Addendum: Defective Dock2 expression in a subset of ASC-deficient mouse lines

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Published reports suggest that the inflammasome adaptor ASC regulates immune responses independently of its well-known role in inflammasomes. In this context, ASC-deficient mice (*Pycard*$^{-/-}$ mice; called '*Asc*$^{-/-}$ mice' here) have been found to be resistant to antigen- and collagen-induced arthritis and experimental autoimmune encephalomyelitis, but mice deficient in the cytoplasmic receptor NLRP3 (*Nlrp3*$^{-/-}$ mice) or caspase-1 (*Casp1*$^{-/-}$; as noted below, these mice are actually doubly deficient in caspase-1 and caspase-11)[1] are not[2–4]. In addition, granuloma formation and host defense during chronic infection with *Mycobacterium tuberculosis* has been suggested to require ASC but not NLRP3 or caspase-1 (ref. 1). In our article, we reported defective expression of the guanine nucleotide–exchange factor Dock2 in the bone marrow–derived macrophages (BMDMs), bone marrow–derived dendritic cells (BMDCs), T cells and B cells of mice with targeted deletion of *Asc*[5], thereby providing a possible explanation for the previously reported defects in antigen presentation and lymphocyte migration in these mice[4]. In this context, Dock2 has already been established as a central regulator of the migration of lymphocytes and plasmacytoid dendritic cells[6–8]. In addition, we demonstrated defective antigen uptake in Dock2-deficient BMDCs similar to that of ASC-deficient cells and that ectopic expression of Dock2 in ASC-deficient BMDCs and lymphocytes restored antigen uptake and lymphocyte migration, respectively[5].

In our facility, we have specifically observed defective Dock2 expression in *Asc*$^{-/-}$ mice, but not in mice deficient in the kinase RICK (*Ripk2*$^{-/-}$ mice), the cytosolic pattern-recognition receptor Nod2 (*Nod2*$^{-/-}$ mice), the adaptors CARD9 (*Card9*$^{-/-}$ mice), TRIF (*Ticam1*$^{-/-}$ mice; called '*Trif*$^{-/-}$ mice' here) or MyD88 (*Myd88*$^{-/-}$ mice), the cytosolic pattern-recognition receptors Nod1 and Nod2 (*Nod1*$^{-/-}$*Nod2*$^{-/-}$ mice), the signaling adaptor MAVS (*Mavs*$^{-/-}$ mice), or the pattern-recognition receptor NLRC4 (*Nlrc4*$^{-/-}$ mice) or in *Nlrp3*$^{-/-}$ mice (**Fig. 1a**). Moreover, defective Dock2 expression has been verified in BMDMs and BMDCs from two independently generated lines of ASC-deficient mice housed in two separate facilities (St Jude Children's Research Hospital, Memphis, Tennessee, USA, and Ghent University, Ghent, Belgium)[5], which makes it unlikely that this is a strain-specific anomaly. The two lines of ASC-deficient mice available to us were generated through the use of embryonic stem cells from mice of the 129 strain[9,10]. Notably, a published study has shown that *Casp1*$^{-/-}$ mice also lack caspase-11 expression because of a mutation in the locus encoding caspase-11 (*Casp4*; called '*Casp11*' here) in the 129 embryonic stem cells used to generate these mice[11]. However, unlike the *Casp1* and *Casp11* loci, the *Asc* and *Dock2* loci reside on separate chromosomes. Moreover, genome-wide single-nucleotide polymorphism (SNP) analysis has confirmed that both of our ASC-deficient mouse lines are approximately 98% identical to C57BL/6 mice (**Fig. 1b**). In addition, we have found normal expression of Dock2 in BMDCs of C57BL/6, 129S1/SvImJ and BALB/c mice (**Fig. 1c**), which suggests that the defective Dock2 expression in these ASC-deficient mice cannot be attributed to their overall genetic background. However, when we examined Dock2 expression in *Asc*$^{-/-}$ mice from other investigators' facilities, we unexpectedly observed defective Dock2 expression in BMDCs and BMDMs of some, but not all, ASC-deficient mouse lines (**Fig. 1d**,**e**).

