Coevolution of Leukemia and Host Immune Cells in Chronic Lymphocytic Leukemia

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Cumulative studies on the dissection of changes in driver genetic lesions in cancer across the course of the disease have provided powerful insights into the adaptive mechanisms of tumors in response to the selective pressures of therapy and environmental changes. In particular, the advent of next-generation-sequencing (NGS)-based technologies and its implementation for the large-scale comprehensive analyses of cancers have greatly advanced our understanding of cancer as a complex dynamic system wherein genetically distinct subclones interact and compete during tumor evolution. Aside from genetic evolution arising from interactions intrinsic to the cell subpopulations within tumors, it is increasingly appreciated that reciprocal interactions between the tumor cell and cellular constituents of the microenvironment further exert selective pressures on specific clones that can impact the balance between tumor immunity and immunologic evasion and escape. Herein, we review the evidence supporting these concepts, with a particular focus on chronic lymphocytic leukemia (CLL), a disease that has been highly amenable to genomic interrogation and studies of clonal heterogeneity and evolution. Better knowledge of the basis for immune escape has an important clinical impact on prognostic stratification and on the pursuit of new therapeutic opportunities.

For the most part, the underlying biology of cancers has been largely considered from a purely cell-autonomous disease point of view. Within this framework, genetic defects accumulate progressively in one (or a few) cells, with the occasional somatic mutation affecting a gene or regulatory element that would drive the cell to preferential growth and escape from signals that would otherwise enforce permanent growth arrest or self-destruction (Hanahan and Wein-

berg 2000). Recent next-generation-sequencing (NGS)-based technologies have shown the complex heterogeneous genetic landscapes of tumors and the potential impact of tumor heterogeneity on treatment response and resistance, cancer progression, and the risk of disease relapse (Alexandrov et al. 2013; Lawrence et al. 2013, 2014; Giannakis et al. 2016) (Fig. 1, top). These genomic studies have also provided evidence that tumors evolve through a process of

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Figure 1. Tumor and immune cells coevolve over time. Arrows denote acquisition of cancer-driving mutations.

clonal evolution, involving genetically distinct subclones that compete over resources and adapt to external pressures (Greaves and Maley 2012; Martincorena et al. 2015).

A direct corollary of this renewed understanding of the role of intratumoral heterogeneity on tumor evolution is an appreciation that successful outgrowth of tumors is also impacted by microenvironmental elements, such as the extracellular matrix, the tumor vascular network, and immune cells (Fig. 1, bottom) (Marusyk et al. 2014). Indeed, immune cellular elements in direct contact with the neoplastic cell have the potential to be protective against cancer through immunosurveillance mechanisms (Smyth et al. 2000; Girardi et al. 2001; Shankaran et al. 2001; Street et al. 2002). In turn, to subvert these physiological immune responses, tumor cells can either generate an immunosuppressive environment or escape from immune recognition (reviewed in Dunn et al. 2002, 2004; Zitvogel et al. 2006). Thus, reciprocal interactions between tumor cells and its microenvironment clearly influence cancer progression, and likely its response to cancer therapy (Fridman et al. 2012; Lion et al. 2012; Kroemer et al. 2015). In parallel with this conceptual shift in mechanisms impacting tumor evolution is the exciting

emergence of clinically effective anticancer immunotherapies, which have further shown the potent impact of reestablishing immunological control over neoplastic cells (Schuster et al. 2011; Pardoll 2012; Porter et al. 2015).

In this review, we explore the mechanisms that govern tumor and immune cells coevolution, focusing on studies of chronic lymphocytic leukemia (CLL). Several key features have made CLL an extraordinary model system to assess these questions. First, its relative slow disease progression kinetics has enabled extended longitudinal sampling from individual patients during disease progression and after treatment. Second, highly pure tumor cells are easily accessible from peripheral blood. These unique disease features along with the recent availability and relative affordability of NGS-based technologies have vastly facilitated the evolutionary dissection of the CLL genome over the course of the disease and therapy highlighting the impact of driver events on disease relapse and clinical outcome. Finally, CLL is considered a prototype of a microenvironment-dependent tumor in which neoplastic cells coevolve together with host immune cells within specific tissue microenvironments, such as bone marrow or lymph nodes. Importantly, targeting pathways

involved in the cross talk between CLL and its microenvironment has already shown potent clinical efficacy (Herman et al. 2013; Brown et al. 2014; O'Brien et al. 2014; Byrd et al. 2015).

