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An ENU mutagenesis approach to dissect "self"-induced immune responses

Unraveling the genetic footprint of immunosurveillance

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The immune system exerts a critical function as it recognizes and eliminates transformed or neoplastic cells, a process also referred to as immunosurveillance. NK cells play a particularly important role in that they are able to recognize tumor cells via "missing-self"—i.e., the absence of major histocompatibility complex Class I on target cells. Moreover, recent studies suggest that NK cells also participate in the onset and regulation of adaptive immune responses. The exact molecular pathways by which this occurs, however, remain poorly understood. To obtain further insight into the genes that are required for self-induced immune responses via NK cell-mediated cell death, our laboratory initiated a forward genetic approach using N-ethyl-N-nitrosourea (ENU) as a mutagen. Specifically, we tested the ability of NK cells from G3 ENU germline mice to recognize missing-self target cells and induce CD8$^+$ T-cell responses following immunization with irradiated tumor cells. Here we present two ENU germline mutants, designated *Ace* and *Chip,* that are defective in the recognition of β-2 microglobulin-deficient target cells, yet exhibit improved clearance of B16 melanoma cells in vivo. Coarse mapping and whole genome sequencing of the *Chip* mutation revealed a missense mutation causing a T→A amino acid substitution in the highly conserved third immuno-receptor tyrosine-based switch motif of CD244 (2B4). The forward genetic approach described here promises to reveal important insight into critical genes that are required for host responses involved in anticancer immunity.

Introduction

The interaction between the immune system and cancer development is a bidirectional relationship involving elimination and adaptation. The critical role of the immune system in the elimination of transformed or neoplastic cells is best illustrated by the increased spontaneous tumor incidence observed in immunodeficient mice, such as SCID, *Rag2*$^{-/-}$, *Perforin*$^{-/-}$, *Il12rb2*$^{-/-}$ or *Ifng*$^{-/-}$ mice (reviewed in ref. 1). Generally, the onset of tumor development in immunodeficient mice is late, with most models developing tumors at > 12 mo of age. The presence of a functional immune system is capable of eliminating transformed cells. However, transformed cells do possess the ability to subvert and evade the immune system. This entire process is referred to as immunoediting and is thought to be comprised of three biological processes: (1) elimination, the extrinsic suppression of tumor cells through immunosurveillance; (2) equilibrium, limiting the expansion of transformed cells by the host immune system; and (3) escape, the development of tumor cell variants with reduced immunogenicity or immunomodulatory capability, resulting in cancer.[2-4] The exact role of specific immune cell populations and the inflammatory pathways in immuno-editing is poorly understood and currently the subject of intense research.

Immunosurveillance is mediated in part by innate immune effector cells—predominantly natural killer (NK) cells—but also involves adaptive immune cells, such as T and/or B cells.[1] Immunosurveillance requires overcoming significant challenges as tumor cells normally represent self-tissue and thus effective adaptive immune responses need to break immunological tolerance. In the innate recognition of tumor cells, NK cells are capable of recognizing and killing various types of tumor targets.[5-7] They are able to sense the expression of a wide variety of surface molecules that can be classified as (1) constitutive self, (2) stress-induced self and (3) pathogen-derived. For tumor recognition, constitutive self and stress-induced self are of particular relevance. Previous studies have revealed that mouse NK cells can recognize major histocompatibility complex (MHC) Class I molecules either directly or indirectly via surface receptors, leading to signals that inhibit NK cell cytolytic functions.[6,8-10] Direct recognition of MHC Class I molecules is mediated by members of the Ly49 family of C-type lectin Type II transmembrane proteins.[11] Alternatively, indirect recognition occurs through CD94/NKG2A receptor binding of MHC-derived leader peptides

