in wt mice. In MSR−/− mice, it was expressed at significantly lower levels at 16 hpO (P = 1.1E-04, 2.4-fold) and 48 hpO (P = 8.8E-03, 1.7-fold).

Secretion of TGF-β, the prototypical anti-inflammatory mediator released when møs engulf apoptotic cells, was indicated by the upregulated expression of at least two genes induced by TGF-β (e.g., Tgfb1, Fig. 11C; the growth factor-related Tgfb14, not shown). In wt mice, Tgfb1, a mediator of epithelial and endothelial cell adhesion, was significantly upregulated at 16 hpO (P = 0.012, 1.9-fold) and 48 hpO (P = 6.5E-04, 3.3-fold). In contrast, in MSR−/− mice, there was no upregulation of the gene, with resultant significant differences in expression compared with wt mice at both time points (16 hpO, P = 8.0E-03, 1.6-fold; 48 hpO, P = 1.7E-03, 3.0-fold). Slpi (Fig. 11D), a serine protease inhibitor with anti-inflammatory activity whose secretion is induced by the binding of møs to apoptotic cells, was significantly upregulated over its levels in shams in both

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**Fig. 10. Expression patterns in response to OBX for engulfment signal genes and scavenger receptors.**

- **A:** “eat me” signals. Plscr1 (phospholipid scramblase 1) was significantly upregulated at 8, 16, and 48 hpO in wt mice; it was expressed at significantly higher level in 0 h sham MSR−/− mice and was upregulated as in wt mice at 8 and 16 hpO but decreased significantly at 48 hpO. Similarly, Anxal (annexin A1) was significantly upregulated at 8, 16, and 48 hpO in wt mice, with similar upregulation at 8 and 16 hpO in MSR−/− mice, but decreased significantly at 48 hpO. C, D: scavenger receptors. Mean hybridization signals for Scarb1 (scavenger receptor class B, member 1) increased significantly at 48 hpO in wt mice. Expression pattern was significantly different in MSR−/− mice, with significantly lower expression at 2 hpO and significantly higher expression at 48 hpO. The scavenger receptor Cd68 (CD68, macrophilin; D) was significantly upregulated at 48 hpO in wt mice, with similar pattern of expression in MSR−/− mice.
wt and MSR−/− mice at 48 hPO (wt, P = 1.2E-03, 10.7-fold; MSR−/−, P = 2.2E-03, 11.2-fold), but there was a statistically significant difference between the levels of expression in the two strains (P = 4.8E-05, 2.2-fold), consistent with reduced mφ activation in the MSR−/− mice. Anti-inflammatory mediator gene Tgfbi (TGF-β induced, C) was upregulated in wt mice at 16 and 48 hPO; its pattern of expression in MSR−/− mice was significantly different at both 16 and 48 hPO. Slpi (secretory leukocyte protease inhibitor, D), another anti-inflammatory mediator gene, was significantly upregulated by OBX at 48 hPO in wt but not MSR−/− mice. E, F: mφ genes. Arg1 (arginase 1, E), an indicator of alternative mφ activation, was significantly upregulated at 48 hPO in wt mice but not in MSR−/− mice. Mφ maturation indicator Chi3l1 (chitinase 3-like 1, F) was significantly upregulated in wt mice at 48 hPO; its pattern of expression in MSR−/− mice was significantly different at both 16 and 48 hPO.

**Other macrophage genes.** In addition to chemokines, cytokines, and phagocytosis mediators, mφs synthesize other characteristic products whose gene expression levels may indicate their maturation or activation state; those regulated by OBX are...
listed in Supplemental Table 7. For example, Ch311 (chitinase 3-like 1, Fig. 11F), a gene induced as mβs near maturation from monocytes, was significantly upregulated at 48 hPo in wt mice (P = 4.1E-03, 3.5-fold); in MSR−/− mice, the gene was expressed at significantly lower levels at both 16 hPo (P = 9.7E-03, 2.3-fold) and 48 hPo (P = 9.1E-03, 4.8-fold). Other genes induced as mβs mature, such as Cd53 and S100a9, were also expressed at significantly lower levels in MSR−/− compared with wt mice. Activation state may also be indicated by gene expression levels. Mβs metabolize arginine via two pathways: by using inducible nitric oxide synthase (NOS2) to yield the highly reactive NO as well as L-citrulline (classical activation), and by using arginase 1 to yield ornithine, a precursor of growth-promoting factors, and urea (alternative activation). In both wt and MSR−/− mice, Nos2 was “always absent” at all time points. In contrast, Arg1 (Fig. 11E) was upregulated in wt mice at 48 hPo (P = 0.014, 7.6-fold), indicating that OE mβs exhibited an alternatively rather than classically activated phenotype in response to OBX (50). In MSR−/− mice, Arg1 was expressed at significantly lower levels than in wt mice at 48 hPo (P = 4.9E-05, 44.6-fold), which, in the absence of Nos2 upregulation, suggests lack of mβ activation rather than classical activation. Clecsf8 (C-type lectin, superfamily member 8), an endocytic receptor expressed primarily by activated mβs, showed the same pattern of regulation and of differences between the two strains.