CLL: A CLINICAL AND BIOLOGICAL HETEROGENEOUS ENTITY

CLL, the most common type of adult leukemia in Western countries, is characterized by the proliferation and accumulation of mature

CD5⁺ CD19⁺ B lymphocytes (Chiorazzi et al. 2005). A precursor state to CLL, monoclonal B-cell lymphocytosis (MBL), has been defined as the presence of clonal B cells in peripheral blood in the absence of other features of CLL (Landgren et al. 2009). Conventionally, patients with early-stage CLL are not treated until they become symptomatic or display evidence of rapid disease progression (Fig. 2A). A hallmark of CLL is its striking variable clinical course among patients, with some individuals surviving for

Figure 2. The mutational landscape of chronic lymphocytic leukemia (CLL) evolves with disease progression. (A) A typical CLL disease course. The CLL patient cohorts primarily characterized in Landau et al. (2015) (blue) and Puente et al. (2015) (red) are marked. (B) The frequency of most recurrently mutated genes in these two CLL cohorts, showing the commonalities and differences in genetic findings between the two. CLL, Chronic lymphocytic leukemia; MBL, monoclonal B-cell lymphocytosis; DFCI, Dana-Farber Cancer Institute; ICGC, International Cancer Genome Consortium.

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many years without therapy and eventually succumbing to unrelated illnesses, and with others having a rapidly fatal disease despite diverse aggressive therapies (Chiorazzi et al. 2005).

In addition to its highly variable clinical features, CLL has been also shown to be remarkably heterogeneous from the standpoint of biological characteristics. This heterogeneity is partly reflected by differences in the mutation status of immunoglobulin variable heavy chain (*IgVH*) genes. According to this criterion, CLL cases can be categorized as having either rare or absence of mutations in the *IgVH* locus (“unmutated”) or as having clonally rearranged *IgVH* genes with 2% or more *IgVH* mutations (“mutated” CLL) (Oscier et al. 1997; Fais et al. 1998). Although patients with the latter tend to have a generally indolent course, the former tend to have an aggressive, more rapidly fatal course (Damle et al. 1999; Hamblin et al. 1999). Biologic heterogeneity has also been discerned through the detection of chromosomal aberrations, with 80% of CLLs carrying at least one of four commonly recurrent events, readily detectable by fluorescence in situ hybridization (FISH) (Döhner et al. 2000). The most common cytogenetic lesion is deletion of chromosome 13q14.3 (*del*[13q]), occurring in more than 50% of cases over time, associated with favorable prognosis. This deleted region contains the *DLEU2/MIR15-16* cluster, which regulates B-cell apoptosis and cell cycle (Klein et al. 2010). The other common events, *del*(11q) trisomy 12 and *del*(17p), occur in 18%, 16%, and 7% of patients, respectively. Patients with *del*(17p), which disrupts the expression of the tumor suppressor *TP53* (Zenz et al. 2010), or *del*(11q), which affects the *ATM* locus (Savitsky et al. 1995), have more advanced disease and more rapid disease progression. Historically, the median time from diagnosis to first treatment in these two groups has been only 9 and 13 months, respectively (Döhner et al. 2000).

GENETIC HETEROGENEITY IN CLL REVEALED BY NGS

The advent of NGS and the application of whole-exome sequencing (WES) to CLL have

transformed our understanding of the genetic heterogeneity of CLL. The earliest studies, studying up to 100 subjects, revealed the high degree of genetic variability among CLL patients, although the overall somatic mutation rate was significantly lower than in solid tumors (Plesance et al. 2010; Fabbri et al. 2011; Puente et al. 2011; Wang et al. 2011). These studies also highlighted several key somatic mutations in CLL, including the transcription factor *NOTCH1* and the splicing factor 3b subunit 1 (*SF3B1*), enabled the reconstruction of the genetic pathways underlying disease pathogenesis, and revealed the clinical impact of genetic events (Wang et al. 2011; Quesada et al. 2012).