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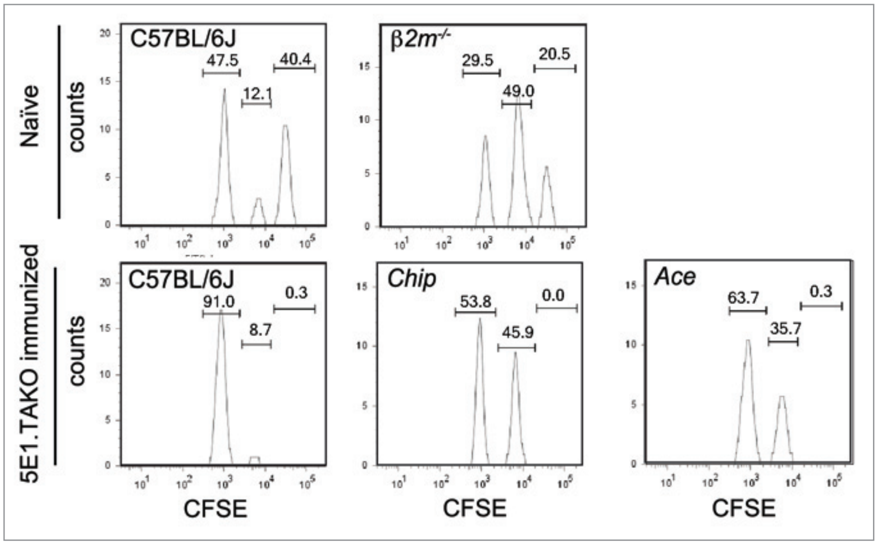

Figure 1. Identification of ENU germline mutants with impaired NK- and/or $CD8^+$ T cell-mediated target cell eradication in vivo. C57BL/6J and G3 ENU germline mice were left untreated (naïve) or were immunized with 5×10^6 irradiated 5E1.TAKO i.p.. Eight days after immunization, mice were injected i.v. with 3×10^6 high CFSE $E1B_{192-200}$ peptide loaded C57BL/6J splenocytes ($CD8^+$ T-cell targets), 3×10^6 medium CFSE $\beta 2M^{-/-}$ splenocytes (NK cell targets) and 3×10^6 low CFSE C57BL/6J splenocytes (control non-target cells). After 48 h, ocular blood samples were collected and analyzed for the number of fluorescent cells in each CFSE population. Both *Chip* and *Ace* mutants exhibited impaired NK yet normal $CD8^+$ T cell-mediated killing of target cells compared with C57BL/6J controls.

expressed by Qa1—a non-classical MHC Class I molecule. When these inhibitory receptors are not engaged by MHC Class I molecules—a condition also referred to as "missing self"—the activation of NK cell effector functions occurs and target cell killing ensues. This is believed to be a major mechanism by which tumor cells, which often exhibit an abrogated or reduced expression of MHC Class I surface molecules, are targeted.[10,12] In addition to missing-self, tumor cells often express stress molecules such as Rae-1, Mult-1 and H60, which are recognized by activating NK cell receptors including NKG2D (reviewed in ref. 13). Ligation of activating NK cell receptors results in tyrosine phosphorylation of adaptor molecules containing immuno-receptor tyrosine-based activation motifs (ITAM) via Src family kinases (reviewed in ref. 14). On the other hand, inhibitory NK cell receptors, bearing immuno-receptor tyrosine-based inhibitory motifs (ITIM), recruit the lipid phosphatase SHIP-1 and the tyrosine phosphatases (SHP)-1 and SHP-2.[14] The latter phosphatases inhibit NK cell killing by targeting tyrosine phosphorylation within the ITAM motifs. Currently, our knowledge on the complex interaction between activating and inhibitory receptor-transduced signals during tumor cell recognition remains incomplete, though the topic elicits widespread interest.

Apart from the innate recognition of tumor cells, recent data suggests a strong link between NK cells and their counterparts from the adaptive immune system.[15] Previous studies in our laboratory suggest that NK cell-mediated cytotoxicity of target cells (lacking MHC Class I) can induce robust T and B cell responses, which can be readily measured following immunization with as few as 10^4 target cells.[15] Priming of T cells in this model was shown to be mediated by dendritic cells that (cross-) present antigens, requiring both Type I and II interferons (IFNs).[15] NK cells can directly induce interferonγ (IFNγ), but the source of Type I IFNs is less clear. We have also previously shown that Type I IFNs can be induced directly by dying cells.[16] In addition, the type of cell death is believed to be a critical factor in the onset of innate immune responses and/or determines the immunogenicity of target cells.[16-18] The molecular/cellular requirements underlying cell death-mediated immune responses remain largely elusive.