**DC genes.** Mβs and DCs both express SR-A, are found at mucosal surfaces, phagocytose apoptotic cells, and express an overlapping repertoire of activation markers, cytokines, chemokines, and other function-related molecules; therefore, it is possible that some differences in the response to OBX in wt and MSR−/− mice were due to differences in DC function. To evaluate this possibility, the expression profiles of two genes that are markers for DCs were analyzed. Ly75 (lymphocyte antigen 75, DC205) and Adam19 (a disintegrin and metalloproteinase domain 19) were expressed in the OE and were regulated by OBX (Supplemental Table 8). In wt mice, Ly75, a marker of immature DCs, was significantly upregulated at 48 hPo (P = 5.5E-03, 1.5-fold); in MSR−/− mice, Ly75 was not regulated by OBX and at 48 hPo was expressed at significantly lower levels than in wt mice (P = 0.037, 1.4-fold), suggesting that the absence of SR-A affected DC function in response to OBX. Adam19 was significantly upregulated at 16 hPo in wt mice (P = 0.043, 1.4-fold) and was a main-effect gene.

**Secretory immune system genes.** Activated B cells that produce polymeric IgA are components of the secretory immune system in the olfactory mucosa (23, 57). The expression of Pigr (polymeric immunoglobulin receptor), the transporter of polymeric IgA in Bowman’s glands, was significantly upregulated in wt mice at 48 hPo (P = 4.9E-03, 2-fold); in MSR−/− mice, it was expressed at a significantly higher level at 2 hPo (P = 0.034, 1.8-fold) and at a significantly lower level at 48 hPo (P = 0.030, 1.8-fold). Igj (immunoglobulin joining chain), a B lymphocyte product that links molecules of IgA into pentamers, was a main-effect gene that was upregulated in wt mice at 2 hPo (P = 0.025, 3.8-fold).

**Molecular validation using real-time RT-PCR.** Using real-time RT-PCR to determine the expression levels of Msr1, we confirmed its significant upregulation by OBX in wt mice at 48 hPo (Fig. 12). The expression of Msr1 was negligible in the MSR−/− mice, with no significant change in expression as a result of OBX.

In addition, we used real-time RT-PCR to determine the direction and magnitude of changes in expression levels for the interleukin gene Il1rn (Fig. 7D), the chemokine gene Cxcl1, the scavenger receptor gene Scarb1 (Fig. 10C), and the phagosome-associated gene H2-D1 (Fig. 11B) at time points at which the microarray data indicated that there were statistically significant differences in expression in the two strains (Table 1). In all cases, the direction of change in the microarray data and the real-time RT-PCR data was the same. Although fold changes determined by real-time RT-PCR are considerably larger for Il1rn and Cxcl1 compared with those obtained from microarray data, these genes also had relatively large fold changes compared with other genes in the microarray data. Because the expression levels of Scarb1 in the wt and MSR−/− mice as determined by real-time RT-PCR were not significantly different although the direction of change was the same, we validated the upregulated expression of Scarb1 mRNA with in situ hybridization.

**Molecular validation using in situ hybridization.** The expression of Scarb1 mRNA was evaluated in the OE of 0 hPo sham and 48 hPo MSR−/− mice. In sham MSR−/− mice (Fig. 13A), there was a faint band of hybridization signals localized over the OE (Fig. 13B). At 48 hPo, the OE was noticeably thinner (Fig. 13C), and hybridization signals increased markedly (Fig. 13D).

There was a very sparse, unlocalized distribution of radioactivity when the sense probe was used in place of the antisense probe in the OE from sham MSR−/− mice, and there was
no change in the OE from 48 hpO MSR\textsuperscript{−/−} mice (not shown). The stronger signal with the antisense probe in the OE of the 48-hpO MSR\textsuperscript{−/−} mice confirms the upregulation of Scarb1 at 48 hpO as determined by the microarray analysis (Fig. 10C).

