

Letter to the Editor

Genetic variation in the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway affects contact hypersensitivity responses

To the Editor:

Allergic contact dermatitis (ACD) is a T-cell-mediated delayed hypersensitivity reaction characterized by the occurrence of inflammatory skin lesions following contact with a particular allergen.¹ Nonprotein allergens, or haptens, capable of eliciting contact dermatitis include a wide range of substances encountered within occupational or domestic settings.² ACD has a reported median prevalence of approximately 20% across Europe and North America and is continuing to increase with limited therapeutic options addressing its burden.³

The development of ACD requires an initial sensitization event where cutaneous exposure to a hapten results in an innate immune response. The initial inflammatory response is suppressed by CD4⁺ regulatory T cells; however, upon a subsequent immune challenge by the same hapten, an inflammatory reaction mediated by CD8⁺ T cells results in the typical pathology of ACD.²

Repeated exposure to haptens has been identified as an occupational risk that can lead to the development of contact dermatitis.³ However, it is clear that there is variation in individual susceptibility to develop clinically significant allergic reactions. More precisely, susceptibility to the development of ACD is related to the presence of genetic variations such as single nucleotide polymorphisms in genes relevant to this process.³ Our current knowledge regarding the impact of genetic polymorphisms on contact dermatitis is largely a result of multiple candidate gene-specific studies, with limited whole-genome expression analyses and genome-wide association study approaches.^{3,4} Importantly, most previous studies have addressed the genetic susceptibility in sensitization, but the ensuing development of dermatitis has seldom been interrogated. Determining the underlying genetics of the subsequent inflammatory response is of particular relevance, because this is directly responsible for patients' symptoms.

Recently, the development of a large panel of recombinant inbred mouse lines known as the Collaborative Cross (CC) has provided a new model to study complex genetic traits. The genome of each CC strain has been comprehensively characterized and therefore we hypothesized that quantitative trait loci (QTL) can be determined for contact hypersensitivity (CHS) by investigating this particular trait across multiple strains.⁵ In this study, we used the CC to investigate the genetic factors underlying CHS, the murine model of ACD in which hapten sensitization and subsequent immune challenge can be fully controlled and standardized.^{1,2}

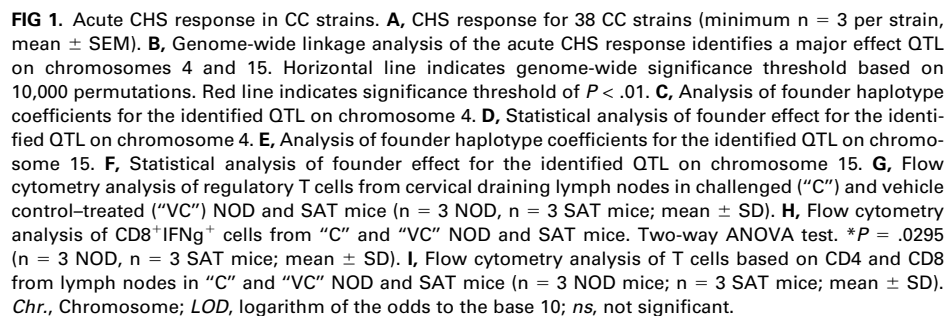
We assessed CHS in 38 strains of CC mice by measuring the increase in ear thickness at various time points after a single standardized oxazolone challenge (see this article's [Methods](#) section in the Online Repository at www.jacionline.org). At each time point, there were marked differences in response between CC strains in both the acute response to immune challenge and postchallenge recovery (see [Results and Discussion](#) sections

and [Fig E1](#) in this article's Online Repository at www.jacionline.org). To determine QTL that control the acute CHS response, we first performed genome-wide linkage analysis on the basis of increase in ear thickness at D1 postchallenge ([Fig 1, A](#); see the [Methods](#) section in the Online Repository). We identified a major effect QTL on mouse chromosome 4 ($-\text{Log}_{10}(P) = 10.1$) in the interval from 19.7 to 33.1 megabases (Mb) ([Fig 1, B](#)). Examination of the founder haplotype coefficients through the linked interval on chromosome 4 showed that the causal variant was derived from the 129SvJv founder strain (hereafter termed 129S) ([Fig 1, C and D](#)).

To determine candidate genes within this interval, DNA variants between susceptible and resistant strains were identified from the Sanger Mouse Genomes database.⁶ No 129S-specific missense variants were identified in any gene within this locus; however, a search of the ENCODE database revealed 34 possible 129S-specific regulatory variants affecting 9 coding regions. Of these, *Bach2* was the most plausible biological candidate associated with the observed phenotype (for further discussion, see [Results and Discussion](#) sections in the Online Repository). Indeed, we found this causal candidate to be carried by strains within which the most rapid ear-swelling responses were observed. When comparing the SAT strain carrying the 129S haplotype and mounting rapid and intense CHS at D1 to NOD that carried a different haplotype at this locus, we observed a tendency toward reduced regulatory T cells at baseline ($P = .06$) ([Fig 1, G](#)) and significantly increased IFN- γ -secreting CD8⁺ T cells in the former ([Fig 1, H](#)). The strains did not differ in total levels of CD4 or CD8 ([Fig 1, I](#)). This shows the importance of the CD8⁺ T-cell response in the phenotype. Of importance, the strains also differed in their macrophage but not neutrophil response, with SAT mice increasing massively the proportion of macrophages (see [Fig E2](#) in this article's Online Repository at www.jacionline.org).