To obtain critical insight into the molecular components required for self-induced immune responses via (NK cell-mediated) cell death, our laboratory initiated a forward genetic approach using N-ethyl-N-nitrosourea (ENU) as a mutagen. Specifically, we examined NK cells from G3 ENU germline mice for their ability to; (1) recognize missing-self target cells and (2) induce $CD8^+$ T-cell responses following immunization with irradiated antigen-expressing cells. Here, we present two ENU-generated germline mutants, designated *Ace* and *Chip,* which are defective in the recognition of β-2 microglobulin (β-2 min)-deficient target cells and exhibit increased clearance of B16 melanoma in vivo. We believe that applying an ENU mutagenesis approach, combined with the probing of immunological pathways driving self-induced immune responses, will provide valuable insight into the genetic footprint underlying immunosurveillance.

Results

Identification of ENU germline mutants with impaired cytolytic effector function. Among ~4,500 G3 ENU germline mice screened for functional NK and $CD8^+$ T cells in vivo, we identified a total of 12 germline mutations from different pedigrees that showed abnormal cell killing by NK and/or $CD8^+$ T cells. Of them, at least 2 mutants were identified with a particular relevance to cancer. These were designated *Chip* and *Ace* and both showed a reduced NK cell capacity to recognize and eliminate β-2m-deficient "missing-self" targets (**Figs. 1 and 2A**). In contrast, both mutants showed a normal killing of antigen-specific target populations by $CD8^+$ T cells. The G1 pedigrees of these germline mutants were selected to establish a homozygote colony used for genetic analysis and in depth phenotypic characterization. The *Ace* mutations behaved as a strictly recessive trait, in that normal cytolytic effector functions were observed in heterozygote mutant mice. Conversely, the *Chip* mutation behaved as a co-dominant trait where heterozygotes for the *Chip* locus displayed an intermediate phenotype with regard to killing of β-*2m*-deficient target cells in vivo (results not shown).