Over time, as a growing collection of sample cohorts have been studied worldwide (reviewed in Guièze and Wu 2015), variation in the detection of somatic mutations and their frequencies has been observed, and can be attributed to (1) limited size of any single cohort that has impeded an effective statistical inference of cancer drivers; (2) differences in cohort composition with respect to their clinical and biological features; and (3) the relative lack of longitudinal sampling and thus a diminished ability to delineate the complex network among driver genes over the course of the disease and therapy. Two recent seminal studies have largely addressed these challenges (Fig. 2B) (Landau et al. 2015; Puente et al. 2015). Both studies sequenced whole exomes or genomes from more than 500 CLL-normal pairs, thus providing highly comprehensive characterizations of CLL. The clinicobiologic features of the two studies were notably distinct. Landau et al. included matched pretreatment and relapse samples from subjects enrolled on the phase-III CLL8 study of the German CLL Study Group (GCLLSG) and who were treated with the combination of fludarabine and cyclophosphamide, with or without rituximab as first-line therapy (Landau et al. 2015). In contrast, Puente et al. studied “earlier” CLL cohorts, more representative of the natural history of CLL and samples from subjects with MBL (Puente et al. 2015). As shown in Figure 2B, both studies identified common CLL mutated drivers but at different frequencies. Although *NOTCH1* (12.6%), *ATM* (11%), *SF3B1*

(8.6%), and *BIRC3* (8.8%) were the most frequently mutated genes detected by Puente et al., mutations in *SF3B1* (21%), *ATM* (15%), and *TP53* (7%) were markedly more abundant in the Landau et al. cohort, with reduced frequencies of mutations in *NOTCH1* (6%) and *BIRC3* (4%) (Landau et al. 2015; Puente et al. 2015). The large sample cohorts of these studies have also allowed the discovery of novel candidate cancer genes and, thus, novel cellular processes and pathways affected in CLL.

NGS has been also highly transformative in unraveling intratumoral heterogeneity in CLL by providing a comprehensive approach to the detection of subclones at high resolution and their tracking over time (Schuh et al. 2012). By integrating somatic mutation and copy number alteration data, which enabled estimation of the fraction of cancer cells harboring a mutation, Landau et al. established that certain somatic mutations (i.e., in *MYD88*, trisomy 12, and *del*[13q]) were detected preferentially as clonal events while others (*SF3B1* and *TP53*) as subclonal events, corresponding to earlier and later events in CLL evolution (Landau et al. 2013). These have been consistent findings within the recently characterized larger sample cohorts that have enabled the generation of network models (Wang et al. 2014a; Landau et al. 2015). Importantly, an increased frequency of subclonal driver events were observed in treated relative to untreated patients, supporting the idea that treatment removes dominant subclones and eliminates competition for growth, thereby allowing for the expansion of one or more fit subclones harboring a higher number of mutated driver genes. Indeed, the studies of the CLL8 cohort showed large clonal shifts between pre- and postchemotherapy relapse samples that were distributed into three main patterns: predominantly stable events (trisomy 12, *del*[13q], *del*[11q]), predominantly increasing on relapse (*TP53* mutations and *del*[17p]), and shifting cancer cell fractions over time (i.e., *ATM*, *SF3B1*, *POT1*) (Landau et al. 2015). Landau et al. further showed that future evolutionary trajectories were already present in the pretreatment sample in one-third of cases. Notably, these results suggest that, within this

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therapeutic context, *TP53* mutation or *del*(17p) provide the strongest fitness advantage compared to CLL drivers. Throughout these studies, the presence of subclonal driver mutations adversely impacted clinical outcome, underscoring the importance of the evolutionary landscape as the engine driving cancer relapse.

IMPACT OF EPIGENETIC HETEROGENEITY ON CLL CLONAL EVOLUTION

Together with genetic heterogeneity, epigenetic modifications play a crucial role in cancer development. Methylation status has been the best characterized of epigenetic alterations, and has been the focus of multiple studies in CLL. In particular, the epigenomic landscape of CLL has been characterized to harbor global hypomethylation combined with local hypermethylation, as described in other cancers (Wahlfors et al. 1992; Cahill and Rosenquist 2013; Ziller et al. 2013). By mapping the similarity between CLLs and normal B cells at various stages of differentiation, a recent study revealed a continuum in the spectrum of originating B cells from which malignant transformation has occurred (Oakes et al. 2016), likely contributing to the biologic and phenotypic heterogeneity of this disease. Recently, comprehensive methylation profiling by genome-wide arrays or by massively parallel sequencing-based approaches in CLL has shown vast intratumoral methylation heterogeneity (Kulis et al. 2012; Pei et al. 2012; Landau et al. 2014; Oakes et al. 2014; Puente et al. 2015; Queirós et al. 2015). Increasing methylation heterogeneity has been consistently associated with advanced genetic subclonal complexity over time and across sample cohorts, thus linking genetic and methylome evolution in CLL (Landau et al. 2014; Oakes et al. 2014; Puente et al. 2015). Landau et al. identified that CLL cells have pervasive increased locally disordered methylation compared to normal B cells, and that expression heterogeneity is associated with methylation heterogeneity. This study further suggested that locally disordered methylation creates the potential to enhance the evolutionary adaptive capacity of CLL cells by increasing the background noise of the genome,

