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Combined Genetic Inactivation of *β2-Microglobulin* and *CD58* Reveals Frequent Escape from Immune Recognition in Diffuse Large B Cell Lymphoma

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SUMMARY

We report that diffuse large B cell lymphoma (DLBCL) commonly fails to express cell-surface molecules necessary for the recognition of tumor cells by immune-effector cells. In 29% of cases, mutations and deletions inactivate the *β2-Microglobulin* gene, thus preventing the cell-surface expression of the HLA class-I (HLA-I) complex that is necessary for recognition by CD8$^+$ cytotoxic T cells. In 21% of cases, analogous lesions involve the *CD58* gene, which encodes a molecule involved in T and natural killer cell-mediated responses. In addition to gene inactivation, alternative mechanisms lead to aberrant expression of HLA-I and CD58 in >60% of DLBCL. These two events are significantly associated in this disease, suggesting that they are coselected during lymphomagenesis for their combined role in escape from immune-surveillance.

INTRODUCTION

Diffuse large B cell lymphoma (DLBCL) is the most common form of adult non-Hodgkin lymphoma (NHL), accounting for 30%–40% of cases (Abramson and Shipp, 2005). Based on gene expression profile studies, three main subtypes have been identified, namely activated B cell-like (ABC), germinal center B cell-like (GCB), and primary mediastinal B cell lymphoma (PMBCL) (Staudt and Dave, 2005). These three subgroups appear to derive from distinct cells of origin, are associated with common as well as distinct genetic lesions, and, most notably, differ in their clinical response to conventional therapeutic regimens (Lenz and Staudt, 2010). Despite the significant progress in the identification of several key genetic lesions and associated deregulated pathways (Klein and Dalla-Favera, 2008; Lenz and Staudt, 2010), a sizable fraction of DLBCL remains incurable, suggesting that further understanding in the pathogenesis of this disease is needed in order to develop more specific therapeutic approaches.

The recent availability of technologies such as next-generation sequencing and SNP array analysis is leading to the identification of a large number of genetic alterations of possible pathogenic significance in DLBCL (Morin et al., 2011; Pasqualucci et al., 2011b). These studies have confirmed that GCB-type DLBCLs are preferentially associated with t(14;18) translocations deregulating *BCL2* (Huang et al., 2002), mutations within the *BCL6* autoregulatory domain (Iqbal et al., 2007; Pasqualucci et al., 2003), and mutations of the chromatin modifier gene *EZH2* (Morin et al., 2010). Conversely, alterations preferentially

Significance

DLBCL is the most common type of lymphoma and includes several subtypes with distinct genotypic, phenotypic and clinical features. We report that the majority of cases, including the two major subtypes, fail to express B2M and CD58. These two molecules are involved in the immune recognition of tumor cells by two important arms of cellular immunity, namely CTL and NK cells. The frequency of these defects, the heterogeneity of the involved mechanisms, including direct gene inactivation, and the significant co-occurrence of defects leading to the immune-escape from both CTL and NK cells, all suggest a strong selective pressure during lymphomagenesis. Overall, these findings implicate the evasion of immune-recognition as an important component of DLBCL pathogenesis.

associated with ABC-DLBCLs include mutations leading to the constitutive activation of NF-κB (Compagno et al., 2009; Davis et al., 2010; Lenz et al., 2008; Ngo et al., 2011), translocations deregulating *BCL6* (Iqbal et al., 2007; Ye et al., 1993), or inactivation of *BLIMP1* (Mandelbaum et al., 2010; Pasqualucci et al., 2006). In addition, genome-wide sequence and copy-number analyses have identified lesions common to all DLBCL subtypes, including the frequent inactivation of the acetyltransferase genes *CREBBP* and *EP300* (Pasqualucci et al., 2011a) and of the trimethyltransferase gene *MLL2* (Morin et al., 2011; Pasqualucci et al., 2011b). Among the many altered genes, we found *β2-Microglobulin* (*B2M*) and *CD58*, which were selected for further analysis given their potential role in the recognition of tumor cells by immune-surveillance mechanisms.

B2M is an invariant subunit required for the assembly of the major histocompatibility complex (MHC) class I, together with the human leukocyte antigen (HLA) heavy chain. The HLA-I complex is present on the plasma membrane of most nucleated cells (Bjorkman et al., 1987) and is involved in the presentation of antigenic peptides derived from the degradation of endogenous self or nonself proteins (Peaper and Cresswell, 2008), including viral- or tumor-associated antigens (Townsend et al., 1985; Zinkernagel and Doherty, 1974). These peptides are then recognized by the T cell receptors of $CD8^+$ cytotoxic T lymphocytes (CTL), leading to the destruction of the target cells that present nonself peptides. Several cancers, including colorectal carcinoma, melanoma, and cervical carcinoma, lack cell surface HLA-I expression due to heterogeneous mechanisms, thus allowing their escape from immune recognition by CTLs (Garrido et al., 2010; Hicklin et al., 1998). In particular, *B2M* gene lesions associated with defective HLA-I expression have been reported in a small number of lymphomas originating from the testis or the central nervous system (Jordanova et al., 2003).

CD58, a member of the immunoglobulin superfamily, is a highly glycosylated cell adhesion molecule that is expressed in diverse cell types as a transmembrane or glycosylphosphatidylinositol-membrane-anchored form (Dustin et al., 1987; Springer et al., 1987). It acts as a ligand for the CD2 receptor, which is present on T cells and most natural killer (NK) cells, and is required for their adhesion and activation (Bolhuis et al., 1986; Kanner et al., 1992; Wang et al., 1999), as documented by the observation that CD58 monoclonal antibodies lead to the diminished recognition and cytolysis of the target cells by both CTLs and NK cells (Altomonte et al., 1993; Gwin et al., 1996; Sanchez-Madrid et al., 1982). Although certain cancers have been observed to downregulate CD58 (Billaud et al., 1990), the mechanisms underlying the lack of expression are largely unknown.

The present study reports the comprehensive characterization of a large panel of DLBCLs for the presence of *B2M* and *CD58* genetic lesions as well as for the expression of the corresponding proteins. The observed alterations have consequences for the recognition of DLBCL by immune effector cells.

RESULTS

The *B2M* Gene Is Targeted by Mutations and Deletions in DLBCL

Following the initial finding of *B2M* mutations in a "discovery panel" of six DLBCL cases (Pasqualucci et al., 2011b), we performed mutation analysis of the *B2M* coding exons in 126 additional DLBCL samples, including 105 primary biopsies and 21 cell lines (total n, including discovery cases = 132). We identified 25 sequence variants distributed in 14/111 (12.6%) DLBCL biopsies and 3/21 (14.2%) cell lines (Figure 1A; Table S1 available online). Among these variants, 12 correspond to inactivating frameshift insertions/deletions (n = 9) or nonsense mutations (n = 3), resulting in transcripts that encode truncated B2M proteins. Of the remaining 13 missense variants, 38% (n = 5) affect the initiator methionine and convert it to arginine, lysine, or threonine (ATG → AGG/AAG/ACG), thus abrogating protein expression, as previously documented in the Burkitt's lymphoma cell line DAUDI (Rosa et al., 1983) (Figure S1A). Five additional missense mutations are expected to inactivate the protein function based on the PolyPhen prediction algorithm (Sunyaev et al., 2001), whereas the remaining three amino acid changes were located in the same allele carrying a premature stop codon, and may thus represent passenger events. The analysis of paired normal DNA in a subset of cases and the screening of several databases of population polymorphisms, including over 1,000 normal individuals (see Experimental Procedures), indicate that the observed mutations represent somatic events, overall accounting for 12.8% (n = 17/132) of the DLBCL samples analyzed.

B2M mutations are present in both the GCB (n = 10/65) and ABC/nonclassified (NC) (n = 7/67) DLBCL subtype (Figure 1B), but were not found in other mature B-NHL analyzed (n = 108 cases, including 37 follicular lymphomas, 16 Burkitt's lymphomas, 44 chronic lymphocytic leukemias, and 11 marginal zone lymphomas). These results suggest a specific role in the pathogenesis of DLBCL.