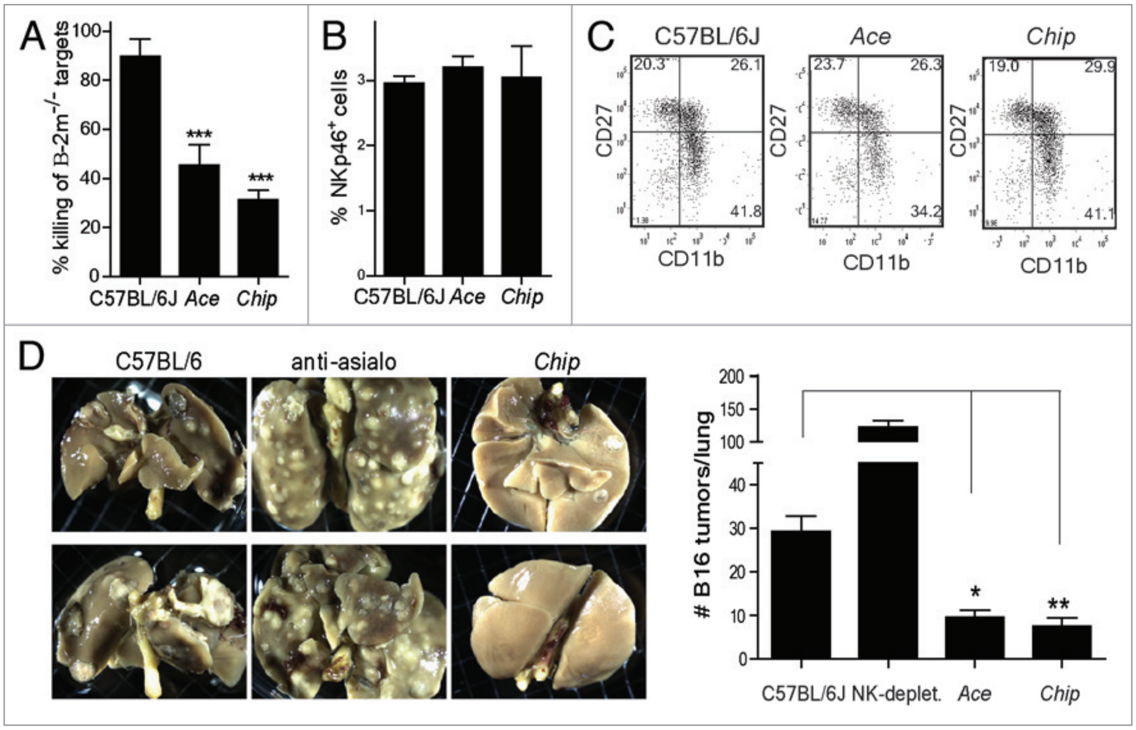

Figure 2. Improved B16 melanoma cell clearance in *Ace* and *Chip* mutants. (**A**) Reduced clearance of CFSE labeled β2m-deficient splenocytes in *Ace* and *Chip* mutants compared with C57BL/6J control mice in vivo. Twenty-four hours after transfer, blood samples were collected and analyzed for the presence of wildtypes plenocytes (low-CFSE) and Kb-deficient splenocytes (medium-CFSE). The percentage killing is calculated from the ratio between β-2m-deficient and C57BL/6J cells administered to β-2m-deficient and control C57BL/6J recipients. Numbers in graph represent the mean percent killing ± SD (n = 4). (**B and C**) The percentage of NKp46+ cells in C57BL/6J and mutant mice and their maturation (**C**) as measured by CD27 and CD11b surface expression (NKp46-gated) (n ≥ 3). (**D**) C57BL/6J control, anti-asialo treated C57BL/6J and homozygote *Ace* and *Chip* mutants were injected 1×10^5 B16 melanoma cells i.v. Only male mice were used in this expt. After 3 weeks, mice were sacrificed and the number of lung tumor nodules were determined. *p < 0.05; **p < 0.01; ***p < 0.001

Increased B16 melanoma tumor clearance in chip and Ace mutants. Characterization of the hematopoietic development in *Ace* and *Chip* mice showed no gross abnormalities in the number of peripheral lymphoid or myeloid cell populations, including the number of $CD4^+$ T cells, $CD8^+$ T cells and B cells, as well as macrophages and dendritic cells (results not shown). Specific analysis of the NK cell population in both mutants revealed normal numbers of NK cells ($NKp46^+$) that appeared to undergo normal maturation as measured by CD27 and CD11b expression (**Fig. 2B and C**). Overall, these data suggest that the *Ace* and *Chip* mutations specifically affect NK cell function yet appear not to influence normal development.

The unique ability of NK cells to recognize cells that express reduced levels of cell surface MHC-I is considered to be an important mechanism by which tumor cells are eliminated. Given the aberrant recognition of MHC Class I-deficient target cells, we sought to examine whether the *Chip* and *Ace* mutants were capable of eliminating B16 melanoma cells. This tumor cell line was derived from a C57BL/6 mouse and is known to express low MHC Class I, a feature critical for their elimination by NK cells.[19-21] Control C57BL/6J, NK cell-depleted (by anti-asialo treatment) and *Chip* and *Ace* mutants were injected with 1×10^5 B16 melanoma cells per mouse i.v. After 3 weeks, mice were sacrificed, their lungs excised and the number of tumor nodules enumerated. As expected, depletion of NK cells caused a vast increase in the number of lung tumors thereby confirming the critical role of NK cells in the elimination of B16 melanoma cells. Surprisingly, both *Chip* and *Ace* mutants displayed significantly enhanced clearance of B16 melanoma cells compared with C57BL/6J control mice (**Fig. 2D**). Overall, the similarities between the *Chip* and *Ace* mutant mice are striking with an impaired recognition of MHC-deficient target cells, yet an improved ability to eradicate B16 tumor cells.

***Chip*; a missense mutation in the ITSM domain of CD244.** The causative mutation in *Chip* mice was identified by coarse mapping, exonic sequence capture and subsequent next generation sequencing of enriched DNA. For coarse mapping, *Chip* C57BL/6J homozygotes males were outcrossed to C57BL/10J females and resulting female offspring were subsequently backcrossed to the homozygote males. A total of 26 offspring (11 *Chip* mutant and 15 wild type phenotypes) were analyzed for both phenotype and genotype. Genotyping was performed using a genome wide custom-made 150-SNP map distinguishing C57BL/6J and C57BL/10J genetic backgrounds. Coarse mapping revealed a single peak with a LOD score of ~6.3 for SNP rs13476182 located on the distal end of chromosome 1 (**Fig. 3A**). The entire critical region was defined by proximal marker rs13476182 and distal marker rs13476295 and consisted of ~36.8 Mb genomic DNA