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and thereby providing increased opportunities for genetic diversification. In agreement with this notion, an association between methylation evolution and adverse clinical outcome has been repeatedly shown (Landau et al. 2014; Oakes et al. 2014; Puente et al. 2015).

COEVOLUTION OF CANCER AND HOST-IMMUNE CELLS

Malignant cells are constantly interacting with the immune system even before becoming clinically evident. Understanding the evolutionary capacity of both tumor and immune cells and the selective pressure they mutually exert on each other during cancer development is emerging as a crucial factor in defining cancer trajectories. Conceptually, when these two evolving systems interact, both cell populations experience pressures that reciprocally shape tumor immunogenicity and lead to success or failure of immunosurveillance (Fig. 3), consistent with the process of cancer immune editing, which consists of three phases: elimination, equilibrium, and escape (Schreiber et al. 2011).

In this process, one can conceive that the tumor, with its expression of tumor-specific antigens, could provoke an antitumor immune response. The mechanisms by which the immune system is alerted to the presence of a developing tumor are not fully understood. It is speculated that among the possibilities are the creation of immunogenic antigens by tumor cells, which together with the release of immunogenic signals by tumor cells (i.e., tumor necrosis factor α [TNF- α], interleukin 1 [IL-1], and interferon α [IFN- α]) lead to the priming and induction of effector T cells capable of recognizing these antigens. In turn, this could lead to cytolysis of target tumor cells, also known as the elimination phase. Evidence for the elimination phase in humans comes from the observation of expanded clonal T cells in patients with spontaneously regressing melanoma lesions (Ferradini et al. 1993; Zorn and Hercend 1999); tumor infiltration by CD8⁺ T cells, natural killer (NK) cells, or natural killer T (NKT) cells has been associated with an improved prognosis for a number of different tumor types (reviewed in Vesely et al. 2011); and immunosuppression has been often associated with increased risk of cancer (re-

Figure 3. Model of tumor and host immune cell coevolution. Clonal diversification of chronic lymphocytic leukemia (CLL) cells may result from cell-autonomous mechanisms ultimately leading to the emergence of fitter subclones competing within bulk CLL. Host immune cells coevolve with CLL cells by initially creating an antitumor immune response. CLL cells might promote immune evasion mechanisms, such as altered antigen presentation or induction of an immunosuppressive microenvironment.

viewed in Dunn et al. 2002; Vesely et al. 2011). There is less evidence for the equilibrium phase, during which tumor cells and antitumor response coevolve restraining cancer growth for extended time periods. Critical and opposing roles of IL-23/IL-12 or TNFR1/INF- γ in maintaining cancer cells in a state of immune-mediated dormancy have been described (Koebel et al. 2007; Müller-Hermelink et al. 2008; Teng et al. 2012; Ngiew et al. 2013).

As a consequence of persistent immune selection pressure placed on genetically plastic tumor cells, it can be expected that subpopulations of tumor cells circumvent immune recognition and/or destruction leading to tumor progression, which is known as tumor immune escape. As opposed to the equilibrium phase, a myriad of studies have assessed the mechanisms of immune escape in cancer, which can be classified into three categories: (1) reduced immune recognition and immune cell stimulation through down-regulation of the expression of tumor antigens and antigen-presenting machinery or decreased priming of dendritic cells or T cells; (2) up-regulation of resistance mechanisms by increased mutated oncogenes; and (3) establishment of an immunosuppressive tumor microenvironment (reviewed in Dunn et al. 2004; Schreiber et al. 2011; Vesely et al. 2011; Teng et al. 2015).