To investigate whether the *B2M* locus is also a target of copy number losses, we analyzed the 21 DLBCL cell lines and 80 of the 111 DLBCL biopsies (for which adequate material was available) by fluorescence in situ hybridization (FISH) and high-density SNP array analysis, respectively. Using these approaches, we observed recurrent deletions affecting the *B2M* locus in 24.7% (n = 25/101) of the cases (Figure 1C for representative examples; Figures S1B and S1C). Notably, 40% (n = 10/25) of the affected samples harbored homozygous deletions (Figure 1C and Figure S1C), an event only rarely observed in other cancers with *B2M* lesions (Garrido et al., 2010). The majority of the biallelic losses (n = 9) are <200 kb in size and identify a minimal region of deletion centered on the *B2M* locus. In fact, the smallest deletion (sample 2126) spans only ~30 Kb and selectively targets the *B2M* gene (Figure 1C; Table S1). Fifteen additional samples showed heterozygous *B2M* deletions, of which four likely represent subclonal events.

The combined analysis of both sequencing and copy-number data revealed that 12.9% of cases (n = 17/132) have lost both *B2M* alleles due to homozygous deletions (n = 9), biallelic mutations (n = 4), or hemizygous deletions with mutations affecting the second allele (n = 4), whereas 13.6% of cases (n = 18/132) harbor monoallelic deletions (n = 12) or mutations (n = 6) (Figure 1E; Table S2). In three additional cases carrying truncating mutations, the status of the second allele could not be determined due to the lack of suitable material for SNP array or FISH analysis. The distribution frequency of *B2M* aberrations is

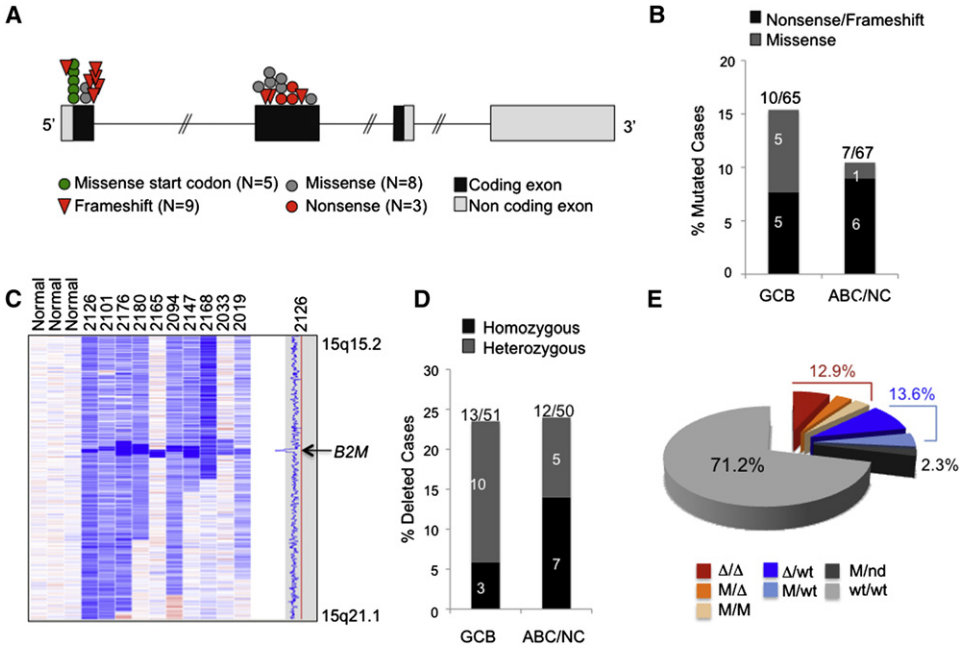

Figure 1. The *B2M* Gene Is Targeted by Genetic Alterations in DLBCL

(A) Schematic representing the distribution of missense, nonsense, and frameshift mutations affecting the *B2M* genomic region in DLBCL biopsies and cell lines (see also Table S1).

(B) Percentage distribution of *B2M* mutated samples in major DLBCL subtypes.

(C) dChip copy number heat map illustrating focal deletions affecting the *B2M* locus (arrow) on chromosome 15 in representative DLBCL samples. The first three columns represent normal (diploid) samples (see also Figure S1 and Table S1).

(D) Distribution of homozygous and heterozygous deletions in GCB- and ABC/NC-DLBCL subtypes.

(E) Overall frequency and allelic distribution of *B2M* alterations, including mutations and deletions, in DLBCL.

Δ, deletion; M, mutation; WT, wild-type; nd, not determined.

comparable in GCB- and ABC/NC-DLBCLs, accounting for 32.3% and 25.3% of the cases, respectively (Figure S1D). Thus, overall 29% (n = 38/132) of all DLBCLs harbor genetic lesions affecting the *B2M* locus, suggesting a critical role for this gene in the pathogenesis of the disease.

B2M Missense Mutations Affect Protein Stability

Although the majority of the *B2M* mutations identified were represented by unambiguously inactivating events, four of the biallelically-affected samples harbored a missense mutation in one of the two alleles (in the absence of secondary frameshift or nonsense mutations in *cis*) (Table S2). These four samples lack B2M expression by immunohistochemical (IHC) analysis (Figures 2A and 2B; Table S2), despite the presence of detectable B2M RNA expression (data not shown). These observations prompted us to investigate whether *B2M* missense mutations induce protein instability. Toward this end, we introduced lentiviral vectors encoding WT or missense mutant *B2M* alleles into WSU, a DLBCL cell line lacking B2M protein expression due to biallelic *B2M* gene inactivation (Table S2). All *B2M* missense variant alleles expressed significantly less protein relative to the WT allele, despite the presence of comparable B2M mRNA levels, as measured by qRT-PCR analysis (Figure 2C). Taken together, the results of the IHC and transient transfection analysis indicate that missense mutations cause the production of unstable B2M proteins.

Seventy-Five Percent of DLBCL Display Aberrant B2M Protein Expression

To examine the functional consequences of *B2M* structural alterations, we analyzed B2M protein expression in normal lymphoid tissues, as well as in 53 DLBCL biopsies and 21 cell lines by IHC analysis. In normal B cells, including germinal-center (GC) B cells, B2M is predominantly expressed on the cell surface as part of the MHC class I complex (Figure S2). However, 75% of DLBCL samples either lacked B2M expression (n = 29/53) or displayed a variety of aberrant expression patterns, including cytosolic (n = 8/53) or perinuclear Golgi (n = 3/53) localization (Figures 3A and 3B). Although biopsies and cell lines with biallelic *B2M* gene alterations exhibit negative staining, as expected, a significant fraction of cases with monoallelic *B2M* inactivation (n = 7/10) also displayed absent or mislocalized protein expression, suggesting silencing of the normal allele or the presence of alternative mechanisms of B2M inactivation by improper subcellular localization (Figures 3B–3D; Table S2). Furthermore, 11 cases with normal *B2M* alleles were also negative for membrane B2M expression. Thus, the majority of DLBCL samples lack cell surface B2M.

Defects in B2M Expression Are Associated with Loss of Cell Surface HLA-I Expression

To investigate whether *B2M* genetic alterations result in perturbed cell surface HLA-I expression, we analyzed the same

