Clonal Expansion and Epigenetic Inheritance Shape Long-Lasting NK cell Memory

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Zusammenfassung

Die Selektion klonal expandierender Zellen mit einzigartigen, somatisch rekombinierten Antigen-Rezeptoren und die Langlebigkeit der daraus hervorgehenden Gedächtniszellen sind definierende Eigenschaften adaptiver Immunität. Dahingegen ist das angeborene Immunsystem darauf programmiert, mittels einer breiten Palette konservierter Rezeptoren möglichst schnell auf Pathogene zu reagieren und wird dabei auf Populationsebene epigenetisch geprägt. In dieser Arbeit möchte ich dieses Paradigma auf der Basis von Natürlichem Killer (NK) Zell-Gedächtnis an das humane Zytomegalievirus (HCMV) als Beispiel für Pathogen-spezifische Anpassung innerhalb des angeborenen Immunsystems herausfordern. Indem wir multiparametrische Einzelzellanalysen zur Kartierung von *ex vivo* NK Zellen mit endogenen Barcodes in Form von somatischen Mutationen in mitochondrialer DNA (mtDNA) verknüpfen, können wir drastische klonale Expansionen adaptiver NK Zellen in $HCMV^+$ Spendern nachweisen. NK-Zell-Klonotypen waren durch eine ihnen gemeinsame, inflammatorische Gedächtnissignatur mit AP1 Motiven gekennzeichnet, die eine Reihe einzigartiger Chromatin-Regionen mit Klon-spezifischer Zugänglichkeit überlagerte. NK-Zell-Klone wurden über einen Zeitraum von bis zu 19 Monaten stabil aufrechterhalten und behielten dabei ihre charakteristischen, Klon-spezifischen epigenetischen Signaturen, was die entscheidende Rolle klonaler Vererbung von Chromatin-Zugänglichkeit für die Prägung des epigenetischen Gedächtnis-Repertoires unterstreicht. Insgesamt identifiziert diese Arbeit zum ersten Mal klonale Expansion und Persistenz innerhalb des angeborenen Immunsystems im Menschen und deutet daraufhin, dass diese zentralen Mechanismen zur Ausbildung von immunologischem Gedächtnis evolutionär unabhängig von diversifizierten Antigen-Rezeptoren entstanden sind.

Summary

A hallmark of adaptive immunity is the clonal selection and expansion of cells with somatically diversified receptors and their long-term maintenance as memory cells. The innate immune system, in contrast, is wired to rapidly respond to pathogens via a broad set of germline-encoded receptors, acquiring epigenetic imprinting at the population level. The presented work challenges this paradigm by studying Natural Killer (NK) cell memory to human Cytomegalovirus (HCMV) infection as an example of pathogen-specific adaptation within the innate immune system. Leveraging single-cell multi-omic maps of *ex vivo* NK cells and somatic mitochondrial DNA (mtDNA) mutations as endogenous barcodes, we reveal drastic clonal expansions of adaptive NK cells in HCMV$^+$ individuals. NK cell clonotypes were characterized by a convergent inflammatory memory signature driven by AP1 transcription factor activity, superimposed on a private set of clone-specific accessible chromatin regions. Strikingly, NK cell clones were stably maintained in their specific epigenetic states for up to 19 months, revealing that clonal inheritance of chromatin accessibility shapes the epigenetic memory repertoire. Together, this work presents the first identification of clonal expansion and persistence within the human innate immune system, suggesting these central mechanisms of immune memory have evolved independently of antigen-receptor diversification.