DISCUSSION

Our results demonstrate that the immune response to OBX in the OE of wt and MSR\textsuperscript{−/−} mice differed in several important ways, including: 1) altered cellular parameters such as the infiltration of fewer m\textregistereds, the presence of fewer proliferative cells, and the apparent persistence of apoptotic bodies in MSR\textsuperscript{−/−} compared with wt mice; 2) different temporal patterns of expression of many categories of genes in the two strains of mice following OBX, particularly at 2 and 48 hpO; and 3) different temporal patterns of expression of immune response genes that were significantly up- or downregulated by OBX in the wt mice, particularly at 16 and 48 hpO, in contrast to OSN-specific genes and genes in apoptotic pathways that were generally expressed at similar levels in the two strains at 16 and 48 hpO. Taken together, these results indicate that SR-A plays a key role in the immune response to OBX in the OE.

The absence of functional SR-A had no significant effect on OE height in 0-h sham mice, suggesting that approximately the same numbers of OSNs were present in both strains before OBX. OE height was significantly reduced by OBX in both strains (57% in wt mice, 68% in MSR\textsuperscript{−/−} mice), indicating a consistent loss of mature OSNs in both strains. This is consistent with the finding that most OSN-specific genes show NSD in expression levels at 0 hpO and that OBX resulted in the significant loss of OSN-specific mRNA transcripts to approximately the same level in both strains at 48 hpO. We currently have no data that account for the small (26%) but significantly lower OE height in MSR\textsuperscript{−/−} compared with wt mice at 3 days post-OBX. The disruption of SR-A expression did result in significantly reduced m\textregistered infiltration and an apparent reduction in the clearance of apoptotic bodies in response to OBX, strongly suggesting that SR-A was involved in m\textregistered recruitment and/or infiltration and in phagocytosis of apoptotic OSNs. Previous studies from our laboratory demonstrated that m\textregistereds are a source of growth factors that promote neurogenesis (25, 26, 61), so the lower numbers of BrdU\textsuperscript{+} cells in MSR\textsuperscript{−/−} mice post-OBX was consistent with reduced m\textregistered infiltration in this strain. The significantly greater number of BrdU\textsuperscript{+} cells in the 0-h sham MSR\textsuperscript{−/−} vs. wt mice (Fig. 2D) may be due to the presence of oxidative stress, a factor known to initiate cell proliferation (e.g., Refs. 38, 77), in the OE of MSR\textsuperscript{−/−} mice as indicated by the EASE analysis of overrepresented categories of genes with a significant difference at 0 h.

Table 1. Real-time RT-PCR validation of microarray data

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Time, hpO</th>
<th>MSR\textsuperscript{−/−} vs. wt Cr</th>
<th>Real-time RT-PCR FC</th>
<th>P</th>
<th>Microarray Data FC</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1m</td>
<td>48</td>
<td>25.4±0.1</td>
<td>−27.0</td>
<td>2.8E-06</td>
<td>−14.3</td>
<td>2.3E-11</td>
</tr>
<tr>
<td>Cxcl1</td>
<td>48</td>
<td>28.2±0.2</td>
<td>−43.0</td>
<td>4.7E-05</td>
<td>−16.0</td>
<td>2.2E-05</td>
</tr>
<tr>
<td>Scarb1</td>
<td>48</td>
<td>25.4±0.7</td>
<td>1.2</td>
<td>0.750</td>
<td>1.4</td>
<td>0.013</td>
</tr>
<tr>
<td>H2-D1</td>
<td>16</td>
<td>22.5±0.3</td>
<td>−2.5</td>
<td>4.3E-03</td>
<td>−2.4</td>
<td>5.9E-05</td>
</tr>
</tbody>
</table>

Threshold cycle (C\textsubscript{T}) values are means ± SD. Real-time RT-PCR values are shown in bold. Cxcl1, chemokine (C-X-C motif) ligand 1; FC, fold change; H2-D1, histocompatibility 2, D region locus 1; hpO, hours postolfactory bulbectomy; B1m, interleukin 1 receptor antagonist; MSR\textsuperscript{−/−}, scavenger receptor class A-deficient mice; Scarb1, scavenger receptor class B, member 1; wt, wild-type C57BL/6 mice.