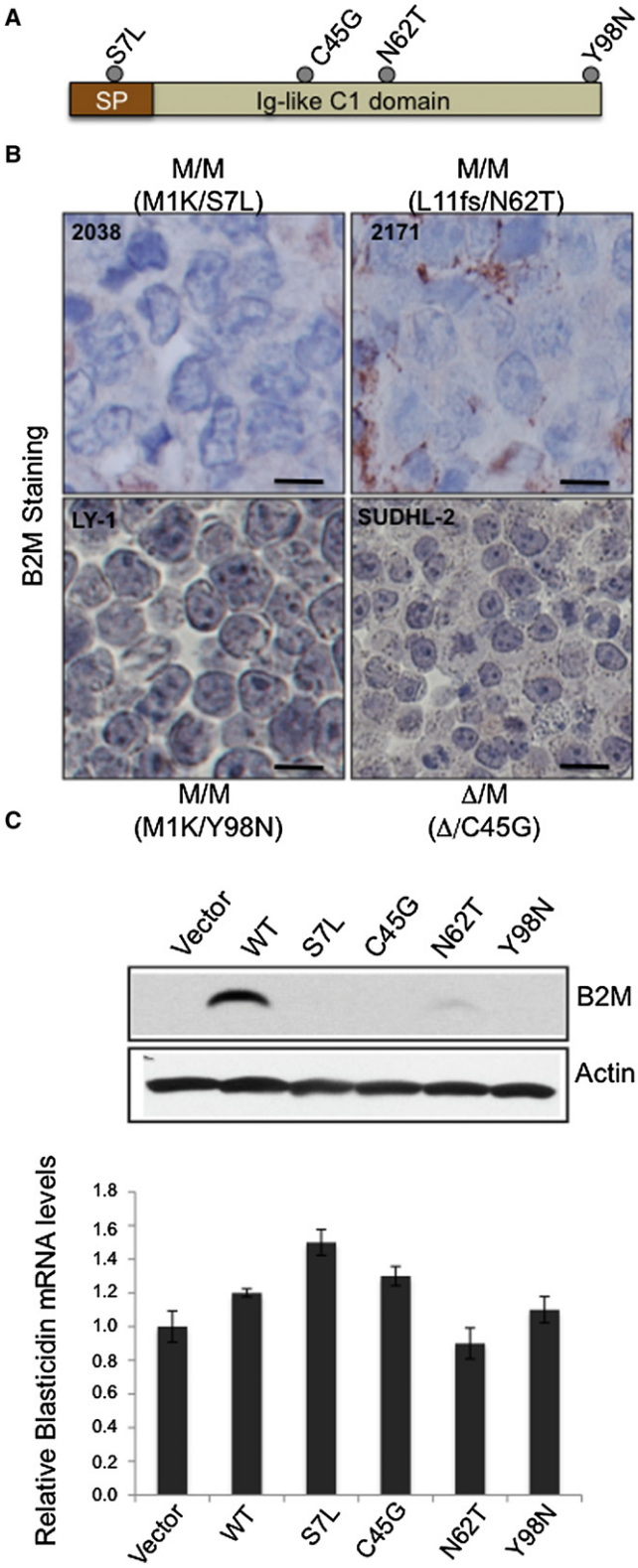

Figure 2. *B2M* Missense Mutations Affect Protein Stability

(A) Schematic representation of the B2M protein, with its N-terminal signal peptide (SP) and C-terminal immunoglobulin (Ig)-like C1 domain. Missense mutations are depicted by gray circles.

(B) Immunohistochemistry analysis of B2M in DLBCL biopsies (top panel) and cell lines (bottom panel) harboring missense mutant alleles. The specific alterations affecting each sample are indicated (Δ, deletion; M, mutation). Scale bar represents 100 μm.

(C) Immunoblot analysis of B2M expression in WSU cells transduced with bicistronic lentiviral vectors encoding WT or missense variants of B2M and blasticidin downstream of an IRES element (actin, loading control). The bar graph below shows the qRT-PCR quantification of the relative blasticidin mRNA levels (mean +/− SEM), which reflect the abundance of B2M transcripts, in the transduced cells.

panel of DLBCL biopsies (n = 53) by IHC using the HC-10 antibody, which recognizes HLA-I (A, B, C) (Stam et al., 1990). We observed positive, negative, or mislocalized HLA-I staining patterns, directly mirroring the pattern of B2M (Figures 4A and 4B). In particular, all samples with normal cell surface B2M expression show positive HLA-I staining (Figure 4C), whereas cases with mislocalized B2M protein (n = 11) have either cytosolic or no HLA-I, indicating that the mechanisms responsible for the perturbation of the normal B2M subcellular localization can also affect the localization or stability of HLA-I proteins. The remaining 29 samples, which lack detectable B2M expression, exhibited negative staining for HLA-I, with the exception of two cases where the HLA-I protein was mislocalized. These observations confirm that loss of cell surface B2M expression, through genomic alterations or other yet uncharacterized mechanisms, is associated with lack of membrane HLA-I in DLBCL. Thus, overall, 75% (40/53) of DLBCL samples lack cell membrane HLA-I expression.

Re-expression of Normal, but Not Mutant, B2M Proteins Results in Restoration of Cell Surface HLA-I Expression

To confirm that the loss of HLA-I is indeed caused by absent or mislocalized B2M expression, we designed experiments to reintroduce functional or missense *B2M* alleles in HLA-I negative DLBCL cell lines. These experiments were also intended to investigate whether lack of B2M expression had other effects on the transformed phenotype of DLBCL cells, as suggested by the proposed role of B2M in transducing the signal of the B cell receptor (BCR), a major survival and proliferation signal for B cells (Colonna et al., 1997; Takai, 2005). Flow cytometric analysis using the pan-HLA-I antibody W6/32 (Parham et al., 1979) showed that, similar to DLBCL biopsies, the cell lines with *B2M* biallelic alterations have significantly reduced (LY-1 and SUDHL2) or absent (WSU) cell surface HLA-I expression (Figure 5A; Table S2). Transduction of these three cell lines with lentiviral vectors expressing WT B2M alleles led to either restoration (WSU) or a significant increase (LY-1 and SUDHL-2) in cell surface HLA-I expression (Figure 5B). Conversely, transduction of B2M missense mutants had no effects on HLA-I expression, thus confirming the nonfunctional nature of these mutants (Figure 5C). No other consequences on the transformed phenotype, including proliferation and survival, were observed (not shown). Taken together, these results demonstrate that *B2M* inactivation is the underlying cause for the lack of HLA-I plasma membrane localization in DLBCL.

Alterations Inactivating the *CD58* Gene in DLBCL

The detection of recurrent copy number changes in *CD58*, a gene known to be involved in the adhesion and activation of

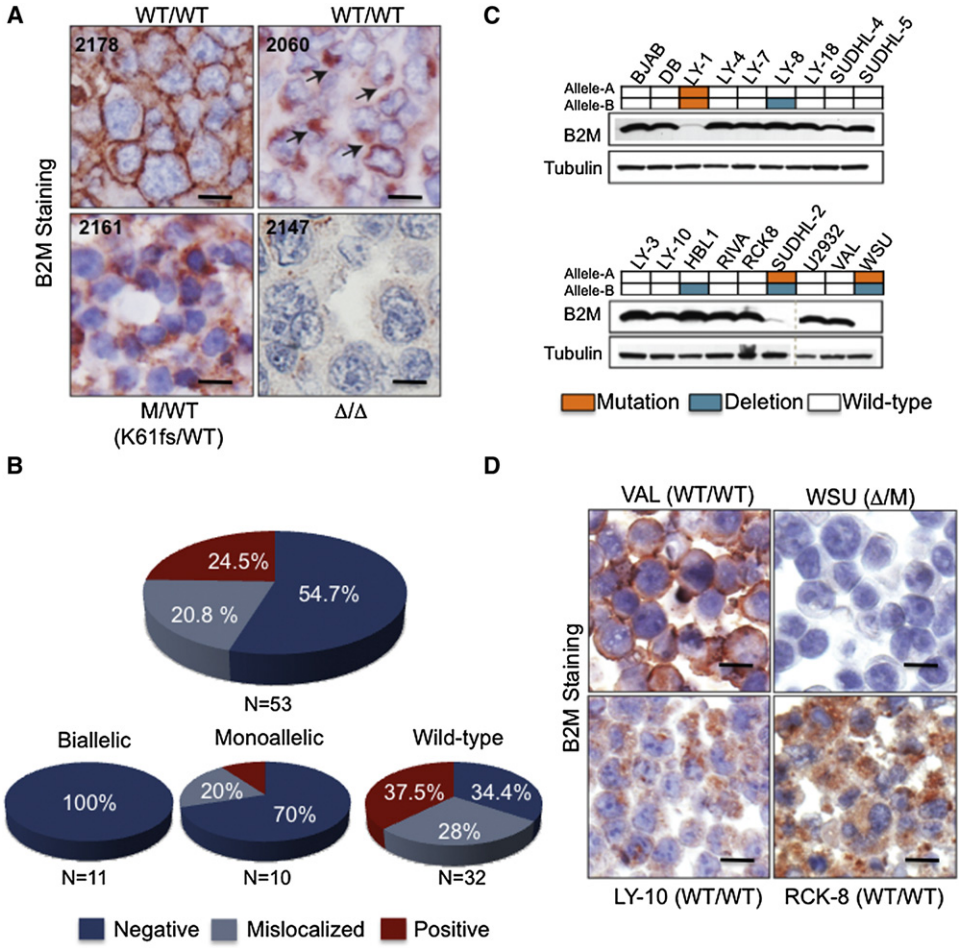

Figure 3. Frequent Loss of B2M Protein Expression in DLBCL

(A) Immunohistochemistry analysis of B2M expression in DLBCL biopsies. Shown are representative examples of the different subcellular localization patterns including membrane (normal), perinuclear/Golgi (indicated by arrows), cytosolic, and no detectable staining (see also Table S2 and Figure S2). The genetic configuration of the two *B2M* alleles is indicated for each case. Scale bar represents 100 μm.