We also mapped a second QTL to chromosome 15 ($-\text{Log}_{10}(P) = 7.8$), in the interval between 32.2 and 55.4Mb, and determined the causal variant to be derived from the CAST founder strain ([Fig 1, E and F](#)). Within this interval we identified *Tnfrsf11b*, *Ebag9*, and *Klf10* as candidate genes, with the CAST haplotype displaying unique noncoding variants of *Klf10* and *Ebag9* within this locus (for further discussion, see [Results and Discussion](#) sections in the Online Repository).

We next analyzed the genetic control over the rate of recovery of ear swelling following immune challenge ([Fig 2, A](#)). Recovery from dermatitis is an important clinical phenomenon because in its absence ACD lesions tend to spread locally and systemically beyond the initial area of contact with the allergen. Remarkably, in several strains the inflammation following immune challenge was maintained over 7 days or worsened despite limiting the challenge to a single application of oxazolone (see [Fig E1](#)). We again calculated the genome-wide significance threshold and identified a bifurcated peak on chromosome X that was highly significant ($-\text{Log}_{10}(P) = 17.0$) ([Fig 2, B](#)) with a 2-logarithm of the odds to the base 10 (LOD) drop between 137.2 and 141.8 Mb. Examination of the founder haplotype coefficients through the linked interval on chromosome X identified that the causal variant was derived from the CAST