Fig. 13. Validation of Scarb1 upregulation by OBX in OE of MSR\textsuperscript{−/−} mice with in situ hybridization. A: section of nasal tissue from 0-h sham stained with Giemsa stain. OE is located between parallel rows of asterisks. B: autoradiograph of same section shows faint hybridization signals for Scarb1 over OE. C: section of nasal tissue at 48 hpO stained with Giemsa stain. OE thickness between parallel rows of asterisks is noticeably reduced. D: autoradiograph of same section shows strong hybridization signals for Scarb1 over OE. L, lumen of nasal cavity; S, nasal septum. Scale bar (in A), 50 μm for A–D.
The global gene analysis demonstrated that about half of the genes with $P < 3.2E-03$ were expressed at similar levels in the 0-h shams of wt and MSR$^{-/-}$ strains, and about half were expressed at significantly different levels. This suggests that there were biological differences between the two strains that might influence the immune response to OBX. Among the immune response genes with $P < 3.2E-03$ shown in Supplemental Tables 4–8, 82% had NSD in their initial level of expression in 0 h shams. In the EASE analysis, the greatest number of categories of genes that were expressed with significantly different temporal patterns in the two strains with a significant difference at 0 h occurred at 2 hpo (10/15 categories), and the greatest number containing genes with NSD at 0 h occurred at 48 hpo (12/15 categories) and included categories related to immune response. These results based on categorical analysis and individual gene analyses suggest that though there were differences in the baseline levels of gene expression between the two strains, the greatest impact of these differences was early in the response to OBX and was less likely to involve immune response genes.

A large number of immune response genes were significantly up- or downregulated by OBX in wt mice, suggesting the importance of this response in neurogenesis and epithelial remodeling. A recent report on gene expression induced by bilateral OBX at 1, 5, and 7 days post-OBX using wt mice of the same strain, sex, and age and the same microarrays also found immune response genes to be a significantly regulated category (68). Many of the individual genes that were upregulated at 1 day post-OBX were also upregulated at 16 and/or 48 hpo in our study. The overlap in immune response genes identified in these two studies and the finding that many of the same genes are upregulated at 5 and 7 days post-OBX indicates that the ongoing immune response continues to play a major role in neurogenesis and epithelial remodeling at up to at least 1 wk post-OBX.

In the absence of functional SR-A, most of the genes directly related to immune response that we identified in this study, including those encoding cytokines, chemokines and mΦ activation and response genes, were expressed with significantly different temporal patterns in wt vs. MSR$^{-/-}$ mice, with most differences occurring at 16 and 48 hpo, and overwhelmingly in a direction indicating impairment of the immune response and mΦ activation in MSR$^{-/-}$ mice. The fact that there were 50% fewer mΦs in the OE of MSR$^{-/-}$ mice undoubtedly contributed to the lower level of expression of these genes but did not account for the reduction in gene expression to baseline levels in most cases. For example, two genes, Ccl2 and Ccl3, which we previously demonstrated to play key roles in monocyte recruitment following OBX and to be expressed exclusively in infiltrating mΦs at 16 hpo and 3 days post-OBX (27), were highly significantly upregulated by $~24$- and 5-fold, respectively, in wt mice; in MSR$^{-/-}$ mice, the mean expression levels at 0 h vs. 48 hpo were not significantly different for either gene and were significantly lower at 48 hpo than 50% of the mean expression level at 48 hpo in wt mice. This suggests that, in MSR$^{-/-}$ mice, the infiltrating mΦs were not responding to local signals that induced Ccl2 and Ccl3 expression in wt mice or that these signals were not present at 48 hpo. The early significant upregulation of Ccl2 expression at 8 hpo suggests that resident mΦs were capable of responding (i.e., were mature) and/or received a local signal that induced Ccl2. Our data support both interpretations; for example, Il1b, an inducer of Ccl2 (8, 34), was significantly upregulated at 2 hpo, before the peak of both Ccl2 upregulation and mΦ infiltration, in both strains, but its mean expression level at 48 hpo in MSR$^{-/-}$ mice was not significantly different from 0 h sham levels. The significantly lower levels of expression of many genes indicative of mΦ maturation and activation, such as Arg1 and Chi3l1, at 48 hpo in MSR$^{-/-}$ mice compared with wt mice also strongly suggests that the mΦs that infiltrated the OE in MSR$^{-/-}$ mice were functionally impaired, accounting to a large extent for the dysregulation in the immune response to OBX.

We have suggested, on the basis of its pattern of expression, that the product of the Il1b gene may potentially initiate the local immune response to OBX. IL-1β is expressed by neurons and its expression and release are upregulated very rapidly after injury (e.g., 1, 3, 35). IL-1β is induced by S100B (53), which in this study was a main-effect gene significantly upregulated at 2 hpo in wt mice. IL-1β induces MCP-1 as cited above. In view of the temporal pattern of expression of Il1b and the concordance of this gene expression data with genes that induce and are induced by IL-1β, further experiments will be performed to clarify the role of IL-1β in the immune response to OBX.