(B) Top panel: overall percentage of DLBCL primary biopsies showing absent (negative), mislocalized, or normal (membrane positive) B2M expression. Bottom panel: percent distribution of B2M expression, according to *B2M* genotype. Note that the "monoallelic" cases also include three samples where the status of the second allele could not be determined. The total number of cases analyzed in each group is specified at the bottom.

(C) Immunoblot analysis for B2M and tubulin (loading control) in DLBCL cell lines carrying WT or genetically altered *B2M* alleles, as indicated.

(D) Immunohistochemistry analysis of B2M expression in representative DLBCL cell lines showing various subcellular distribution patterns. The status of the *B2M* locus is indicated. Scale bar represents 100 μm.

most NK cells as well as CTLs, prompted the targeted sequencing of the *CD58* coding exons in the same extended panel of 132 DLBCL samples. This analysis revealed a total of ten sequence variants, affecting 6.8% (n = 9/132) of the samples (Figure 6A). Of these variants, 80% (n = 8/10) are nonsense mutations, frameshift deletions/insertions or splice site mutations that result in aberrant transcripts encoding for truncated proteins, all of which lack the CD58 transmembrane domain (Figure S3A; Table S3). The remaining two variants were in-frame deletions, which result in the loss of either Tyr119 or Ser98 in the extracellular domain of CD58, with presently unclear functional consequences (Figure 6A; Table S3). *CD58* mutations are distributed at comparable frequency in both GCB- and ABC/NC-DLBCL (6.2% and 7.5%, respectively) (Figure 6B).

High-density SNP array analysis revealed the presence of biallelic or monoallelic deletions affecting the *CD58* locus in 19.8% of the samples analyzed (n = 17/80 biopsies and 3/21 cell lines) (Figure 6C; Figure S3B; Table S3). Notably, the deleted regions in two samples encompass only the *CD58* locus, strongly indicating that *CD58* is the specific target of aberration in this region (Figure S3B). Even though *CD58* deletions were identified in both DLBCL phenotypic subtypes, their frequency was significantly higher in ABC/NC-DLBCL, where they account for 30% (n = 15/50) of samples, as compared to 9.8% (n = 5/51) in GCB-DLBCL ($p < 0.01$) (Figure 6D).

Based on the combined analysis of sequencing data and copy number data, 21.2% (n = 28/132) of DLBCL samples harbor either biallelic (n = 7/132, 5.3%) or monoallelic (n = 21/132, 15.9%) genetic lesions disrupting the *CD58* gene (Figure 6E).

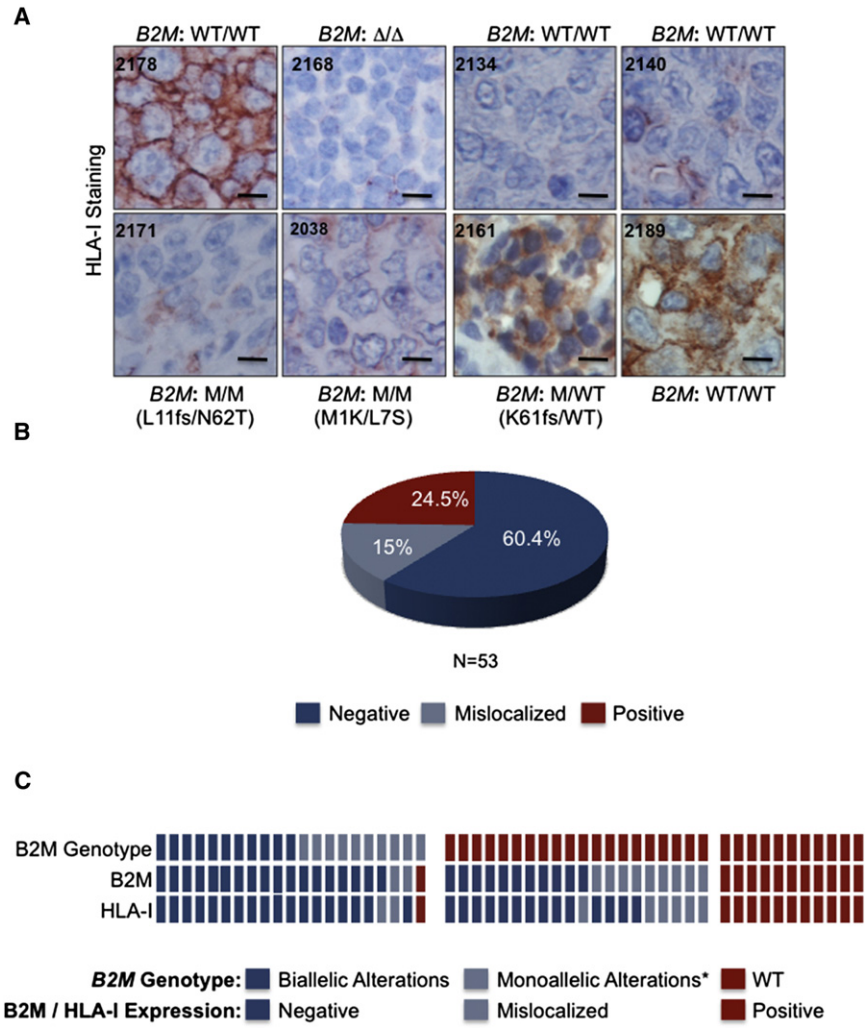

Figure 4. Defects in B2M Expression Associate with the Lack of Cell Surface HLA-I

(A) Immunohistochemistry analysis of HLA-I in DLBCL samples using the HC-10 antibody, which recognizes HLA-A, -B, and -C. The genetic status of *B2M* is indicated for each sample. Scale bar represents 100 μm.

(B) Percentage distribution of DLBCL samples with negative, mislocalized, and positive HLA-I cell surface expression.

(C) Relationship between *B2M* genetic status and expression of the B2M and HLA-I proteins in individual DLBCL biopsies. In the heatmap, each column represents one DLBCL sample; the genotype and staining patterns are color-coded as indicated. *This category includes three mutated samples where the status of the second allele could not be determined.

These alterations are more prevalent in ABC-DLBCLs (67.9% of the lesions found) than in GCB-DLBCLs (32.1%) (Figure S3C). Similar to *B2M*, PCR-amplified exon sequencing analysis of other mature B-NHLs failed to detect any mutation, suggesting that *CD58* genetic lesions may be specifically selected during DLBCL pathogenesis.

Frequent Loss of Cell Surface CD58 Expression in DLBCL

Because DLBCL arises due to malignant transformation of germinal center (GC) B cells, we first sought to determine the expression pattern of CD58 in normal GC B cells. Gene expression profile analysis of purified tonsillar GC and naive B cells, together with flow-cytometric analysis of reactive tonsils, confirmed that CD58 RNA and protein expression were high in GC B cells relative to naive B cells (Figures S4A and S4B). Accordingly, immunofluorescence analysis of human tonsillar sections showed CD58 protein expression in GC B cells, but not in naive B cells in the mantle zone (Figure S4C). In contrast to normal GC B cells, IHC analysis of DLBCL biopsies showed frequent negative or mislocalized protein expression (Figure 7A). Among the cases with biallelic gene alterations, 75% (n = 3/4) exhibited no protein expression (Figure 7B; Table S4); the remaining sample, which harbors two disruptive mutations, showed an abnormal cytosolic localization pattern in the absence of membrane staining, likely due to the expression of truncated proteins that lack the transmembrane domain, but which can be nonetheless recognized by the polyclonal CD58 antibody. Interestingly, 87% of the monoallelically altered and 54% of the WT samples also showed negative or mislocalized CD58 staining patterns, strongly suggesting alternative pathogenic mechanisms for CD58 inactivation. As in the primary biopsies, lack of surface CD58 protein expression was observed in all of the DLBCL cell lines harboring biallelic alterations (Figures S4D and S4E; Table S4).