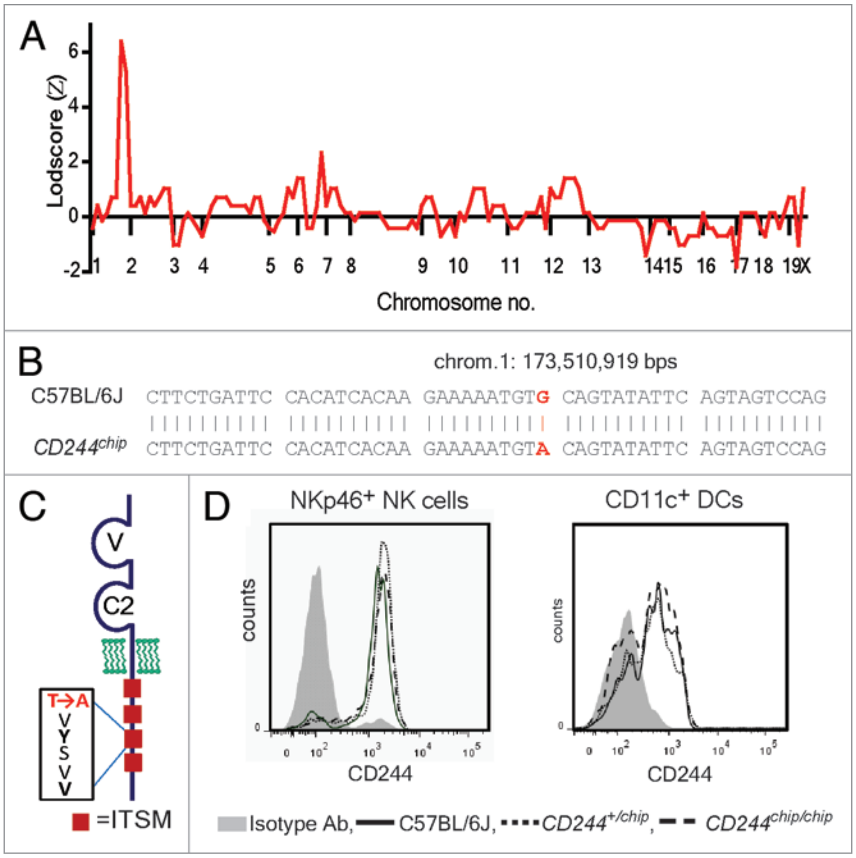

Figure 3. Impaired missing self-recognition in *Chip* mutants is the result of a missense mutation in the third ITSM motif of CD244. (**A**) Coarse mapping of the *Chip* mutation based on 26 mice and a panel of 150 SNPs covering the entire genome. The *Chip* phenotype was linked to the distal site of chromosome 1. (**B**) The G→A missense mutation at nucleotide 173,510919 bps on chromosome 1 (**C**) causes a single amino acid substitution (T→A) in the third ITSM motif of CD244. (**D**) CD244 surface protein expression is unaffected on NK cells ($NKp46^+$) and dendritic cells ($CD11c^+$) of homozygote and heterozygote CD244 mice, as determined by flow cytometry.

containing 523 annotated genes. The genomic coordinates for the region-specific coding sequences were defined based on the NCBI build 37.2 database (*Mus musculus*). The defined region was submitted to NimbleGen for the design of a sequence capture array. Ultimately, 3082 exons were targeted, covering 946.1 kb of total genomic DNA of which 97.1% was covered by sequence capture probes. DNA that could not be targeted by the sequence capture probe predominantly represented low copy repeats and non-coding bases (data not shown). The enriched genomic DNA was submitted for next-generation sequencing and the resulting sequence was compared with the reference sequence derived from the NCBI build 37.2database. A total of 4 homozygous nucleotide changes were identified (**Table 1**). Two of the homozygous mutations represented intronic mutations within *cep170* and *GM1305* genes. A third nucleotide change caused a non-synonymous amino-acid change (L→P) in the Lamin B receptor (*Lbr*) and a fourth nucleotide change (G→A) represented a non-synonymous mutation resulting in a single amino-acid substitution (T→A) in the third immuno-receptor tyrosine-based switch motif (ITSM) of CD244 (**Fig. 3B and C**). The intronic mutations in *Cep170* and *GM1305* are highly unlikely to affect gene expression or function. Previously, *Lbr* mutants and targeted knockout mice were found to exhibit abnormal skin and hair development and showed impaired growth—phenotypes not observed in *Chip* mutants. On the other hand, CD244 is expressed on NK cells and previous reports have indicated an important regulatory role for this receptor in NK cell signaling,[14] Moreover, the missense mutation replaces a highly conserved threonine in the ITSM motif of CD244 with an alanine. We therefore considered the mutation in *Cd244* to be responsible for the *Chip* phenotype and from here on mutant mice are referred to as *Cd244^{chip}* mice. Importantly, the missense mutation did not affect CD244 surface expression on NK cells and dendritic cells compared with C57BL/6J control mice as measured by flow cytometry (**Fig. 3D**).