Many of the immune response genes regulated by OBX may be associated with more than one relevant function and/or may be expressed by multiple cell types. For example, some cytoskeletal genes associated with phagocytosis may also be regulated because of other processes that occur in the OE during this time span as a result of OBX, such as diapedesis, OSN degeneration, loss of contacts between epithelial cells as OSNs die, and activation of cells in the progenitor cell compartment (25). Another example is the large number of genes that are expressed by both mΦs and DCs, including SR-A. Our limited data on specific markers of DCs suggest that they participate in the immune response to OBX, in agreement with previous results (68) and, further, that the absence of SR-A affects DC gene expression. DCs do not, to our knowledge, secrete growth factors as mΦs do; thus DCs are unlikely to be involved in the regulation of neurogenesis in the OE. A more likely role is for immature DCs to act as antigen presenters via class I MHC in the induction of mucosal and systemic tolerance for self-antigens derived from apoptotic cells (19, 69). Ly75, a marker of immature DCs (71), was upregulated in wt mice at 48 hpo (this study) and at 5 days post-OBX (68), suggesting that DCs that have ingested apoptotic OSNs remain intact in the OE and present antigens derived from apoptotic OSNs. The lack of upregulation of Ly75 in MSR$^{-/-}$ mice may result from impairment of DC binding to apoptotic cells as suggested for mΦs below. Thus DCs are likely to play a very different role from that of mΦs in the immune response to OBX.

The MSR$^{-/-}$ model was utilized because SR-A is a key receptor in several mΦ functions that are relevant to the epithelial immune response to OBX. It is difficult to ascribe the reduced numbers of recruited/infiltrating monocytes/mΦs and the impressive dysregulation in the expression of genes related to mΦs and mΦ-associated functions to a single function of SR-A. However, our results strongly indicate that in both strains of mice, resident mΦ activation occurred at 2–8 hpo. The reduced mΦ infiltration into the OE in MSR$^{-/-}$ mice most
likely resulted from the loss of the adhesion function of SR-A, resulting in a temporal pattern of gene regulation that strongly suggests a lack of activation beginning with endothelial tethering/diapedesis. The resultant lack of monocyte maturation and subsequent mΦ activation led to reduced secretion of mΦ chemokines and cytokines. In addition, the lack of functional SR-A may have reduced the binding of mΦs to apoptotic cells and caused a defect in phagocytosis for which for SR-B and CD68 partially compensated. As a result of defective phagocytosis, the upregulation of genes encoding anti-inflammatory mediators was also reduced. Yet there were no histological signs of inflammatory infiltrates or gene regulation to suggest the induction of a proinflammatory response, supporting our interpretation of the lack of mΦ activation in the MSR \textsuperscript{−/−} mice.

OBX induced OSN apoptosis and the resultant mΦ infiltration into the OE. Given the low number of mΦs present in the OE of wt 0-h sham mice and reports that OSNs release factors that regulate neurogenesis (e.g., 79), how relevant are experiments in which mΦs are our mice. However, in humans documented to be free of nasal disease, immune system cells, including mΦs, and immune factors are normal components of the olfactory mucosa (e.g., 24, 39–41, 57). This indicates that in the “real world,” immune surveillance and immune response are active processes in the OE, which is exposed to ambient particulates, pathogens, pollutants, allergens, etc. and which provides a direct pathway into the brain (e.g., Ref. 31). Nasal disease and associated olfactory dysfunction result in a marked increase in the number of immune system cells; smell dysfunction and recovery correlate with OSN loss and neurogenesis, respectively (4, 24, 41, 42, 54). Taken together, these studies emphasize the importance of mΦs and the innate immune response to the study of olfactory epithelial dynamics and neurogenesis.

In summary, our results confirmed that a robust immune response is initiated in the OE shortly after OBX and that mΦs play a key role in the recruitment and activation of infiltrating mΦs, the phagocytosis of apoptotic OSNs, and neurogenesis. By utilizing MSR \textsuperscript{−/−} and Mip1-\alpha \textsuperscript{−/−} transgenic mouse models in which mΦ infiltration/function is impaired in response to OBX (this study and Ref. 50), our laboratory has provided convincing evidence that activated mΦs promote olfactory neurogenesis, and, conversely, that in their absence, neurogenesis is reduced.

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