Overall, 67% of DLBCL biopsies and 19% of DLBCL cell lines lack CD58 cell surface expression. Despite that genetic lesions of *CD58* were significantly more frequent in ABC/NC-DLBCL, the percentage of cases showing absent or aberrant CD58 expression was similar in the two subtypes (GCB-DLBCL, 65%; ABC/NC-DLBCL, 68%) (Figure 7C; Figure S3C). Thus, loss of CD58 surface protein expression is a frequent event across all DLBCLs.

CD58 Contributes to NK Cell-Mediated Cytolysis of DLBCL

To determine whether CD58 loss affects NK cell-mediated lysis in DLBCL, we reintroduced the *CD58* coding sequence (long/transmembrane isoform), or control empty vectors, into the HBL1 DLBCL cell line, which harbors a homozygous deletion of the *CD58* locus and hence lacks CD58 expression. Reconstituted HBL1 cells were then tested as targets in NK-mediated cytotoxicity assays. As shown in Figure 7D, re-expression of CD58 induced a significant increase in the percentage of cytolysis (30%–50%, $p < 0.001$), when compared to

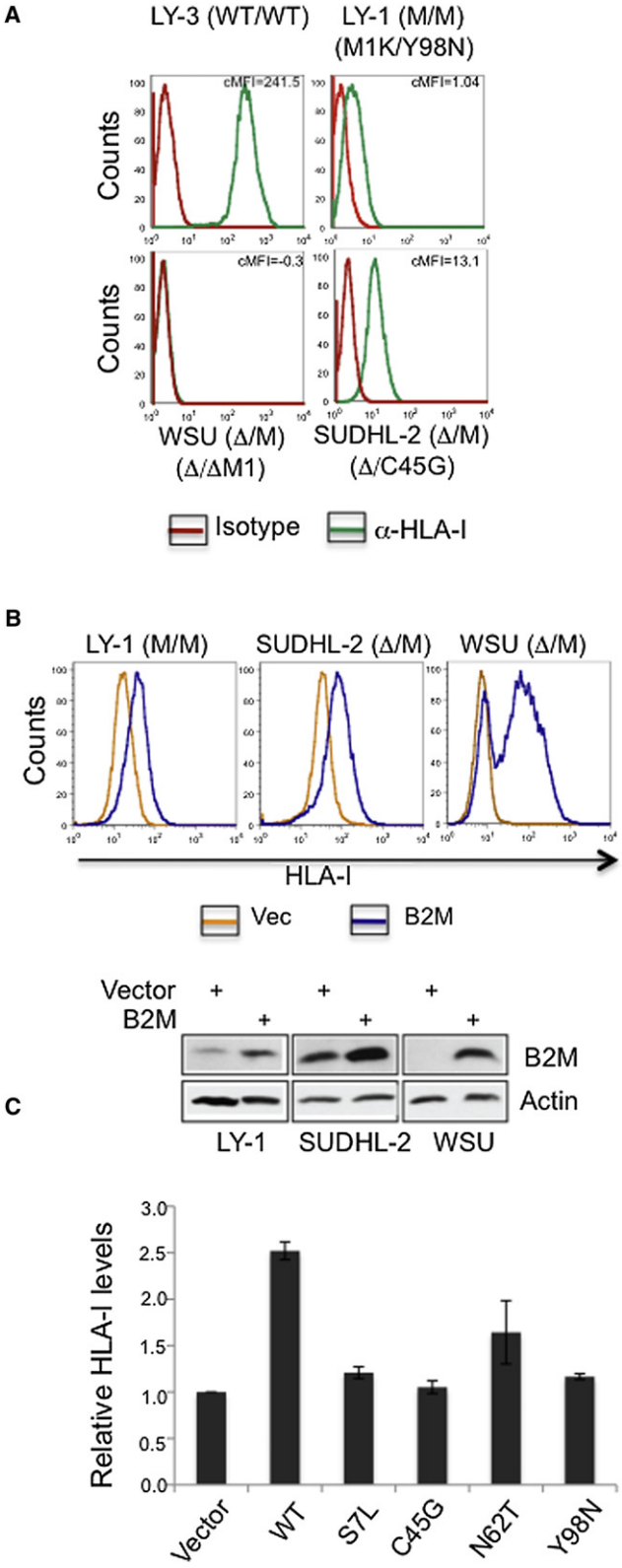

Figure 5. Reintroduction of WT B2M, but Not Mutant Alleles Result in Restoration of Cell Surface HLA-I

(A) Flow cytometric analysis of cell surface HLA-I expression in three DLBCL cell lines harboring biallelic *B2M* lesions, and a *B2M* WT cell line. The corrected mean fluorescence intensities (cMFI = HLA-I antibody MFI minus isotype control MFI) are indicated. Note the complete lack of expression in WSU, and the significantly reduced levels in two additional cell lines carrying a *B2M* missense mutation with genetic loss of the second allele.

(B) HLA-I expression in the three DLBCL cell lines upon reintroduction of WT B2M (blue), as compared to empty vector control (yellow). Western blot analysis of B2M expression in the same cells confirms the presence of exogenous B2M protein. Actin, loading control.

(C) Relative cell surface HLA-I levels (mean +/− SEM), assessed by flow cytometry, in the WSU cell line reconstituted with vectors expressing WT or missense mutants of B2M, as compared to empty vector (arbitrarily set as 1).

vector-transduced cells. Conversely, blocking cell surface CD58 with the anti-CD58 TS2/9 antibody (Le et al., 1990) in the RIVA cell line, which expresses CD58, decreased the susceptibility to NK lysis by 20%–26% ($p < 0.001$) (Figure 7E; Figures S4F and S4G), whereas TS2/9 had no effect on the *CD58*-null HBL1 cell line (Figure S4H). Together, these data demonstrate that CD58 loss is involved in the recognition of DLBCL by NK cells.

Most DLBCL Cases Lack Expression of both HLA Class I and CD58 Proteins

Effective evasion of immune surveillance requires avoidance of both CTL and NK cell recognition. Therefore, it is conceivable that tumors would evolve to lose both HLA-I and CD58 and thus escape the recognition by both arms of cellular immunity. Consistent with this notion, 61% of the DLBCL cases analyzed concurrently lack HLA-I and CD58 on the cell surface (Figures 8A and 8B) ($p < 0.042$), suggesting that these alterations may be coselected during lymphomagenesis for their complementary roles in protecting from CTL- and NK cell-mediated lysis.

DISCUSSION

B2M and *CD58* Gene Inactivation in DLBCL

Our results indicate that direct inactivation of the *B2M* gene by deletion and/or mutation occurs in a sizable fraction of DLBCLs. *B2M* inactivation, whether biallelic or monoallelic, is often associated with loss of protein expression. The type and distribution of these lesions are analogous to those occasionally found in melanoma (Hicklin et al., 1998) and cervical carcinoma (Koopman et al., 2000). In addition, *B2M* genetic lesions have been previously reported in a small number of DLBCL cases originating from the testis or the central nervous system (n = 2 of 15 analyzed) (Jordanova et al., 2003) and in the Burkitt's lymphoma cell line Daudi (Rosa et al., 1983). Our results show that *B2M* gene inactivation is a recurrent event in nodal DLBCLs of both the GCB- and ABC-type, suggesting a pathogenetic role in this disease (see below).

Inactivation of the *CD58* gene by structural alterations had not been previously appreciated in DLBCL and has not been reported in other types of cancer. These lesions include truncating mutations and focal deletions, often disrupting both alleles, and affect 21% of DLBCL cases. As in the case of *B2M*, *CD58* lesions can be found in both major subtypes of DLBCL, but not in other common lymphoid malignancies derived from mature B cells, suggesting that they represent pathogenetic mechanisms rather specific for this type of tumor.