Discussion

By taking a forward genetic approach using ENU mutagenesis we have identified a number of germline mutants with impaired recognition of "missing-self" targets. Two independent mutants (*Ace* and *Chip*) exhibit similar phenotypes in that they show aberrant recognition of "missing-self" β-*2m*-deficient target cells, yet an improved clearance of B16 melanoma cells in vivo. Taken together, these observations seem to contrast with the idea that reduced MHC Class I expression mediates protection against B16 tumor growth. The causative mutation in the *Chip* mutant was identified as a missense mutation in the third cytosolic ITSM motif of CD244, whereas the *Ace* mutation localized on chromosome 11 and remains to be identified. The B16 tumor data in *Cd244^{chip}* mice are in agreement with previous findings observed in Cd244-targeted knockout mice,[22] in that improved B16 melanoma tumor clearance was observed in male but not female mice (*Cd244^{chip}* female data not shown). Nonetheless, these findings suggest a paradox between the reduced clearance of β-2m-deficient target cells and an improved clearance of B16 tumor cells, a tumor cell-line expressing low Class I MHC. Although these observations could imply a role for other cell types such as $CD8^+$ T cells, injection of B16 melanoma cells in $CD8^+$ T cell-depleted hosts had limited effect on the clearance of B16 melanoma cells in vivo (results not shown). Together with the high tumor burden in NK cell-depleted cells, these data suggest that clearance of B16 melanoma cells is mediated by NK cells and that the increased elimination of tumor cells in both mutant lines result from a hyper-activation of NK cells. An important difference between both target cells is that the β-2 min target cells in our in vivo cytotoxicity assay are of hematopoietic origin and express CD48 (the ligand for

Table 1. Homozygote mutations identified in the *Chip* critical region

Position (Chrom. 1)	Ref.*	Alt.*	Depth* (per-allele)	Gene	Effect	Predicted impact	AA change
173510919	A	G	24 (0,24)	*Cd244*	Non-synonymous	high	T/A
178718539	A	T	28 (0,28)	*Cep170*	Intron	low	-
181664487	C	A	14 (0,14)	*Gm1305*	Intron	low	-
183759000	A	G	24 (0,24)	*Lbr*	Non-synonymous	Moderate	L/P

*Depth, the number of reads for the specified nucleotide; Ref., reference nucleotide as annotated in the NCBI build 37.2 database; Alt., alternate nucleotide observed.

CD244), whereas B16 melanoma cells lack expression of CD48. Since the immunoreceptor tyrosine switch motif in the CD244 cytosolic domain has been reported to mediate both activating and inhibitory signals, interaction of CD244 with CD48-ligand may well determine the hypo-/hyper-activation of NK cells observed in both models. For *Ace* mutants, identification of the causative mutation may reveal insight into these opposing effects with regard to missing-self recognition of hematopoietic or non-hematopoietic cells. Ultimately, the genetic deficiencies underlying these phenotypes will provide new insight into the molecular requirements for immunosurveillance.