Contents

1. Introduction

One of the first documentations of immunological memory as a concept is almost 2500 years old, when Greek historian Thucydides had noted during the plague in Athens that "*it was with those who had recovered from the disease that the sick and the dying found most compassion. These knew what it was from experience, and had now no fear for themselves; for the same man was never attacked twice – never at least fatally*" (Thucydides, reprinted 1951). Two-and-a-half millennia later we find ourselves yet again in the middle of a pandemic in which we strive to induce (and maintain) immunological memory. Thankfully, basic research has taught us a lot about the fundamental mechanisms that enable immune systems to remember, thus establishing a theoretical framework to explain immune memory. At its very core, Darwinistic evolutionary mechanisms structure adaptive immune responses into three phases: (1) The generation of diverse specificities, (2) Clonal competition under different selection pressures and (3) Long-term survival of the fittest. Here, I would like to discuss these concepts in the context of the fascinating biology of virus-specific NK cells, highlighting parallels and differences to classical adaptive immunity, as well as gaps in our understanding that motivated the present work.

1.1 Diversification of immune specificity

1.1.1 Innate and adaptive immune specificity

At the beginning of any immune response lies the recognition of the pathogen via immune receptors. While these receptors can be classified based on structural homology, the cell types on which they are expressed, or the ligands which they recognize, their classification into germline-encoded and somatically-rearranged receptors is of conceptual importance for immunology as a whole, as it also divides the vertebrate immune system into an innate and adaptive arm. The innate immune system, in particular the myeloid lineage, is equipped with a broad panel of different germline-encoded pattern-recognition receptors (PRR) that detect conserved, oftentimes central structural elements shared by various pathogens, so-called pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). Prominent examples belong to the toll-like receptor (TLR) family that sense various microbial components, of which especially TLR3, TLR7, TLR8, and TLR9 are important for viral infection as they detect double-stranded RNA (TLR3), single-stranded RNA (TLR7/8) and unmethylated DNA (TLR9) during the life cycle of different viruses (Alexopoulou et al., 2001; Heil et al., 2004, 2004). PRRs are crucially involved in the initiation of the immune response, as they enable quick sensing of pathogenic structures to induce a first

effector response by phagocytes that engulf the pathogen and produce mediators to recruit, activate, and direct other immune cells towards the most efficient type of response for containing and eliminating the respective pathogen. Therefore, the specific recognition of pathogen-derived structures is crucial for the success of the consequent immune response. Millions of years of evolution have shaped the recognition mechanisms of PRRs, which are broad but still highly specific, as highlighted by the efficient recognition of lipopolysaccharide from most terrestrial microbes by TLR4, contrasted by a complete lack of responses to cell wall components of some deep-sea microbes (Gauthier et al., 2021). This specificity showcases how the evolutionary selection of immune receptors shapes the repertoire of germline receptors to produce what could be called evolutionary immune memory (Farber et al., 2016). However, as specific as these receptors might be to their respective ligands, these structures are shared by a wide range of different species and typically not referred to when immune specificity is being discussed.

True antigen-specificity has initially been established by extensive studies on serological reactions early in the 20th century, in particular to protein-coupled haptens, establishing the fine specificity of immunoglobulins as B cell receptors (BCRs) and neutralizing agents (Goebel and Avery, 1929; Klopstock and Selter, 1928; Landsteiner and Lampl, 1918; Landsteiner and van der Scheer, 1931). Until the late 1950s, the prevailing theoretical framework to explain the generation of different antibody specificities was the "instruction theory", as originally proposed by Paul Ehrlich (Lindsten, 1999) and further developed by Felix Haurowitz (Haurowitz, 1960), placing the antigen as a template in the center of complementary antibody formation. It was only after the structural basis of antigen-binding was resolved, revealing that antibodies harbor variable as well as crystallizable constant regions (Edelman, 1959; Porter, 1959), that theoretical work by Nils Jerne, David Talmage, and Sir Frank Macfarlane Burnet shaped the "selection theory". They postulated that diverse antigen receptors are expressed independently of antigen exposure, anticipating selection by specific antigens. Due to the lack of a secretory form, initial difficulties to raise specific antisera, and the biochemical nature of peptides presented on major histocompatibility complex (MHC) as ligands, it took until the early 1980s when several elegant studies on T cell clonotypes established the T cell receptor (TCR) as specific antigen receptor (Acuto et al., 1983; Meuer et al., 1982, 1983a, 1983b; Reinherz et al., 1982), whose genetic transfer was sufficient to transfer antigen- and MHC-specificity (Dembić et al., 1986; Saito et al., 1987). For the clonal strategy to be successful, the vast diversity of constantly evolving pathogens from different phylae needs to be met by a similar diversity of immune receptors. Rather than being germline-encoded, these extensive numbers of unique antigen receptors are generated through somatic recombination (Davis and Bjorkman, 1988; Tonegawa, 1983). In jawed vertebrates, this process is catalyzed by the RAG-1 and RAG-2 enzymes, which cooperate to introduce DNA double-strand