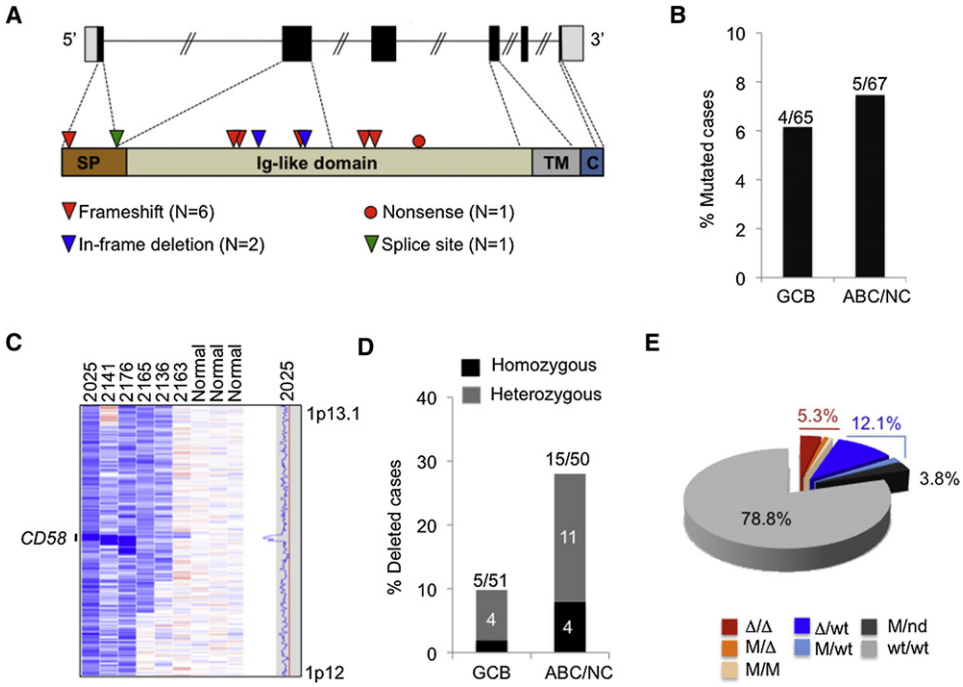

Figure 6. The *CD58* Gene Is a Target of Genetic Alterations in DLBCL

(A) Schematic diagram of the *CD58* gene (top) and protein (bottom), with its known functional domains (SP, signal peptide; Ig, Immunoglobulin single-pass type I membrane; TM, transmembrane; C, cytoplasmic). Color-coded symbols depict distinct types of mutations (see also Table S3).

(B) Percentage of mutated samples in the GCB and ABC/NC-DLBCL subtypes.

(C) dChip SNP inferred copy number heatmap of the 1p13.1 region encompassing *CD58* in representative DLBCL primary cases and three normal (diploid) DNAs (see also Figure S3 and Table S3).

(D) Percentage of samples harboring *CD58* deletions in GCB and ABC/NC DLBCL subtypes. The actual number of affected samples over total analyzed is shown on top.

(E) Overall frequency of *CD58* structural alterations in DLBCL (Δ, deletion; M, mutation; nd, not determined).

Additional Mechanisms Lead to the Lack of Functional B2M and CD58 Protein Expression in DLBCL

A notable finding of this study is that direct genetic lesions of the *B2M* and *CD58* genes represent only "the tip of the iceberg" of heterogeneous defects in the production of functional B2M and CD58 polypeptides in DLBCL. In fact, additional mechanisms lead to the complete lack of cell surface expression of these molecules in over half of DLBCL cases. These mechanisms include the lack of expression of the second allele in cases with monoallelic gene inactivation, which is commonly observed in tumor suppressor genes and may be a consequence of promoter hypermethylation or other epigenetic mechanisms. More unexpected was the finding of mislocalized protein expression in cases with either monoallelic gene inactivation or WT alleles, observed for both B2M and CD58. The basis for this functional alteration is unknown, and may involve yet unidentified genetic lesions affecting proteins that participate in the intracellular transport of B2M and CD58. B2M mislocalization may also be due to defects in HLA-I expression or assembly, which is required for cell surface localization of B2M (Hughes et al., 1997). However, no recurrent copy number alterations, mutations or abnormalities in RNA expression were detected in any of the candidate genes involved in this process, including *TAP1*, *TAP2*, *LMP2*, and *LMP7*. Thus, the identification of the mechanisms leading to mislocalized B2M and CD58 expression will require further genomic and/or functional screenings.

Loss of Functional B2M Leads to Lack of HLA-I Expression and Escape from CTL

As expected, all cases lacking B2M cell surface expression were also devoid of membrane HLA-I, independent of the mechanism underlying the B2M defect (in total, 75% of all DLBCLs). Because HLA-I is misfolded and subsequently degraded in the absence of functional B2M (Hughes et al., 1997), the lack of HLA-I expression in these cases represents a consequence of B2M defects. Consistent with this notion, restoration of B2M expression in *B2M* null cell lines led to reexpression of HLA-I. In rare cases, where the HLA-I protein is absent whereas the B2M protein is mislocalized, the defects may reside in the HLA-I heavy chain genes or in the transport machinery, because both are required for proper B2M localization (Hughes et al., 1997; Peaper and Cresswell, 2008). Indeed, deletions of various portions of the HLA-I locus have been reported in DLBCL (Riemersma et al., 2000), although direct evidence for the specific targeting of HLA-I genes is missing. Overall, our results provide a genetic basis and a mechanistic explanation to previous observations on the lack of HLA-I expression in DLBCL.

The HLA-I complex presents antigenic peptides to CTL, which then proceed to the destruction of the target cells. Thus, HLA-I

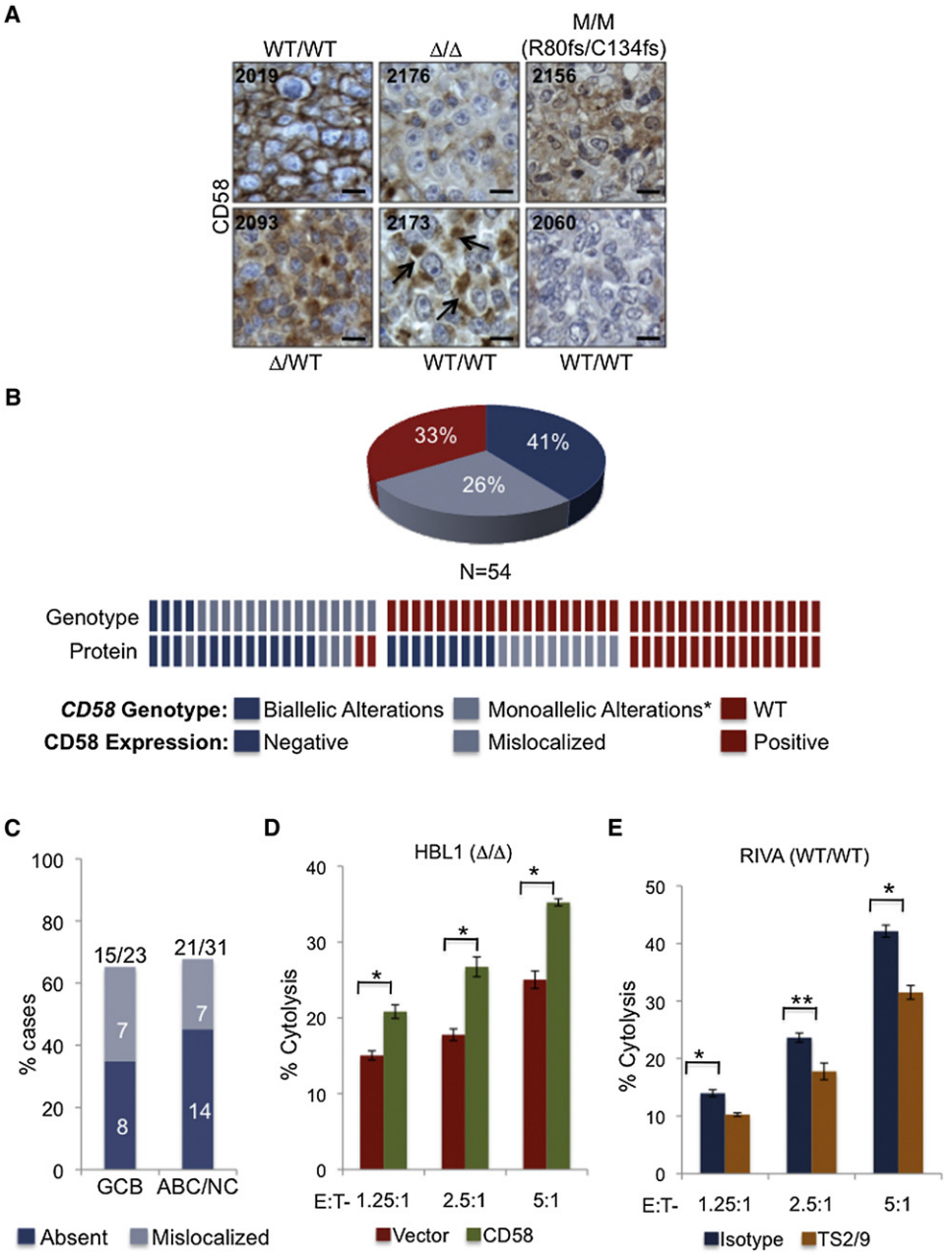

Figure 7. Aberrant Expression of the CD58 Protein in DLBCL Affects NK Cell-Mediated Cytolysis

(A) CD58 protein expression in representative DLBCL biopsies (genomic status as indicated: Δ, deletion; M, mutated; WT, wild-type) (see also Table S4). Scale bar represents 100 μm.