As our results were highly reproducible, we believe the most probable explanation for the differences in Dock2 expression in different ASC-deficient lines may be subtle differences in genetic background and/or environmental factors. In this context, *Asc*$^{-/-}$ mice have been shown to harbor an altered microflora composition, which leads to the development of a severe dextran sodium sulfate–induced colitis that is transferable to wild-type mice housed together with newborn *Asc*$^{-/-}$ mice that were cross-fostered at birth with wild-type mothers[12]. Alternatively, ASC-deficient mouse lines that fail to express Dock2 may be hemi- or homozygous for an inactivating 'passenger' mutation of *Dock2*. In this context, a spontaneous genomic duplication and frameshift mutation in *Dock2* that reaches homozygosity has been reported to complicate the interpretation of interferon and antibody responses in at least a subset of available mice deficient in the transcription factor IRF5 (ref. 13). Further analysis is warranted, although sequencing of PCR-amplified *Dock2* cDNA isolated from ASC-deficient macrophages with lower Dock2 expression (lines 1 and 2) has failed to identify any missense mutations in *Dock2* (**Fig. 1f**).

In conclusion, the precise reasons for the differences in Dock2 expression in some, but not all, ASC-deficient mouse lines remain unclear and should be explored further in future experiments. Nevertheless, our new experimental data emphasize the proposal that verifying the Dock2-expression status of the various ASC-deficient mouse lines now available is imperative for proper determination and interpretation of the inflammasome-dependent and inflammasome-independent phenotypes of these mice.

ACKNOWLEDGMENTS

We thank R. Flavell (Yale University School of Medicine), G. Nunez (University of Michigan), S. Akira (Osaka University) and V. Dixit (Genentech) for mutant mice, and A. Amer (Ohio State University) and K. Fitzgerald (University of Massachusetts) for femurs from *Asc*$^{-/-}$ mouse lines.

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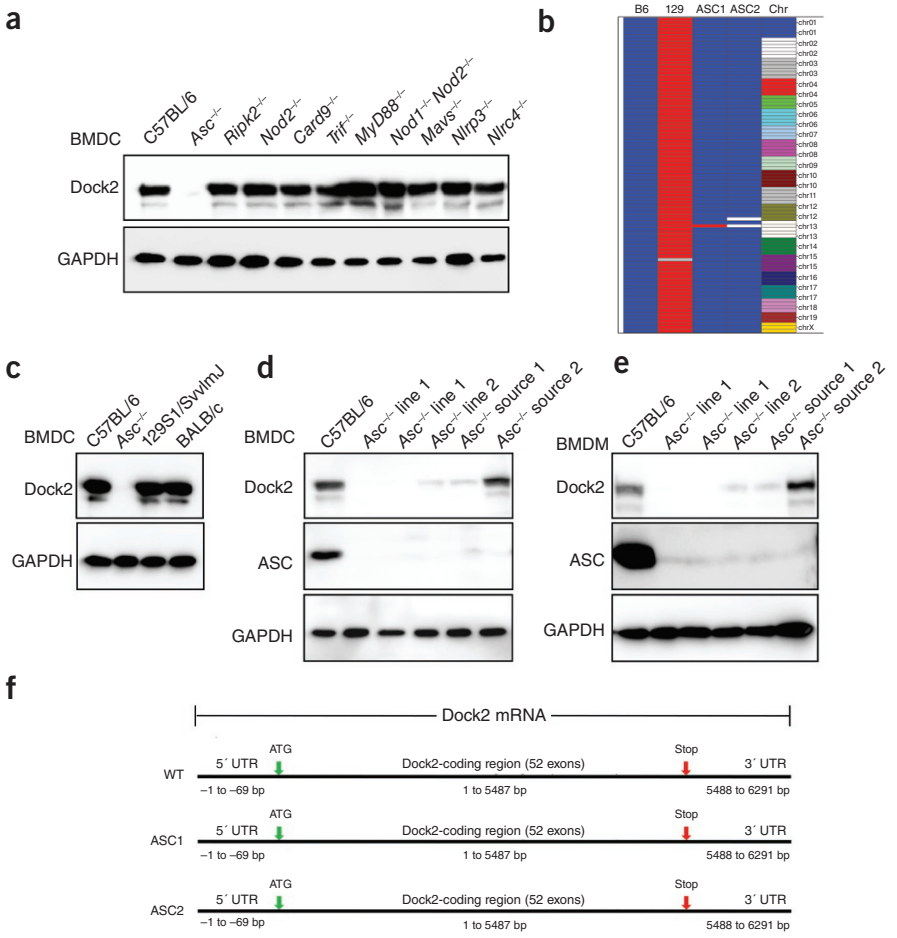