CD244 (also known as 2B4) is a member of the signaling lymphocyte activation molecule (SLAM) receptor family and is predominantly expressed on NK cells, memory $CD8^+$ T cells, monocytes and dendritic cells. As mentioned above, CD244 preferentially binds to CD48, another member of the SLAM receptor family that is expressed on most hematopoietic cells. However, for optimal CD244 ligation, co-engagement with other NK cell receptors such as NKp46 is needed.[23,24] Originally, CD244 was found to function as an activating receptor on NK cells, as it was observed that antibody binding resulted in increased cytotoxicity and IFNγ production.[25,26] Subsequent studies, however, implied that CD244 could also serve as an inhibitory receptor on NK cells.[27] The cytoplasmic domain of CD244 contains a total of four ITSM domains that mediate activating and inhibitory signals following ligation of CD244. ITSM domains have a conserved tyrosine motif consisting of TxYxxV/I (with x representing any amino acid). Receptor-ligation results in tyrosine phosphorylation of the ITSM motifs and recruitment of Src homology 2 (SH2) domain-containing adaptor proteins including SAP (SLAM-associated protein), EWS-Fli1-activated transcript 2 (EAT-2) or EAT-2-related transducer (ERT).[28-31] SAP can interact with the Src kinase Fyn mediating phosphorylation and activation of downstream substrate molecules such as Vav-1 and PLCγ. In contrast, EAT-2 and ERT do not associate with Fyn,[31] thus providing a potential explanation on how activating and inhibitory signals are regulated at the level of the CD244 cytoplasmic domain. Indeed NK cells from Sap-deficient mice exhibit a hypo-response, whereas *Eat-2-* or *Ert*-deficient mice demonstrate augmented NK cell function, providing further support for the decisive roles of these adaptors in mediating activating or inhibitory signals following CD244 ligation.[31,32] In addition, phosphorylated ITSM motifs can recruit the inhibitory kinase Csk and phosphatases such as SHP-1, SHP-2 and SHIP providing inhibitory signals in a phosphatase-dependent manner.[33-35] NK cell inhibitory receptors, through the recognition of MHC Class I, prevent tyrosine phosphorylation in ITSM motifs in CD244.[36] Interestingly, the missense mutation in *Chip* mice affects the conserved threonine residue in the third ITSM domain in the cytoplasmic domain of CD244 (see **Fig. 3C**). Previous work by Watzl's group revealed that this specific ITSM domain uniquely recruits the c-Src kinase Csk, the inositol phosphatase SHIP, and the protein tyrosine phosphatases SHP-1 and SHP-2, whereas all 4 phosphorylated ITSM motifs of CD244 are able to recruit SAP.[33] Although this remains to be confirmed, the missense mutation changing the conserved threonine of this ITSM motif in *Chip* mice is likely to impair binding of phosphatases, c-SRC and SAP. Whether this impairs the overall function of CD244 or whether the mutation results in a hypomorphic phenotype remains to be determined. Importantly, *CD244^{chip}* mice express normal surface levels of CD244 suggesting that bidirectional signaling through CD48 is likely unaffected.

In summary, ENU mutagenesis is a particularly powerful approach for defining the genetic footprint of poorly defined biological processes, including NK cell function.[37,38] Here we applied this approach to obtain deep genetic insight into self-induced innate and adaptive immune responses, i.e., "missing self" recognition and cell death-induced $CD8^+$ T-cell responses. We believe that both these pathways are directly relevant to immunosurveillance, and indeed the identification of two ENU germline mutants (*Ace* and *Chip*) display a better clearance of B16 melanoma than wildtype C57BL/6. The characterization of these mutants and the overall ENU mutagenesis approach will provide profounds insights into the complex immunological pathways underlying immunosurveillance.

Material, Mice and Methods

Mice and reagents. All experiments were performed according to the US National Institutes of Health guidelines and were approved by the IACUCs of The Cincinnati Children's Hospital Medical Center (protocol #8D01008). C57BL/6J and C57BL/10J mice were obtained from Jackson Laboratory. ENU germline mice were generated at CCHMC. All mice were housed under specific pathogen free conditions. ENU Isopac was obtained from Sigma-Aldrich. Anti-CD244 was obtained from eBiosciences.

ENU mutagenesis. ENU mutagenesis was performed as previously described in reference 39. Briefly, male C57BL/6J mice were treated with a weekly dose of 90 mg/kg ENU i.p. for a 3 week period. After a short period of sterility, male mice were bred to C57BL/6J females to generate G_1 and subsequent G_2 females which were backcrossed with the G_1 male to generate G3 mice.

G3 mice were screened for abnormal lymphocyte responses as described below.