(B) Overall proportion of DLBCL biopsies showing defective CD58 surface expression (top). The relationship between *CD58* genetic lesions and protein expression pattern in individual cases is presented below. *This category includes five mutated samples where the status of the second allele could not be determined.

(C) Frequency of cases showing aberrant CD58 surface expression in DLBCL subtypes.

(D) Percent NK cell-mediated cytolysis in HBL1 cells transduced with lentiviral vectors expressing CD58 (or empty vectors) at various effector to target (E/T) ratios (mean +/− SD; *$p < 0.001$). Data shown represents one of three independent experiments performed in triplicate (see also Figure S4).

(E) NK cell-mediated cytolysis in the DLBCL cell line RIVA (*CD58* WT) after blocking of cell surface CD58 by anti-CD58 TS2/9 antibodies (mean +/− SD; *$p < 0.001$; **$p < 0.01$). Data shown represents one of two experiments performed in triplicate (see also Figure S4).

negative DLBCL cells will be unable to present such peptides, and will remain invisible to T cell-mediated immune-surveillance. The nature of the antigenic peptides that DLBCL cells fail to present is unknown. Because these cases are not infected by any known viruses, the involved antigens may be represented by "tumor antigens" generated by the deregulated expression

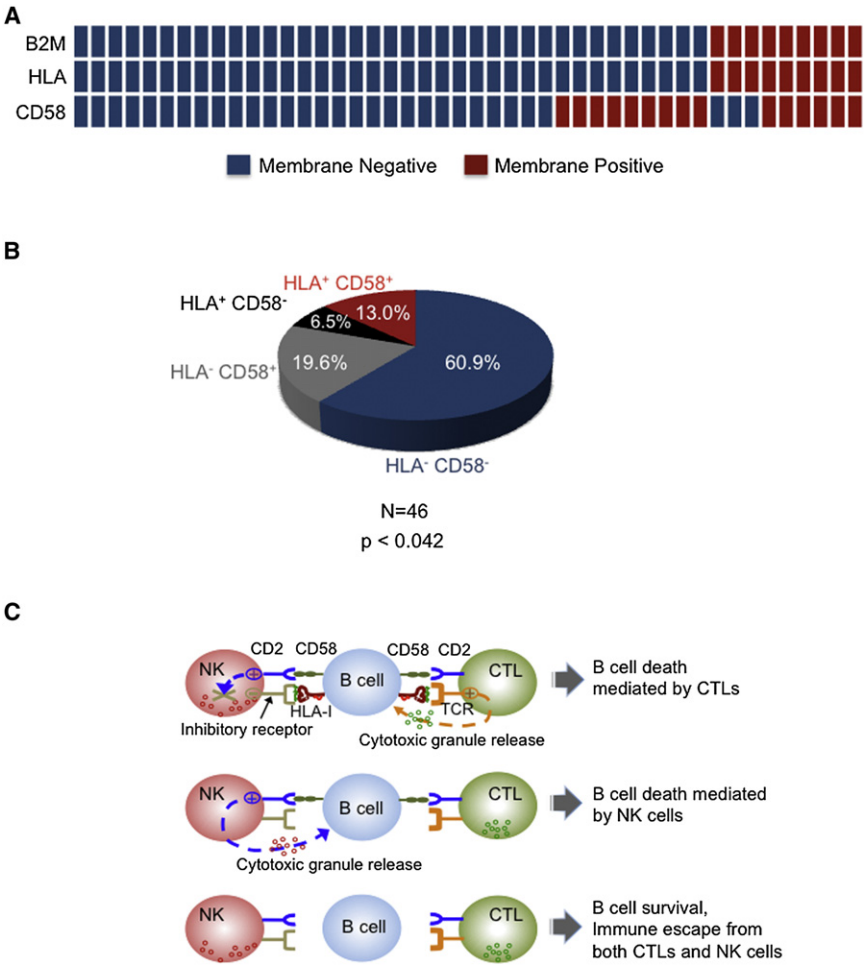

Figure 8. Concurrent Loss of Cell Surface HLA-I and CD58 Is a Common Feature in DLBCL

(A) B2M, HLA-I, and CD58 membrane protein expression in individual DLBCL cases, color coded as indicated. A two-tailed Fisher's exact test was used to determine whether the correlation in the expression patterns of HLA-I and CD58 was statistically significant ($p < 0.042$).

(B) Overall proportion of DLBCL biopsies showing lack of HLA-I and/or CD58 surface protein expression.

(C) Model for the mechanism of immune escape in DLBCL. Lymphoma cells displaying tumor-associated antigens through HLA-I can be recognized and subsequently lysed by CTLs. To evade CTL recognition, tumors often downregulate HLA-I expression (top). However, HLA-I also acts as a ligand for the NK cell inhibitory receptors that transduce signals to counterbalance the activating cues from receptors such as CD2. Thus, tumor cells devoid of HLA-I are vulnerable to NK cell attack via activation of the CD2 receptor by CD58 on the tumor cell (middle). In DLBCL, concurrent loss of HLA-I and CD58 confers protection from both T cell- and NK cell-mediated lysis (bottom).

of proteins or by mutant protein themselves (Boon and van der Bruggen, 1996).

Lack of CD58 Is Relevant for Recognition by Both CTL and NK Cells

By acting as a ligand for the CD2 receptor on T and NK cells, CD58 contributes to the adhesion and activation of both types of immune effector cells. Accordingly, monoclonal antibodies against CD58, which disrupt CD2/CD58 interactions, result in diminished recognition and cytolysis of target cells by both CTLs and NK cells (Altomonte et al., 1993; Gwin et al., 1996; Sanchez-Madrid et al., 1982). The relevance of CD58 in tumorigenesis has been previously suggested based on the observation that CD58 expression is downregulated in several cancer types, although the underlying mechanisms are unknown. The results herein confirm that variations in CD58 expression levels can influence NK cell-mediated cytolysis of DLBCL cells in vitro (Figures 7D and 7E), and suggest that loss of CD58 expression may contribute to decreased recognition of DLBCL cells by NK cells in vivo.

Frequent Combined Loss of HLA-I and CD58 Suggests Coselection during Lymphomagenesis

Although the lack of HLA-I expression in the majority of DLBCL samples confers escape from CTL, the same event will still trigger recognition and lysis by NK cells (Kärre et al., 1986), an event unlikely to be positively selected during lymphomagenesis. This paradox could be explained by the observation that DLBCLs often concomitantly lose the expression of CD58, which may facilitate their escape from both CTL and NK recognition (summarized in Figure 8C). In support of this notion, 75.7% of the DLBCL cases lacking HLA-I expression also lack CD58 expression. The significant association between these two events suggests that they may be coselected during lymphomagenesis for their combined role in the escape from immune recognition.

Implications for DLBCL Pathogenesis and Therapy

The findings herein indicate that escape from recognition by immune effector cells may represent a frequent pathogenetic event in DLBCL, common to both the GCB and ABC subtypes. This mechanism is likely to be an important contributor to the development of DLBCL by complementing the defects in proliferation, differentiation, and survival that are caused by other genetic lesions present in these tumors. Alterations affecting other molecules involved in immune recognition, such as the T cell-recognition ligands *PD-L1* and *PD-L2* as well as the transcriptional coactivator *CIITA* have been reported in PMBCL and Hodgkin lymphoma (Green et al., 2010; Steidl et al., 2011). Additionally, frequent loss of HLA-II in DLBCL has been suggested to play an important role in CTL evasion (Rimsza et al., 2004). These data further corroborate the hypothesis that escape from immune-surveillance through multiple mechanisms is a common feature of all DLBCL subtypes. Evasion from the immune system may protect DLBCL cells from the recognition

of altered polypeptides produced by mutated oncogenes and tumor suppressor genes (Boon and van der Bruggen, 1996). The ability to escape from both the adaptive and innate immune responses should be considered in the development of immunotherapeutic approaches for DLBCL.