CLL AS A MODEL SYSTEM TO STUDY INTERACTIONS BETWEEN TUMOR AND IMMUNE CELLS THROUGH THE COURSE OF THE DISEASE AND THERAPY

The process of cancer immunoediting is difficult to assess in humans. Nevertheless, CLL, in which malignant B cells are in direct apposition with other immune components within specialized lymphoid compartments (Klein and Dalla-Favera 2008), provides an opportunity to study tumor-immune interactions over time. The tight regulation of survival and proliferation of CLL cells by the molecular interactions within lymphoid tissues has been well recognized (Burger et al. 2000; Burger and Kipps 2002). Characteristically, CLL cells residing in lymph nodes

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form distinct structures known as pseudofollicles or proliferation centers (Schmid and Isaacson 1994; Sachanas et al. 2013). It has been suggested that these proliferation centers might be the predominant site for clonal expansion of CLL cells (Caligaris-Cappio and Ghia 2008). Accumulating data now suggests that the interplay between leukemic and immune cells is a dynamic process that not only promotes tumor progression and immune escape, but that also shapes the extent to which malignant B cells are immunogenic and immunostimulatory, as we review below.

The Co-Opting of Normal Immune Functions to Promote CLL

A common feature of malignant cells is their ability to co-opt normal lineage-specific signaling pathways for their prosurvival benefit. In the case of CLL, these leukemic cells have subverted the B-cell programs of normally transient activation mediated by B-cell receptor (BCR) signaling and immune costimulation in the settings of encounters with cognate antigen or microenvironmental cues (Fig. 4). Indeed, these cellular pathways are constitutively activated in CLL even in the absence of discernible extrinsic ligands, thereby leading to increased cell survival, proliferation, and migration of the malignant cell (Stevenson et al. 2011; Dühren-von Minden et al. 2012; Woyach et al. 2012). The essentiality of activated BCR signaling to CLL pathogenesis has been clearly shown, given the potent clinical responses observed with agents that directly target BCR signaling (Byrd et al. 2013; Brown et al. 2014; Furman et al. 2014; O'Brien et al. 2014). Likewise, the importance of signaling through Toll-like receptors (TLRs) to CLL has been highlighted by the discovery of highly recurrent somatic mutations in CLL in the key adaptor protein MYD88 (Puente et al. 2011; Landau et al. 2013; Martínez-Trillos et al. 2014) that bridges TLRs to the activation of downstream interleukin receptor-activated kinases and the transcription factor NF- κ B (Akira and Takeda 2004; Ngo et al. 2011). The recurrent MYD88^{L265P} mutation has been found to drive rapid B-cell proliferation in the absence

Aside from this corruption of normal intrinsic B-cell functions to provide progrowth signals for CLL cells, well-characterized reciprocal interactions between CLL cells and multiple immune cell populations have been described, which, in total, drive the secretion of supportive

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(Zucchetto et al. 2009), and are activating to CLL cells through BAFF and APRIL and through interactions with NOTCH1 and CD38 receptors (Nishio et al. 2005; Zweidler-McKay et al. 2005; Deaglio et al. 2010; Cols et al. 2012); (2) nonclassical monocytes ($CD14^{low}CD16^{2+}$), which are weakly phagocytic, present at higher levels in CLL patients compared to healthy individuals (Maffei et al. 2013), and are promoted to differentiate into NLCs in vitro (Jia et al. 2014); (3) T-cell populations (T_{FH} , T_{H2} , T_{reg}), whose close interaction with CLL cells (which secrete CCL3 and CCL4 as potent T-cell chemo-attractants) in proliferation centers (Burger et al. 2009) induces the secretion of activation molecules by T cells (i.e., CD40 ligand, IL-4, and IL-21) (Buske et al. 1997; Granziero et al. 2001; Ghia et al. 2002; Patten et al. 2008; Bagnara et al. 2011; Ahearne et al. 2013; Os et al. 2013); and (4) follicular dendritic cells (FDCs), which are mandatory participants in B-cell development in germinal centers by attracting and retaining antigen-activated B cells and facilitating their clonal expansion within the FDC network in a T-cell-dependent fashion (Liu et al. 1996). FDCs are found in proliferation centers within the lymph node and marrow of CLL patients (Chilosi et al. 1985; Schmid and Isaacson 1994) and protect CLL cells against apoptosis promoting leukemia progression (Pedersen et al. 2002; Heinig et al. 2014).