breaks within the genetic blocks encoding the V, D, and J segments of antigen receptors (McBlane et al., 1995; Oettinger et al., 1990) that are then joined by the DNA repair machinery (Gellert, 2002). In addition to this combinatorial diversification, heterogeneous end-joining and random addition of nucleotides by terminal deoxynucleotidyl transferase creates junctional diversity (Gilfillan et al., 1993; Komori et al., 1993), resulting in a theoretical potential of over 10^{11} distinct heterodimeric receptor combinations, which exceeds the number of B cells and T cells in adult vertebrates (Davis and Bjorkman, 1988). As the stochastic nature of VDJ recombination inevitably generates receptors with high potential for self-reactivity or incapable of binding potential ligands with high enough affinity, stringent selection prunes the antigen-receptor repertoires. In the case of T cells, positive and negative selection in the thymus trim the receptor repertoire to an acceptable affinity range (Alam et al., 1996). This clonal deletion is a costly process as less than ~5 % of $CD4^+$ $CD8^+$ thymocytes develop into mature CD4 or CD8 T cells, with the majority being lost due to failure to undergo positive selection (Egerton et al., 1990; Huesmann et al., 1991; Shortman et al., 1991). A small fraction of cells with TCRs at the higher end of affinity towards self-peptides develop into regulatory T cells (Jordan et al., 2001; Lee et al., 2012). Together, this process of receptor diversification followed by selection builds an anticipatory pool of naïve cells with functional antigen receptors, while at the same time it ensures central tolerance by deleting the majority of potentially self-reactive cells and is therefore the fundamental basis of adaptive immune responses by T and B cells.

1.1.2 Stochastic combinatorial expression diversifies the NK cell repertoire

The diversification of receptor repertoires by somatic recombination is a strategy that generates a large pool of adaptive immune cells capable to respond to a variety of different pathogens driven by their antigen receptors. In contrast, Natural Killer (NK) cells rely on various innate receptor families with different expression patterns and degrees of pathogen specificity. The original discovery of their priming-independent "natural" but specific lytic activity against certain tumor cells by Ronald Herberman and Rolf Kiessling (Herberman et al., 1975; Kiessling et al., 1975), together with clinical and experimental observations, established an important role for NK cells in the defense against not only tumors or viral infections, but also allogeneic graft rejection (Biron et al., 1989; Kärre et al., 1986; Murphy et al., 1987). This sparked a whole area of studies on their recognition and control mechanisms; and so a plethora of inhibitory and activating NK cell receptors were identified in the 90s in the laboratories of Alessandro and Lorenzo Moretta (Cantoni et al., 1999; Moretta et al., 1990a, 1993; Pende et al., 1999; Pessino et al., 1998; Wagtmann et al., 1994), David Raulet (Diefenbach et al., 2000; Jamieson et al., 2002; Vance et

al., 1998, 1999), Eric Long (Wagtmann et al., 1995a, 1995b), Wayne Yokoyama (Brown et al., 2001; Idris et al., 1999; Karlhofer and Yokoyama, 1991; Karlhofer et al., 1992; Mehta et al., 2001), Lewis Lanier (D'Andrea et al., 1995; Litwin et al., 1994; Smith et al., 1998) and Andrew McMichael (Braud et al., 1998). Their seminal findings laid the basis for studies on the requirements of NK cell activation, revealing that NK cell responses are regulated by integrating activating and inhibitory receptor signals (Leibson, 1997), thereby enabling sensing of pathological situations while remaining self-tolerant.