EXPERIMENTAL PROCEDURES

Primary Samples

Primary biopsies from 111 newly diagnosed, previously untreated DLBCL patients were obtained as paraffin-embedded and/or frozen material from the archives of the Departments of Pathology at Columbia University and Weill Cornell Medical College, after approval by the respective Institutional Review Boards (Exempt Human Subject Research of anonymized/deidentified existing pathological specimens, under regulatory guideline 45 CFR 46.101(b)(4)) and their detailed characterization has been reported (Compagno et al., 2009). Based on prior gene expression profile studies, the DLBCL cohort (cell lines and primary biopsies) comprised 65 GCB-DLBCL and 67 ABC/NC cases. Other B-NHL cases analyzed included 37 follicular lymphomas, 44 chronic lymphocytic leukemias, 16 Burkitt's lymphomas, and 11 marginal zone lymphomas.

Mutation Analysis

Targeted DNA sequencing of the *B2M* and *CD58* coding exons was performed by the Sanger method on PCR products obtained from whole genome amplified DNA (RepliG kit, QIAGEN). The somatic mutations identified through this method were further confirmed by PCR amplification and double-strand DNA sequencing of independent products obtained from high-molecular-weight genomic DNA. The sequencing results were compared to the UCSC Human Genome database reference mRNA sequence with accession number NM_004048.2 for *B2M* and NM_001779 for *CD58*, using the Mutation Surveyor software as described (Pasqualucci et al., 2011b). In cases harboring more than one mutation, their allelic distribution was analyzed by cloning and sequencing of PCR products encompassing both sequence variants, obtained from cDNA and/or high molecular weight genomic DNA. The PCR primers used for genomic and cDNA amplification of *B2M* and *CD58* are listed in the Supplemental Experimental Procedures.

High-Density SNP Array Analysis

Genome-wide DNA profiles of 80 DLBCL primary cases and 11 paired normal DNAs were obtained using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Santa Clara, CA) as part of an independent study (Pasqualucci et al., 2011b) and are available from the database of Genotypes and Phenotypes repository (http://www.ncbi.nlm.nih.gov/gap) under accession number phs000328.v1.p1. Copy number data for the DLBCL cell lines were obtained from the Gene Expression Omnibus database (accession number GSE22208). The detailed procedure for data processing and analysis is described in Pasqualucci et al. (2011b).

Flow Cytometry

Analysis of cell surface HLA-I in DLBCL cell lines was performed using the W6/32-FITC antibody (Abcam) (Parham et al., 1979). Cells were washed twice for 5 min in PBS + 0.5% BSA and incubated for 30 min with 1 μg of W6/32-FITC per million cells in 100 μl of PBS + 0.5% BSA on ice. The unbound antibody was removed by washing the cells twice with PBS+0.5% BSA for 5 min followed by analysis on a FACSCalibur instrument (Becton Dickinson). Data were analyzed using the CellQuest (Becton Dickinson) and FlowJo softwares (TreeStar). Corrected mean fluorescence intensities (cMFIs) were calculated by subtracting the MFI of cells labeled with an isotype control antibody from the MFI of W6/32-FITC labeled cells. Cell surface CD58 expression was assessed using anti CD58-PE antibodies (AICD58, Beckman Coulter) in the same protocol.

Immunohistochemistry

Paraffin embedded DLBCL tissue microarrays (TMAs) were constructed as described (Compagno et al., 2009) and analyzed for B2M, HLA-I, and CD58 protein expression by immunohistochemistry using standard protocols. Briefly, antigen retrieval was performed in sodium citrate buffer (pH 6.0) in a microwave. The sections were incubated with anti-B2M (1:2,000, DAKO), HC-10 (1:100, a generous gift from Dr. J. Neefjes, Netherlands Cancer Institute, Amsterdam) (Stam et al., 1990), or anti-CD58 antibody (5 μg/mL, R&D Systems, AF1689) overnight. For B2M and HLA-I, the sections were incubated with secondary antibodies conjugated to horseradish peroxidase (HRP) (EnVision, Dako) followed by visualization with 3-amino-9-ethylcarbazole (Sigma). For CD58, the sections were incubated with donkey anti-goat-biotin secondary antibody (1:100, Jackson Immuno) followed by streptavidin-HRP (1:100, Vector Lab), which was then visualized by 3,3′-diaminobenzidine substrate (Vector Lab). All sections containing >20% of tumor cells were considered for scoring.

Lentiviral Transductions

For the reconstitution studies, lentiviral vectors encoding B2M and CD58 were generated by replacing the GFP cassette of the pCCL.sin.PPT.hPGK.GFPWpre vector (kindly provided by Dr. Luigi Naldini, Fondazione San Raffaele, Italy) (Dull et al., 1998) with a B2M- or CD58-IRES-Blasticidin cassette, respectively. Lentiviral vectors, along with helper plasmids encoding Δ8.91 and the VSV-G envelope glycoprotein, were cotransfected into HEK293 cells, and the viral supernatant was used to infect DLBCL cell lines according to standard protocols. In the B2M reconstitution experiments, transduced cells were selected in Blasticidin (LY-1, 5 μg/mL; WSU, 1 μg/mL; SUDHL-2, 0.5 μg/mL) for 10 days before analysis. For the NK-mediated cytotoxicity assays, HBL1 cells were used five days after transduction with CD58-expressing vectors, in the absence of selection.

Immunoblotting

The expression of B2M and CD58 in DLBCL cell lines was analyzed by standard immunoblotting protocols, using antibodies specific for anti-B2M (Dako, 1:1,000) and anti-CD58 (AF1689, R&D Systems: 1:500; for the glycosylated form, TS2/9, Biolegend: 1:200) (Le et al., 1990) specific antibodies. As loading controls, the levels of β-actin (1:10,000, Clone AC-15; Sigma) or β-tubulin (1:2,000, Clone B-5-1-2; Sigma) were measured.

NK Cell Isolation and Cytotoxicity Assays

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected buffy coats (New York Blood Center) of healthy donors by Ficoll-Hypaque (GE Healthcare) density gradient centrifugation. NK cells were then isolated from PBMCs using the NK Cell Isolation Kit (130-092-657, Miltenyi Biotec), and maintained in Iscove's medium containing 5% FCS, 10% human AB serum (New York Blood Center), minimal essential amino acids, sodium pyruvate, L-glutamine, and 2.5 U/ml of recombinant human IL-2 (PeproTech) for 12 hr. For the cytotoxicity assays, target DLBCL cell lines (HBL-1 and RIVA) either untransduced or transduced with lentiviral vectors expressing CD58 were first labeled with 2.5 μM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) for 24 hr. Target cells (T) (0.1×10^6) were then incubated with effector NK cells (E) cells at a ratio (E/T) of 0:1, 1.25:1, 2.5:1, and 5:1 for 4 hr at 37°C in 5% CO_2, and the percentage of cell death in the CFSE-labeled target cells was assessed by flow cytometric analysis of 7-aminoactinomycin D (7-AAD) uptake. NK cell-mediated cytotoxicity was measured after subtraction of the background cell death (E/T = 0:1). For the CD58-blocking experiments, RIVA or HBL1 cells were incubated at 4°C for 1 hr with either the anti-CD58 TS2/9 or an isotype control antibody (both from Biolegend; final concentration, 10 μg/ml per million cells). The blocking efficiency was assessed by staining the cells with the anti-CD58 TS2/9-APC antibody (Biolegend) followed by flow cytometric analysis.

ACCESSION NUMBERS

The SNP Array 6.0 data reported in this article have been deposited in the database of Genotypes and Phenotypes under accession number phs000328.v1.p1. Gene expression profile data from the DLBCL primary cases are available from the Gene Expression Omnibus database under accession number GSE12195.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.ccr.2011.11.006.

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