Immune Suppression and Escape

Under healthy conditions, the immune system can suppress tumor progression either by destroying tumor cells or by inhibiting their outgrowth, a process also known as cancer immunosurveillance (Mittal et al. 2014). The continuous selection pressure by the immune system placed on genetically unstable tumor cells has a high likelihood of driving the development of resistance mechanisms to subvert immunosurveillance.

As mentioned above, tumor-immune escape can occur through many different mechanisms, such as induction of an immunosuppressive state within the tumor microenvironment, up-regulation of resistance mechanisms, or re-

duced immune recognition (Schreiber et al. 2011), each of which has been observed in CLL. Studies of gene-expression profiling of $CD4^{+}$ and $CD8^{+}$ T cells from CLL patients have revealed an expression signature of altered immune response, with changes in genes involved in cell differentiation, cytoskeleton formation, cell trafficking, and cytotoxicity. Consistent with these findings, defective immunological synapse formation with antigen-presenting cells (APCs) has been shown in CLL patients (Görgün et al. 2005; Ramsay et al. 2008, 2012). This cellular signaling structure orchestrates the complex communication between the T cell and the APC in a way that ensures detailed antigen recognition and effective T-cell responses (Grakoui et al. 1999). In addition, $CD8^{+}$ T cells from CLL patients express high levels of PD1, CTLA-4, CD160, and CD244, and show functional defects in proliferation and cytotoxicity (Motta et al. 2005; Gassner et al. 2012; Nunes et al. 2012; Riches et al. 2013). These findings are consistent with a phenotype of T-cell exhaustion, although CLL cells retain the ability to degranulate and produce protumor cytokines as opposed to classical exhaustion (Riches et al. 2013). Adding to this picture, T_{reg} -cell populations are elevated in CLL and MBL patients, and appear to play a major role in suppressing immune responses either by direct interaction with other immune cell types, such as effector $Th17$ cells or NK cells, or through immunosuppressive cytokines, such as IL-10 and transforming growth factor β (TGF- β) (Kitabayashi et al. 1995; Bluestone and Abbas 2003; Liu et al. 2006; Gowda et al. 2010; Costello et al. 2012; Jadidi-Niaragh et al. 2012).

Additional molecular mechanisms of evasion have been found to come from dysregulated NK-cell-mediated immune responses in CLL through (1) down-regulation of activating NKG2D receptors and corresponding ligands (Huergo-Zapico et al. 2014); (2) up-regulation of inhibitory killer cell immunoglobulin-like receptors (Junevik et al. 2007; Verheyden et al. 2009); (3) indirect suppression of NK activity exerted by T_{reg} cells (Gowda et al. 2010; Costello et al. 2012; Jadidi-Niaragh et al. 2012) and M2-macrophages (Hasmim et al. 2015). Although

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little is known about the contribution of the monocyte–macrophage compartment to CLL development, constitutive M2 skewing was recently shown in CLL patients, based on high levels of interferon regulatory factor 4 (IRF4) transcription factor and low IL-12 levels, considered M2 and M1 markers, respectively (Audrito et al. 2015). Using the TCL1-tg mouse model, Hanna et al. likewise observed severe skewing of M2 macrophages, aberrantly high PD-L1 and PD-L2 expression, and secretion of multiple immunosuppressive cytokines, such as IL-10, TNF- α , and CXCL9 by M2 macrophages, altogether likely contributing to T-cell exhaustion (Hanna et al. 2015). In addition to macrophages dysfunction, myeloid DCs from CLL patients are also severely defective, unable to effectively drive a Th1 response and display down-regulation of major histocompatibility complex class II (MHC-II) and up-regulation of PD-L1 (Orsini et al. 2003, 2004; Hanna et al. 2015). Another emerging population of suppressive cells, the so-called myeloid-derived suppressor cells (MDSCs), which are characterized as CD14⁺ cells lacking human leukocyte antigen–antigen-D-related (HLA-DR) expression (HLA-DR^{lo}), have been found at increased absolute numbers in CLL patients, with higher levels of MDSCs correlated with disease progression and poorer prognosis (Jitschin et al. 2014; Liu et al. 2015). MDSCs suppressed in vitro T-cell activation and proliferation and induced suppressive regulatory CD4⁺ T cells (T_{reg}) in CLL (Jitschin et al. 2014). Only a few studies have addressed the temporal changes in the quantitative and qualitative aspects of immunologic state of the normal immune cellular components in CLL patients with disease progression. Some investigators have examined the changes in absolute T- and NK-cell counts over time, with the consistent observations of increased counts in early-stage CLL patients as well as in patients with MBL compared to those with more advanced disease (Palmer et al. 2008; Gonzalez-Rodriguez et al. 2010; Christopoulos et al. 2011; D’Arena et al. 2011b; Forconi and Moss 2015). Higher NK, CD8⁺, and CD4⁺ T-cell counts have been also consistently associated with better outcomes (Palmer et al. 2008;