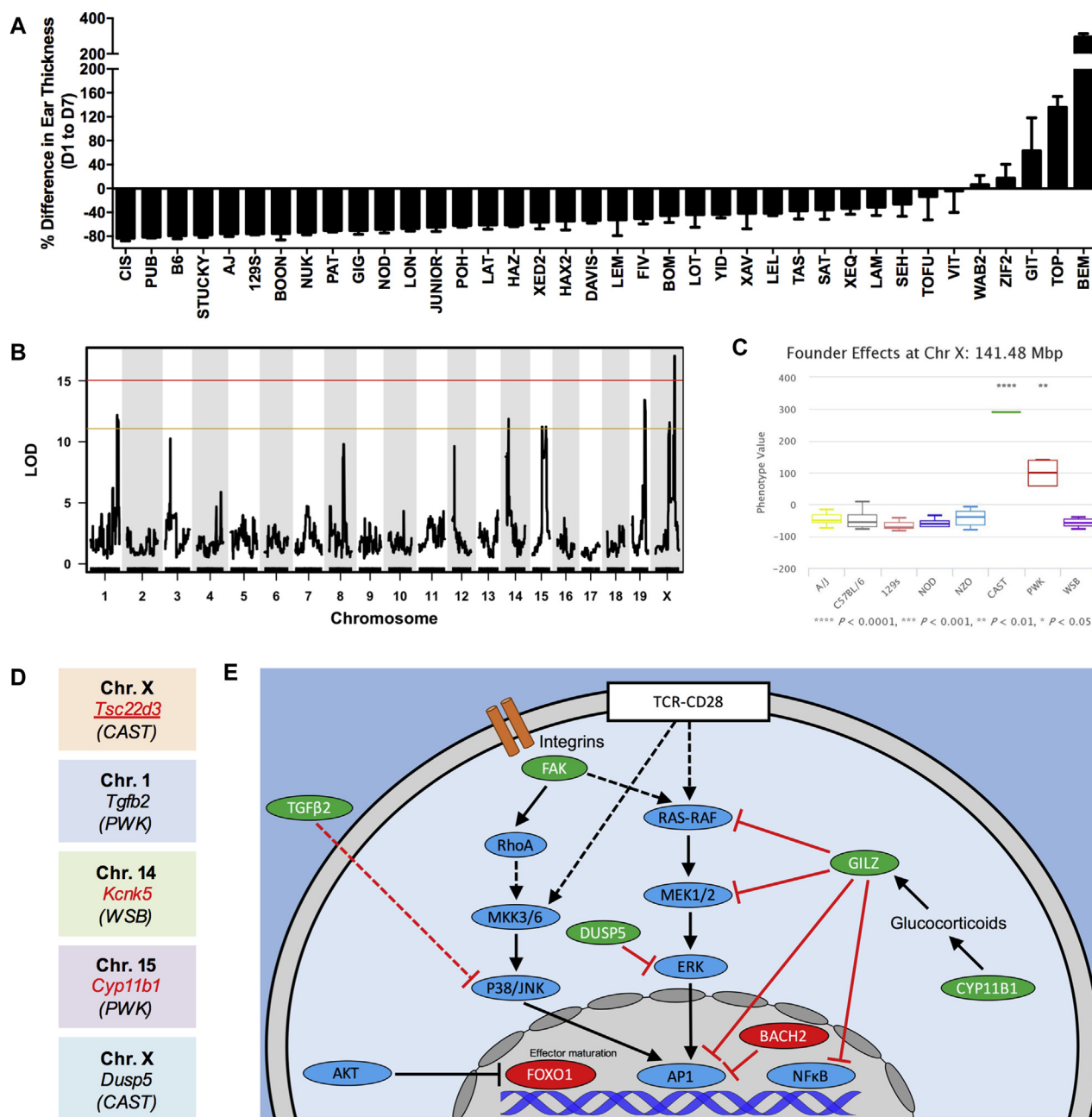


FIG 2. Postchallenge recovery following oxazolone-induced immune challenge in CC strains. **A**, Postchallenge recovery for 38 CC strains (minimum $n = 3$ per strain, mean $\pm$ SEM). **B**, Genome-wide linkage analysis of postchallenge recovery identifies a major effect QTL on chromosome X. Horizontal line indicates genome-wide significance threshold based on 10,000 permutations. Red line indicates permutation-derived significance threshold of $P < .01$, and brown line suggestive linkage ($P < .05$). **C**, Analysis of founder haplotype coefficients for the identified QTL on chromosome X. **D**, Analysis of genomic variants corresponding to founder haplotype (in parentheses) for the significant and suggestive linkages reveal candidate genes that may contribute to the observed phenotype (only the best candidate genes are shown). Variants resulting in missense protein changes are highlighted in red, with those carrying regulatory variants in black. **E**, Schematic depiction of intracellular MAPK/ERK pathway signaling within the T cell, with candidate genes found in this study depicted in green (for recovery phase) and red (for response over time). *Chr.*, Chromosome; *LOD*, logarithm of the odds to the base 10.

founder strain (Fig 2, C). There were only 7 genes with CAST-specific missense variants within this region, as well as 18 genes carrying regulatory variants in the ENCODE database (see Table E1 in this article's Online Repository at www.jacionline.org). Among these genes, we identified *Gilz* (also known as

Tsc22d3) as a biological candidate associated with the recovery following immune challenge given its role in the response to glucocorticoids and IL-10 (Fig 2, D; see the Discussion section in the Online Repository). When considering recovery as an outcome, we observed several other loci with suggestive

linkage, all of which returned LOD scores greater than 10 (Fig 2, B and D; see Table E1). These intervals include chromosome 1, 188.1 to 191.0 Mb; chromosome 14, 19.2 to 21.7 Mb; chromosome 15, 71.6 to 76.1 and 83.3 to 93.2 Mb; and chromosome 19, 50.1 to 53.9 Mb. When examining these intervals and the variants corresponding to the founder haplotype most strongly associated with the phenotype, we identified several biologically relevant candidates that may modify CHS recovery. These included *Tgfb2*, *Kcnk5*, *Lrrk2*, *Ptk2*, *Cyp11b1*, *Shoc2*, and *Dusp5* (for further discussion, see Results and Discussion sections in the Online Repository). Together with the previously identified candidates *Gilz* and *Bach2*, many of these genes were identified as members of the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signaling pathway (Fig 2, E).

Finally, we performed further genome-wide scans for linkage at each day after immune challenge up to D4, also comparing the linkage peaks detected here with those associated with non-recovery at D7. This allowed the confirmation of previously identified loci that failed to reach statistical significance across multiple time points and identification of additional candidate genes (for further discussion, see Fig E2 and Results and Discussion sections in this article's Online Repository at www.jacionline.org).

In this study, we have demonstrated that genetic variability underlies substantial differences in CHS responses and we have identified genetic loci strongly associated with the response to CHS immune challenge in the mouse. The most important associations were with the level of inflammation (acute response to immune challenge), and level of recovery (postchallenge recovery), which both revealed strong gene candidates explaining the observed phenotype. These were identified as *Bach2* and *Gilz*, respectively, and are of significant interest because both are known modifiers of CHS and immune responses in general.^{7,8} Beyond these 2 genes, we have also identified a large number of additional loci and candidate genes possibly associated with the CHS response in multiple independent analyses over time. Altogether, these findings point to the possibility that the MAPK/ERK signaling pathway is a key regulator of the development and recovery of CHS.

In conclusion, our data overwhelmingly support a strong genetic basis for the development of ACD. The intensity of the dermatitis as well as the recovery from the dermatitis were regulated by key loci where candidate genes regulate the MAPK/ERK pathway. These findings have far-reaching implications in the management of ACD and point toward new therapeutic targets.

Julien M. D. Legrand, PhD^a
Edwige Roy, PharmD, PhD^a
Batoul Baz, PhD^a
Pamela Mukhopadhyay, PhD^b
Ho Yi Wong, BSc^a
Ramesh Ram, PhD^c
Grant Morahan, PhD^c
Graeme Walker, PhD^{b*}
Kiarash Khosrotehrani, MD, PhD, FACP^{a*}

From ^athe UQ Diamantina Institute, Translational Research Institute, University of Queensland, Brisbane, Australia; ^bthe QIMR Berghofer Medical Research Institute, Brisbane, Australia; and ^cthe Centre for Diabetes Research, Harry Perkins Institute of Medical Research, Perth, Australia. E-mail: k.khosrotehrani@uq.edu.au.

*These authors contributed equally as senior authors to this work.

Present address: Julien M. D. Legrand is currently at the Australian Regenerative Medicine Institute, Monash University, Clayton, Victoria, Australia.

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ACD is a T-cell-mediated delayed hypersensitivity reaction within the skin and has a high prevalence worldwide.^{E1} ACD occurs following hapten sensitization upon contact with the skin, with subsequent exposures resulting in inflammatory skin lesions, namely, dermatitis or eczema.^{E2} Susceptibility to the development of ACD is clearly variable among individuals and is attributed to polymorphisms in genes associated with the immune response.^{E1} Previous studies have sought to determine the genetic factors underlying hapten sensitization, yet few have examined the genetic determinants driving the different stages of subsequent dermatitis in this context.^{E1}