In vivo cytotoxicity assay for assessment of NK and CD8⁺ T-cell responses. G3 ENU germline mice were assessed in vivo for their ability to recognize and eliminate target cells representing "missing-self" (MHC Class I-deficient; NK cell targets) and/or antigen-specific target cells (CD8⁺ T-cell targets). Specifically, 6 week-old ENU germline mice were immunized with 1×10^6 5E1.TAKO cells i.p. After 1 week, mice were injected i.v. with a mixture of 10×10^6 cells containing three different cell populations distinguishable by carboxyfluoresceinsuccinimidyl ester (CFSE) intensity. The cell populations included a low CFSE C57BL/6J reference population (2 μM CFSE); a medium CFSE C57BL/6J β-*2m*-deficient NK cell target (10 μM CFSE); and a high CFSE antigen-specific CD8⁺ T-cell target population (50 μM CFSE). The latter were C57BL/6J splenocytes previously labeled with 5 μg/ml of the immunodominant $E1B_{192–200}$ peptide for 30 min in complete medium (Iscove's modified Dulbecco medium (IMDM), containing 10% FBS, 1% penicillin/streptomycin and 50 μM L-glutamine). CFSE staining for each population was performed for 10 min at room temperature with the indicated concentrations of CFSE in PBS. Forty-eight hours after injection, a blood sample was taken from each mouse and the presence/absence of the reference and target cell populations were determined by flow cytometry.

SNP genotyping and low resolution whole genome mapping. To establish critical regions for the *Chip* and *Ace* mutations, we performed low resolution mapping using a custom designed genome-wide SNP map with 150 markers informative for the C57BL/6J and C57BL/10J genetic backgrounds. Genotyping was performed using the Illumina Golden Gate Assay.[40] Briefly, a homozygous male mutant was outcrossed to C57BL/10J females. Subsequent hybrid males and females were intercrossed and 20–30 offspring were evaluated for concordance between the presence of the phenotype and homozygosity for the C57BL/6J alleles, and absence of the phenotype and heterozygosity/homozygosity for the C57BL/10J alleles. The LOD (Log Odds Distance) score (Z) was calculated for each marker, and used as an index of linkage.

Genomic DNA capture and deep sequencing. Genomic DNA capture of the *Chip* critical region, which was defined by proximal marker rs13476182 and distal marker rs13476295, was performed as described before in reference 41. Briefly, a custom 385K Roche-NimbleGenSeqCap array was designed to enrich DNA sequence covering all exons including an additional 50 bps upstream/downstream sequence. Exons were defined by the *Mus musculus* NCBI build 37.2 annotation.

A template library for analysis by next generation sequencing was prepared from mouse genomic DNA extracted from tail clips. The library was hybridized to the custom designed NimbleGenSeqCap and the enriched DNA was subsequently sequenced on an Illumina Genome Analyzer IIx using the paired-end protocol and collecting 40 bases from each read. Read alignment against the mouse genome was performed using the CASAVA software from Illumina. The software program SeqMate was used for variant identification a post-alignment for read sequence visualization. Variants were reported based on a number of parameters: depth of coverage, proportion of each base at a given position the number of different reads showing a sequence variation. For the array enrichment of *Chip* exons, parameters were set to: 10× minimum depth of coverage, ignoring all observations with a base quality less than or equal to 25, allowing heterozygotes allelic ratios from 50%/50% to 75%/25% and a minimum of 3 read relative locations per base was required. Ultimately, identified mutations in the *Chip* critical region were confirmed by PCR and Sanger sequencing using an Applied Biosystems 3730x l DNA Analyzer.

B16f10-melanoma tumor challenge in vivo. C57BL/6J control, NK cell-depleted C57BL/6J and homozygote mutant mice were injected 1×10^5 B16F10 tumor cells i.v. Depletion of NK cells in vivo was performed by injecting mice i.p. with 20 μL of anti-asialo GM1 antibody as recommended by the manufacturer (Wako Pure Chemical Industries), 1 d prior and after injection of B16 melanoma cells. After 3 weeks, mice were sacrificed and pulmonary tumor nodules were quantified.

Statistical data analysis. Data were analyzed using the GraphPad Prism4® software (GraphPad Software, San Diego, CA). The statistical significance of the differences among groups was determined from the mean and standard deviation by Student's two-tailed test or by ANOVA followed by Dunnett's test for three or more groups. Data was considered significant when $p < 0.05$.

Disclosure of Potential Conflicts of Interest

The authors have no financial or other conflict of interest relevant to the subject of this article.

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