Gonzalez-Rodriguez et al. 2010; Huergo-Zapico et al. 2014). Altogether, these observations suggest an initial expansion of T and NK cells, consistent with preserved antigen-presenting functions in early CLL cells. At the same time, higher levels of circulating Th17 cells and higher Th1:Th2 ratio have been observed in CLL compared with normal donors and both levels progressively decrease with disease progression (Van den Hove et al. 1998; Podhorecka et al. 2002; Jain et al. 2012; Hus et al. 2013; Jadidi-Niaragh et al. 2013; Sherry et al. 2015). Th1 and Th17 cells have been established as critically important cellular components in antitumor immunosurveillance in CLL (van Sandick et al. 2003; Fridman et al. 2012). An association between higher levels of circulating Th17 cell numbers and longer survival has been described (Jain et al. 2012; Jadidi-Niaragh et al. 2013; Sherry et al. 2015). In addition to Th1/Th2/Th17 T-cell subsets, increased frequency in T_{regs} in CLL and MBL (Badoual et al. 2009), and correlation with disease progression and time to first treatment has been shown in CLL patients (Beyer et al. 2005; Giannopoulos et al. 2008; D’Arena et al. 2011a, 2012; Weiss et al. 2011; Lad et al. 2015). Finally, increased numbers of T_{EH} cells have been consistently observed in CLL patients as compared to normal subjects, although their association with clinical stage has been controversial (Ahearne et al. 2013; Cha et al. 2013).

Higher levels of CTLA-4 and PD-1 have been consistently associated with rapidly progressive disease and worse outcome, supporting the notion of T-cell exhaustion as an immune-escape mechanism during CLL progression (Motta et al. 2005; Ramsay et al. 2008; Nunes et al. 2012; Brusa et al. 2013), even while the association between these features and clinical stage have not been strong (Gassner et al. 2012; Brusa et al. 2013; Grzywnowicz et al. 2015; Novák et al. 2015; Rusak et al. 2015). Recently, antibodies targeting the PD-1/PD-L1 interaction have shown impressive antitumor activity in solid and hematologic malignancies, highlighting the therapeutic opportunity for targeting the modulation of T-cell inhibitory molecules (Topalian et al. 2012; Armand et al. 2013;

Ansell et al. 2015). This therapeutic direction for CLL is supported by recent work in CLL murine models showing that exposure to PD-L1-blocking antibodies prevented CLL development and reactivated immune effector functions (McClanahan et al. 2015a,b).

GENERATION OF CLL-DIRECTED IMMUNE RESPONSES

A key path for tumor control is through the mounting of an effective immune response against the tumor. Central to this endeavor is the ability of the immune system to recognize and drive responses against tumor antigens (Sensi and Anichini 2006).

In CLL, a limited spectrum of leukemia-associated antigens have been identified and tested. These have included fibromodulin, PRAME, MPP11, RHAMM/CD168 (Giannopoulos et al. 2006, 2010), and ROR1 (Hudecek et al. 2010). Prospective vaccination with RHAMM peptide has been tested in CLL patients, with responses limited to examination of patients in early disease (Giannopoulos et al. 2010). Notwithstanding, the identification of aberrantly expressed tumor-associated antigens (TAAs) has historically been time-consuming and laborious. Recent advances in mass spectrometry have facilitated the identification of a vast array of naturally presented HLA class I and class II binding peptides in primary CLL samples, and excite the possibility of expanding the spectrum of targetable entities (Kowalewski et al. 2015).