In this study, we used strains from the CC, a murine resource developed for investigation of complex genetic traits, to evaluate ACD responses using a standard CHS system. The CC was developed from 8 founder strains of mice (A/J, C57BL/6J, 129S1SvImJ, NOD/LtJ, NZO/HILtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) whose genomes have been sequenced.^{E3} The CC mice were developed using a strategy that ensures that each strain has genetic contribution from each of the founder strains, resulting in a population with large phenotypic and genetic diversity that models the amount of variation present in the human population.^{E3} Because the genome of each CC strain has been comprehensively characterized, investigation of a particular trait across multiple strains can determine the underlying QTL associated with the observed phenotype.^{E4} To date, the CC has provided insights into the underlying genetic factors important for a number of conditions, including inflammatory bowel disease,^{E5} melanoma susceptibility,^{E6,E7} and nonalcoholic fatty liver disease.^{E8}

METHODS

CC mice

All CC mouse strains were kindly provided by Geniad Pty Ltd (West Perth, Australia). Mice were bred at Animal Resources Centre (Murdoch, Australia)^{E9} and housed at the University of Queensland Centre for Clinical Research Animal Facility for the duration of the study. All work in this study was conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes, 8th Edition, 2013, and institutional ethical requirements. A minimum of 3 female mice at age 8 weeks for each of 38 strains of the CC were used for CHS experiments.

CHS experiments

Mice were sensitized at age 8 weeks by treating with 150 μ L of 5% oxazolone (4-ethoxymethylene-2-phenyloxazol-5-one) (Sigma-Aldrich, St Louis, Mo), a strong sensitizer, in an 80% acetone/20% corn oil vehicle solution on their abdomen that had been shaved previously using electric clippers. Seven days later, ear-thickness measurements were taken from both ears using a digital micrometer (Mitutoyo, Sakado, Japan). Following initial measurements, mice were challenged with a single application of 20 μ L of 2% oxazolone in vehicle to both surfaces of their left ear, and vehicle-only control to their right ear. This time point was taken as day (D) 0, and subsequent ear-thickness measurements were taken at D1, D2, D3, D4, and D7 postchallenge. Ear swelling is a widely used measure to assess T-cell-mediated delayed-type hypersensitivity reactions including ACD.^{E10-E15}

Flow cytometry

Ears and lymph nodes were harvested. Cells were stained for 20 minutes at 4°C with the fluorophore-conjugated cell surface markers CD3 PB, CD4 PETR, CD8 PerCPCy5.5, CD25 PE, IL10 PE, Ly6c APCCy7, Ly6g PE, and IFN γ FITC. After washing, cells were fixed and then permeabilized for Foxp3 staining according to the manufacturer's protocol (Biolegend, San Diego, Calif). Isotype controls were used to exclude nonspecific fluorescence.

Analysis was performed using a Gallios cytometer (Beckman Coulter, Brea, Calif). The data were analyzed using Kaluza software (Beckman Coulter). Regulatory T cells were analyzed on the basis of CD25 and FoxP3 expression, gating on CD3⁺ population. CD8⁺IFN γ ⁺ cells and T cells based on CD4 and CD8 expression were analyzed gating on CD3⁺ population.

Data analysis

At each time point, ear-thickness measurements for the challenged ear were calculated as a percentage of the vehicle-control-treated ear thickness. These data were plotted over time according to strain. For each strain, the acute response to immune challenge was determined as the percentage increase in ear thickness of the challenged ear compared to the vehicle-control ear thickness at D1. Postchallenge recovery was calculated as the difference between D1 and D7 measurements, which were calculated as a percentage difference between challenged and vehicle-treated ears. Negative values indicated overall recovery, and positive values indicated worsening of inflammation. The means calculated for each strain across each of the investigated phenotypes were used for subsequent QTL mapping.

QTL mapping

The construction of the CC founder haplotypes is based on the method described in Ram et al.^{E4} For mapping, we used a logistic regression matrix model over the reconstructed haplotypes matrix to produce genome-wide distribution of *P* values (ANOVA chi-squared). For QTL mapping, we calculated significance values on the basis of 10,000 permutations, to limit the possibility of type I error.

RESULTS AND DISCUSSION

The CC reveals a wide range of diversity in CHS responses

Striking differences in response for both the acute reaction to immune challenge and postchallenge recovery were observed across the 38 CC strains we analyzed. In the acute response, mean percentage increase in ear thickness was observed to vary between 55% (VIT strain) and 233% (SAT strain) (Fig E1, A). Regarding postchallenge recovery, when comparing ear thickness between D1 and D7, 33 of the 38 strains analyzed were observed to have a negative value for percentage change in ear thickness, demonstrating an overall recovery from the initial inflammatory response (Fig E1, A). However, the remaining 5 strains failed to recover and displayed a worsening response, as demonstrated by an increased ear thickness at D7 compared with D1. In particular, the BEM strain exhibited the worst recovery, with a mean increase of 290% in ear thickness from D1. The variation in response among strains is best demonstrated visually with a heat map of percentage change in ear thickness over time (Fig E1, B). Notably, this illustrates that at each time point the extremes of responses are exhibited by different strains, suggesting that particular genes are likely to be influencing the immune response at different times postchallenge.

Analysis of the acute CHS response in CC strains reveals a major effect QTL on chromosome 4

Genome-wide linkage analysis based on the acute CHS response (defined by increase in ear thickness at D1 postchallenge) identified a major effect QTL on chromosome 4 between 19.7 and 33.1 Mb, with the causal variant found to be derived from the 129S founder strain. Within this interval, we identified *Bach2*

(BTB Domain and CNC Homolog 2) as a candidate gene associated with the acute CHS response.