Figure 1 Dock2 expression in BMDCs and BMDMs. (**a**) Immunoblot analysis of Dock2 in lysates of BMDCs from wild-type (C57BL/6) and mutant mice (genotypes above lanes). GAPDH (glyceraldehyde phosphate dehydrogenase) serves as a loading control throughout. (**b**) Genome-wide SNP analysis of mouse strains ASC1 (St Jude Children's Research Hospital; original source, Millennium Pharmaceuticals) and ASC2 (Ghent University; original source, Genentech), which are 98.9% identical (ASC1) and 97.8% identical (ASC2) to the C57BL/6J (B6) strain. Blue indicates homozygous C57Bl/6J genotype; red indicates 129S1/SvImJ genotype; white indicates heterozygous genotype between strains; gray indicates 'no call' at that position in the genome. Far right, chromosome location(Chr) of SNPs investigated. Heterozygous and homozygous SNPs associated with the 129S1/SvImJ (129) strain are present only on chromosomes 12 and 13. (**c**) Immunoblot analysis of Dock2 in naive BMDCs from C57BL/6, *Asc*$^{-/-}$, 129S1/SvImJ and BALB/c mice. (**d**,**e**) Immunoblot analysis of Dock2 in BMDCs (**d**) and BMDMs (**e**) from wild-type mice and *Asc*$^{-/-}$ mice from the following facilities: line 1, St Jude Children's Research Hospital; line 2, Ghent University; source 1, Ohio State University; source 2, University of Massachusetts Medical School. (**f**) Sequence analysis of Dock2 cDNA in BMDMs from *Asc*$^{-/-}$ mouse lines 1 and 2. Data are representative of three independent experiments.

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METHODS

Mice.
The *Ripk2*$^{-/-}$, *Nod2*$^{-/-}$, *Card9*$^{-/-}$, *Trif*$^{-/-}$, *Myd88*$^{-/-}$, *Nod1*$^{-/-}$*Nod2*$^{-/-}$, *Mavs*$^{-/-}$, *Nlrp3*$^{-/-}$ and *Nlrc4*$^{-/-}$ mice and the four *Asc*$^{-/-}$ mouse lines have been reported before[14–21]. Mice were housed in a pathogen-free facility and animal studies were done according to protocols approved by St. Jude Children's Research Hospital Committee on Use and Care of Animals.

Generation of BMDCs.
Bone marrow cells isolated from femurs of 6- to 12-week-old mice were cultured for 7 d at 37 °C in RPMI medium containing 10% heat-inactivated FBS, 100 μM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml of streptomycin, supplemented with granulocyte-macrophage colony-stimulating factor (20 ng/ml), in a humidified atmosphere containing 5% CO_2. On days 3 and 5, half of the medium was replaced with fresh medium containing 40 ng/ml granulocyte-macrophage colony-stimulating factor. Lysates were prepared on day 7 for immunoblot analysis.

Generation of BMDMs.
Bone marrow isolated from the femurs of 6- to 12-week-old mice was cultured at 37 °C in Iscove's modified Dulbecco's medium containing 10% heat-inactivated FBS, 20% conditioned medium from L cells (mouse fibroblasts lacking thymidine kinase), 100 U/ml of penicillin, and 100 μg/ml of streptomycin, in a humidified atmosphere containing 5% CO_2. After 5–7 d of incubation, cells were collected and plated in six-well plates in Iscove's modified Dulbecco's medium containing 10% heat-inactivated FBS and antibiotics. Macrophages were cultured for an additional 24 h before lysates were prepared for immunoblot analysis.

Immunoblot analysis.
Standardized protein concentrations of cellular lysates were separated by SDS-PAGE. Antibody to Dock2 (09-454; Millipore), to ASC (AL177; Enzo Life Sciences) and to β-actin (4970; Cell Signaling Technology) were used at a final dilution of 1:1,000. Proteins were detected by horseradish peroxidase–based enhanced chemiluminescence (34095; Thermo Scientific).

Genome-wide SNP analysis.
SNPs were assayed through the use of a panel of 93 custom-designed SNPs (median intermarker distance of 22.2 megabases; five markers per chromosome) that discriminate between the C57BL/6J and 129S1/SvImJ mouse strains. DNA from mouse tails was assayed with the Illumina GoldenGate assay on the BeadXpress system according to the manufacturer's recommended procedures (Illumina).

RT-PCR and sequencing.
Total RNA was isolated from the BMDCs with TRIzol RNA-extraction reagent (Invitrogen). Full-length *Dock2* cDNA was amplified from 500 ng total RNA with gene-specific primers (forward, 5′-AAGGCGCCTAACCACCCCAGCCA-3′; reverse, 5′-GGTATCATTTCAAATTGTGCTATCATTCC-3′) and the SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen). The *Dock2* cDNA sequence was analyzed by the Sanger dideoxy sequencing method.