Until recently, the bulk of TAA discovery has relied on the identification of those targets with high expression in leukemia cells compared to normal cells. Recent advances, however, in NGS and peptide epitope prediction algorithms (Lin et al. 2008; Zhang et al. 2011; Cibulskis et al. 2013) have opened the possibility of systematically mining the genome for antigen epitopes, which are products of somatic mutations that generate protein-coding region changes, and predicting whether the resulting modified peptide sequences can potentially bind surface MHC class I molecules so that they can be recognized by the T-cell receptor (TCR) of cytotoxic T cells (Fig. 5) (Sensi and Anichini 2006; Ha-

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cohen et al. 2013). Indeed, application of this strategy to identify so-called tumor neoantigens has led to clinical evidence of natural and therapy-induced antitumor responses in mouse and human across solid tumors (Gubin et al. 2014; Yadav et al. 2014; Carreno et al. 2015; Rizvi et al. 2015; Schumacher and Schreiber 2015; Tran et al. 2015; Van Allen et al. 2015; Giannakis et al. 2016), as well as in CLL (Rajasagi et al. 2014).

A series of discoveries in solid tumors have described tumor escape mechanisms related to reduced immunogenicity of tumor cells. Loss of neoantigen expression has been described as one of these mechanisms, Rooney et al. reported increased cytolytic activity in tumors with higher neoantigen load and that depletion of neoantigen load was unexpectedly found in some tumor types, consistent with immunologic selective pressure (Rooney et al. 2015). Efficient processing of tumor epitopes by cellular proteolytic intermediates is essential for the generation of an effective antitumor cytotoxic response. Impaired antigen processing caused by deregulation of the proteasomal degradation has been consistently revealed as an immune escape mechanism in tumor cells (Sun et al. 2002; Guillaume et al. 2012; Ebstein et al. 2016). One of the most frequent causes of immune escape in human tumors is defects in the antigen-presenting machinery owing to HLA class I molecule down-regulation or loss (Restifo et al. 1993; Seliger et al. 2001, 2002; Paschen et al. 2006), low affinity peptide–MHC interactions (Engels et al. 2013), and, recently, a greater-than-expected frequency of somatic mutations in class I HLA genes, predicted to be loss of function, was discovered across cancers, associated with up-regulation of signatures of cytolytic activity characteristic of tumor infiltration by effector lymphocytes. This was especially pronounced in colorectal cancers (Shukla et al. 2015; Giannakis et al. 2016). The types of resistance mechanisms related to reduced immunogenicity developing in CLL remain to be seen.

CONCLUSIONS AND FUTURE CHALLENGES

Emerging evidence underscores the relevance of deciphering not only the genomic evolution of

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Figure 5. Somatic mutations have the potential to generate neoantigens. Neoantigens are produced as a result of somatic mutations in cancer cells. Major histocompatibility complex (MHC) class I molecules present processed epitopes on the tumor cell surface to effector T cells through the T-cell receptor (TCR). Several mechanisms of immune escape within this process are depicted: (1) loss of neoantigens generation, (2) impairment of antigen processing, and (3) defects in antigens presentation. HLA, Human leukocyte antigen.

tumor cells across time and therapies but also the temporal dissection of the immune composition that may govern these processes and, more importantly, the molecular interplay between both tumor and immune cells. A comprehensive understanding of the mechanisms that influences cancer immunoediting have suggested new immune-related prognostic markers and the biologic rationale for the growing classes of new immune-based cancer treatments, and offer the unique opportunity for refining personalized-based approaches to cancer treatment. Moreover, insights into the complexity of biological networks within the tumor microenvironment give us the chance to consider new mechanisms of treatment resistance that may inform us of new directions for clinical trials.

These concepts have important implications across cancer, including on CLL. In many ways, CLL presents a model system for dissecting out these concepts because of the ready accessibility of both malignant cells and surrounding immune cells, and because of its relatively indolent nature that enables interrogation of samples

over time. We anticipate that new investigations in CLL will address critical questions in this area, including how do specific driver mutations affect the quality and quantity of tumor antigens? How does the burden of CLL neoantigens differ across the course of disease evolution? How does the genomic landscape of a tumor shape antitumor immune responses and how is the genomic landscape shaped by antitumor immune responses? How do driver mutations affect the antitumor immune response? What immune processes modulate CLL elimination and escape? Surely, with the advent of new and more sophisticated technologies and with our expanding understanding of cancer immunity, we can expect rapid progress in our understanding of this biology, with an impact on the development of personalized treatment approaches for this disease over the next few years.

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