BACH2 is a transcription factor involved in B-cell development and differentiation into plasma cells.^{E16} In addition, it has been described as an essential factor regulating T-cell function. It is expressed in CD4⁺ and CD8⁺ memory T cells and acts as a repressor of T-cell superenhancers, mediating many of their effector functions during terminal differentiation.^{E17} As such, constitutional *Bach2* deficiency results in a reduction in regulatory T cells^{E18} and especially the establishment of quiescent effector memory T cells,^{E19} which have both been shown to be essential in CHS.^{E20} Furthermore, *BACH2* gene polymorphisms in humans have been linked to autoimmune diseases such as Addison's or Graves' diseases,^{E21,E22} vitiligo,^{E23} type 1 diabetes,^{E24} and to a lesser extent, allergic asthma.^{E20}

We also identified a second QTL on chromosome 15 between 32.2 and 55.4 Mb and determined the causal variant to be derived from the CAST founder strain. *Tnfrsf11b* (TNF receptor superfamily member 11b), *Ebag9* (Estrogen receptor binding site associated, antigen, 9), and *Klf10* (Kruppel like factor 10) were identified as candidate genes within this region. *Tnfrsf11b* specifically carried a missense variant unique to the CAST haplotype, and loss of this gene has been shown to result in an increase in thymic regulatory T-cell development.^{E25} However, sustained overexpression of this gene has been shown not to alter CHS.^{E26}

Notably, the CAST haplotype displayed unique noncoding variants of *Klf10* and *Ebag9* within this locus. *Ebag9* is a negative regulator of T-cell cytotoxicity and its loss has been associated with increased CD8⁺ granzyme release, which may be of relevance in the pathogenesis of CHS.^{E27} Finally, *Klf10* polymorphisms as identified here may also alter CHS because it regulates TGFβ signaling on effector cells.^{E28}

Recovery following immune challenge is highly variable among CC strains and genome-wide scan reveals a major effect QTL on chromosome X

Analysis of the rate of recovery (defined as the percentage change in ear thickness at D7 relative to D1) following immune challenge and subsequent genome-wide linkage analysis identified a bifurcated peak on chromosome X. We determined that the causal variant for this peak was derived from the CAST founder strain. *Gilz* (glucocorticoid-induced leucine zipper, also known as *Tsc22d3*) was identified as the most relevant biological candidate. *Gilz* has been described as an anti-inflammatory protein and its expression is induced by the activity of glucocorticoids via the glucocorticoid receptor or via IL-10.^{E29} *Gilz* acts through direct inhibition of essential factors such as AP1^{E30} in various cell types, but also through the inhibition of nuclear factor kappa B or MAPK signaling by direct binding to key components.^{E31} Consequently, *Gilz* has been shown to have a significant role in inflammatory disorders through its activity in both the innate and adaptive immune systems.^{E29} Moreover, in accordance with our results, overexpression of *Gilz* has been demonstrated to result in the suppression of delayed-type hypersensitivity.^{E32} The variants D22N and E171Q identified in the CAST haplotype may therefore result in the impairment of GILZ protein function and the consequent severe CHS phenotype observed among some CC strains.

When considering overall recovery as an outcome, we identified several additional loci with suggestive linkage. For

these peaks it is more difficult to obtain absolute significance (despite LOD scores of >10), because the linkage is dominated overwhelmingly by the strains BEM and TOP, in which the inflammation does not resolve. However, given the large difference in phenotypic measurement for BEM in particular (Fig 2, A, and Fig E1), it is conceivable that other genes that modify CHS recovery may be present within these intervals. Candidate genes identified within these intervals included *Tgfb2*, *Kcnk5*, *Lrrk2*, *Ptk2*, *Cyp11b1*, *Shoc2*, and *Dusp5*. Within the chromosome 1 interval, *Tgfb2* (transforming growth factor beta 2) is the most likely candidate associated with CHS recovery. TGFβ2 has long been described as a regulator of antigen presentation to T cells and as an inducer of tolerogenic macrophages and dendritic cells, and can mediate its effects through the MAPK signaling pathway.^{E33-E35} Importantly, TGFβ2 has been implicated in the induction of immunological tolerance in CHS.^{E34}

On chromosome 14, *Kcnk5* (potassium channel subfamily K member 5) was identified as a candidate gene and is known to encode potassium channels that are important for effector T-cell function.^{E36} Chromosome 15 harbored missense variants of *Lrrk2* (leucine-rich repeat kinase 2), a regulator of innate immunity and CHS,^{E37} as well as *Ptk2* (protein tyrosine kinase 2) encoding Focal Adhesion Kinase,^{E38} and *Cyp11b1* (cytochrome P450 family 11 subfamily B member 1), which encodes a component of the extraadrenal glucocorticoid synthesis mechanism.^{E39} Finally, chromosome 19 harbored genes such as *Shoc2* (SHOC2 leucine rich repeat scaffold protein) and *Dusp5* (dual specificity phosphatase 5), both of which are known to be important in MAPK/ERK signaling.^{E40,E41} *Dusp5* is also implicated in T-cell receptor signaling thresholds, as well as in reducing effector cells in favor of memory T-cell populations.^{E41,E42}

Analysis of CHS phenotype over time reveals additional significant genomic associations

Following investigation of the acute CHS response and postchallenge recovery, we took an overarching view of the genetics of CHS, treating the response as a system changing over time (Fig E2). We performed additional genome-wide scans at each day after immune challenge up to D4 to identify additional QTL. We also compared the linkage peaks detected here with those associated with nonrecovery at D7. By determining the location of statistically significant peaks in association, we confirmed most of the loci described above but also identified a number of additional peaks. In particular, we uncovered a locus on chromosome 3 between 50 and 60 Mbps that was not found to reach statistical significance at D1 or D3 postchallenge, but was found to be significant at D2 (Fig E2). Within this locus, *Foxo1* was identified as an essential candidate because its inactivation is essential for the migration of regulatory T cells to peripheral tissues in order to suppress CD8⁺ T-cell responses.^{E43} FOXO1 has also been shown to mediate the alternative activation of M2 macrophages resulting in allergic lung inflammation.^{E44} Interestingly, given the previous implication of *Gilz* in this study, FOXO1 has been shown in other systems to be regulated by GILZ under homeostatic conditions.^{E45}

Within the same locus, we also identified *Postn* (periostin) as a relevant candidate gene. Periostin has been strongly associated with allergic airway diseases and proposed as a biomarker in asthma.^{E46}

CHS and the CC as a model to investigate the genetic variability underlying human ACD

CHS and ACD are complex diseases and suspicion of an underlying genetic susceptibility stems from early family and twin studies.^{E47,E48} A major study where members of the same family were sensitized experimentally with p-nitrosodimethylaniline reported increased sensitization rates of children when both parents were sensitized, strongly supporting an underlying genetic factor.^{E47} Human studies that do not use experimental sensitization (as is often the case in genome-wide association study) are confounded by important factors such as differences in exposure to the hapten, medication, or comorbidity. In addition, the capacity of the hapten to trigger reactions is an important parameter and it has been suggested that a strong sensitizer such as 2,4-dinitrochlorobenzene may bypass the genetic influence as opposed to weak sensitizers such as p-nitrosodimethylaniline or nickel.^{E48}

In most studies to date, 2 questions arise. First, relating to sensitization, it is unclear why only some people react and develop ACD to a given common hapten despite the wide exposure of the population. The second question relates to the development and intensity of the dermatitis that does occur in a subset of patients following sensitization. Experience demonstrates that the first question can be addressed only in the specific context of a given hapten. In this study, we addressed the second question. Oxazolone is a well-established strong sensitizer used in many CHS studies for decades.^{E10-E15} Although modalities of sensitization may vary, choosing this strong sensitizer applied uniformly across different strains allowed us to bypass variability in sensitization and therefore evaluate the level of dermatitis. Indeed, as expected, all mice had an increase in ear thickness, showing that dermatitis and therefore sensitization occurred in all animals.

Many previous studies have adopted a candidate-based approach to identify genetic determinants of ACD. Indeed, TNF- α ,^{E49} IL-16,^{E50} or angiotensin-converting enzyme^{E51} genes among others have been linked to sensitization or polysensitization. Because most of these studies are focused on sensitization, it may be argued that an overlap is not expected. Similarly, none of the genes suggested to be involved in CHS in our study were associated with atopic dermatitis. Of interest, many of the candidates identified in this study are concentrated around the MAPK/ERK pathway (Fig 2, E) and the regulation of this pathway may be of importance in T-cell receptor signaling and the generation of regulatory T cells.^{E52} Indeed, the activation of this pathway occurs upon costimulatory molecule engagement such as CD28, whereas inhibitory signals through CTLA4 or PD1 have been shown to downmodulate ERK activity.^{E53,E54} As such, it could be argued that the findings of our study point toward a novel and unexplored series of therapeutic targets.

The CC is a resource designed to enable rapid mapping of QTL and enables the identification of multiple interacting genes influencing a given phenotype. Hence, this model is ideal for our study and has enabled us to demonstrate dramatic diversity between strains in terms of both the immediate response to immune challenge and the ability of inflammation to return to normal after challenge. One caveat is that although the CC was generated from 8 diverse founder strains, which encompass more than 90% of common genetic variation in the species, it is possible that other alleles exist that may not be present in the CC. However,

it is possible that the CC has revealed new allele interactions influencing CHS, which have been detected in this study.

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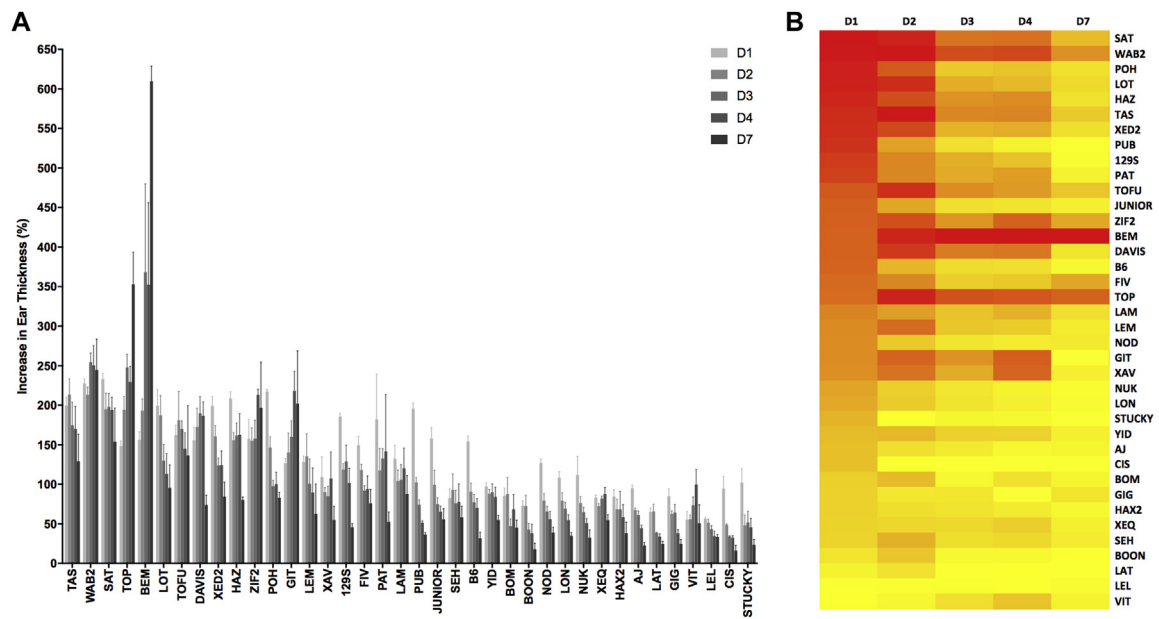


FIG E1. Variation in the CHS response between CC strains. **A**, Mean percentage change in ear thickness following oxazolone sensitization and subsequent challenge over 7 days (minimum $n = 3$ mice per strain, mean $\pm$ SEM). **B**, Heat map depicting mean percentage increase in ear thickness over time for each strain, with red coloring representing the largest increases in ear thickness.

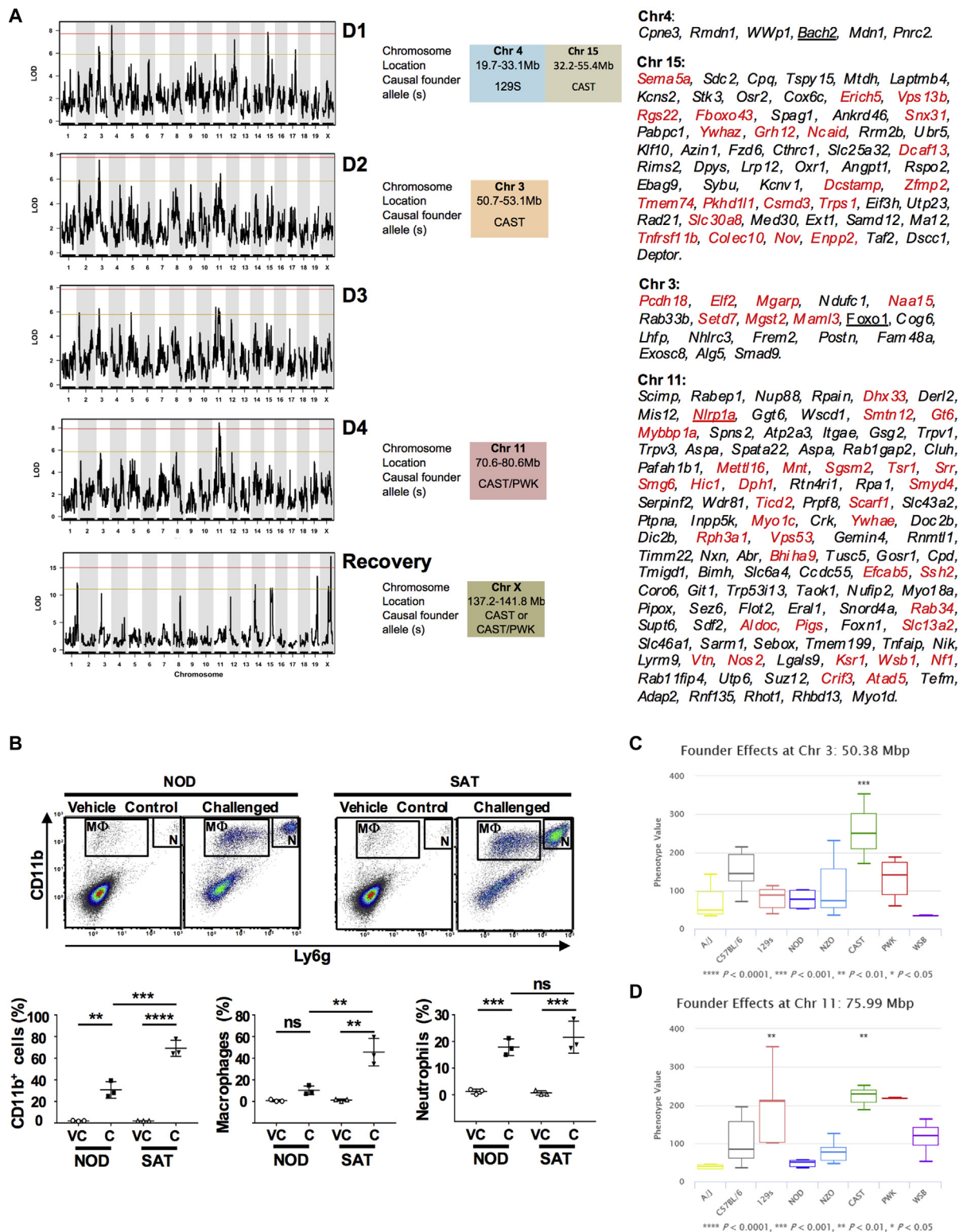


FIG E2. Analysis of CHS response over time following oxazolone-induced immune challenge in CC mice. **A**, Genome scans at D1, D2, D3, D4, and D7 after challenge. Linkage peaks with statistically significant LOD scores are shown schematically on the right, along with the founder strain allele that introduced the putative causal allele. To the right are lists of the known genes within the respective intervals. Red = founder allele carries a missense mutation in that gene; black = founder allele(s) carries a regulatory variant(s). Genes identified as strong candidates on the basis of their function are underlined. **B**, Flow-cytometry plot representation of skin-infiltrating leukocytes at D1 based on CD11b and Ly6g from challenged ("C") and vehicle-control ("VC") NOD and SAT mice. *MΦ*, macrophages; *N*, neutrophils. Graphs depict proportions of cells shown in plots (total CD11b⁺, macrophages, neutrophils). ** $P < .01$, *** $P < .001$, **** $P < .0001$, ns $P > .05$. Two-way ANOVA test. $n = 3$ NOD, $n = 3$ SAT mice. Results shown as mean $\pm$ SD. **C**, Analysis of founder haplotype coefficients for the identified QTL on chromosome 3. **D**, Analysis of founder haplotype coefficients for the identified QTL on chromosome 11. *Chr.*, Chromosome; *LOD*, logarithm of the odds to the base 10; *ns*, not significant.

TABLE E1. Candidate genes identified within QTL associated with post-immune challenge recovery

Chromosome	Founder haplotype (effect, <i>P</i> value)	Candidate genes
X	CAST or CAST/PWK (more, <i>P</i> < .0001)	<i>Prps1</i> , <i>Tsc22d3</i> , <i>Mid2</i> , <i>Eif2c5</i> , <i>Tex13</i> , <i>Vsig1</i> , <i>Psm10</i> , <i>Atg4a</i> , <i>Col4a6</i> , <i>Col4a5</i> , <i>Gucy2f</i> , <i>Nxt2</i> , <i>Kcne1</i> , <i>Acs14</i> , <i>Tmem164</i> , <i>Ammecr1</i> , <i>Rgag1</i> , <i>Chrdl1</i> , <i>Pak3</i> , <i>Capn6</i> , <i>Dcx</i> , <i>Alg13</i> , <i>Trpc5</i> , <i>Zcchc16</i> , <i>Lhfp11</i>
1	PWK (more, <i>P</i> < .0001)	<i>Tgfb2</i> , <i>Rrp15</i> , <i>Spata17</i> , <i>Gpatch2</i> , <i>Esrrg</i> , <i>Kctd3</i>
14	WSB (more, <i>P</i> < .0001)	<i>Nr1d2</i> , <i>Nid2</i> , <i>Gng2</i> , <i>Saysd1</i> , <i>Kcnk5</i> , <i>Kcnk16</i>
15	PWK (more, <i>P</i> < .0001)	<i>Col22a</i> , <i>Kcnk9</i> , <i>Trappc9</i> , <i>Chrac1</i> , <i>Ago2</i> , <i>Ptk2</i> , <i>Dennd3</i> , <i>Slc45a4</i> , <i>Grp20</i> , <i>Ptp4a3</i> , <i>Mroh5</i> , <i>Bai1</i> , <i>Mroh4</i> , <i>Arc</i> , <i>Surp1</i> , <i>Them6</i> , <i>Ly6k</i> , <i>Hem1</i> , <i>Cyp11b1</i> , <i>Cyp11b2</i> , <i>Ly6e</i> , <i>Ly6i</i> , <i>Ly6a</i> , <i>Ly6c1</i> , <i>Ly6c2</i> , <i>Ly6f</i> , <i>Ly6h</i> , <i>Lych</i> , <i>Gpipbp1</i> , <i>Zfp41</i> , <i>Top1mt</i> , <i>Rhpn1</i> , <i>MafaZc3h3</i> , <i>Zdhhc25</i> , <i>Brd1</i> , <i>Zbed2</i> , <i>Alg12</i> , <i>Creld2</i> , <i>Pim3</i> , <i>Till8</i> , <i>Mlc1</i> , <i>Mov1011</i> , <i>Panx2</i> , <i>Trabd</i> , <i>Tubgcp6</i> , <i>Hdac10</i> , <i>Mapk12</i> , <i>Mapk11</i> , <i>Plxnb2</i> , <i>Denn6b</i> , <i>Ppp6r2</i> , <i>Sbfl</i> , <i>Adm2</i> , <i>Miox</i> , <i>Lmf2</i> , <i>Sco2</i> , <i>Tymp</i> , <i>Klhdc7b</i> , <i>Syce3</i> , <i>Cpt1b</i> , <i>Chkb</i> , <i>Mapk8ip2</i> , <i>Shank3</i> , <i>Acr</i> , <i>Rabl2</i> , <i>Syt10</i> , <i>Arsa</i> , <i>Alg10b</i> , <i>Cpne8</i> , <i>Kif21a</i> , <i>Slc2a13</i> , <i>Abcd2</i> , <i>Lrrk2m</i>
19	CAST (more, <i>P</i> < .0001)	<i>Sorcs3</i> , <i>Sorcs1</i> , <i>Ins1</i> , <i>Rbm20</i> , <i>Pdcd4</i> , <i>Xpnpep1</i> , <i>Add3</i> , <i>Mxi1</i> , <i>Smp3</i> , <i>Dusp5</i> , <i>Shoc2</i>

Analysis of genomic variants corresponding to founder haplotype for the significant linkage on chromosome X and suggestive linkages on chromosomes 1, 14, 15, and 19 reveals candidate genes that may contribute to the observed phenotype (only known genes are shown). Variants resulting in missense protein changes are highlighted in boldface, with the remainder carrying regulatory variants. For each founder haplotype, the effect on ear inflammation (more or less) is indicated.