Despite their reliance on germline-encoded receptors, the expression of NK cell receptors at variable frequencies, ranging from widespread expression by the whole pool to the limited presence on small subsets, is a different strategy for repertoire diversification (Hammer et al., 2018a). Most NK cells express receptors for cytokines such as Interleukin-15 (IL-15), IL-12, IL-18, or Interferon-α (IFN-α) and are therefore able to crosstalk with myeloid and stromal cells that release these soluble mediators released upon pathogen sensing via PRRs (Biron et al., 1999). Additionally, the low-affinity IgG receptor FcγRIII or CD16 is expressed by a large proportion of NK cells and enables them to recognize opsonized target cells to engage in antibody-dependent cellular cytotoxicity (ADCC) (Cassatella et al., 1989; Lanier et al., 1988). Widespread expression of the prototypical NK cell receptor NKG2D mediates so-called "induced-self responses", recognizing endogenous ligands upregulated by cellular stress (Diefenbach and Raulet, 2001; Diefenbach et al., 2000; Jamieson et al., 2002). Similarly, most NK cells express NKp46 (Pessino et al., 1998) and (in humans) NKp30 (Pende et al., 1999), which among other ligands bind to viral hemaglutinins (Mandelboim et al., 2001), and to the stress-induced B7-H6 (Brandt et al., 2009), respectively. In contrast, other NK cell receptors are expressed only by a fraction of NK cells. Prime examples are the MHC class I-specific receptors of the murine Ly49 and human Killer immunoglobulin-like receptor (KIR) families which are the molecular determinants of the classical "missing-self response" defined by Klas Kärre and Hans Gustaf Ljunggren (Kärre et al., 1986; Ljunggren and Kärre, 1990). In this setting, specific rejection of MHC class I-devoid or mismatched target cells is due to the lack of "self" signals transmitted via inhibitory KIRs (in humans) or Ly49 receptors (in mice) – a model that was inspired by instructions for civil fishermen to avoid false-alarms of Russian submarine sightings at the Swedish shorelines during the cold war. Observations that human leukocyte antigen B (HLA-B) and HLA-C alleles offered differential protection of susceptible targets against lysis by NK cell clones (Cella et al., 1994; Ciccone et al., 1992; Colonna et al., 1993; Mandelboim et al., 1996) were the starting point for the development of specific reagents and cloning of the many different members of the polymorphic KIR family (Colonna and Samaridis, 1995; D'Andrea et al., 1995; Gumperz et al., 1995; Litwin et al., 1994; Moretta et al., 1993; Wagtmann et al., 1995b). KIRs are classified based on their structure that determines

ligand specificity and signaling (Gumperz et al., 1995; Litwin et al., 1994). Intracellularly, inhibitory KIRs have a long (L) cytoplasmic tail containing immune receptor inhibitory tyrosine-based inhibitory motifs (ITIMs) that recruit inhibitory signaling components such as the tyrosine phosphatase SHP-1 (Burshtyn et al., 1996). Activating KIRs instead, have a short (S) and positively charged cytoplasmic tail that facilitates coupling to the activating signaling adaptor DAP-12 (Lanier et al., 1998a; Olcese et al., 1997). Further, their specificity to groups of HLA class I alleles is determined by varying numbers of structurally distinct extracellular immunoglobulin superfamily (IgSF) domains, specified by the first digit in their nomenclature (Long et al., 1996). Prominent examples are the KIR2D receptors that mostly bind different HLA-C alleles (Ciccone et al., 1992; Mandelboim et al., 1996; Moretta et al., 1993), whereas KIR3DL1 recognizes alleles containing HLA-Bw4 motifs. The observation that clonal lines of NK cells had consistent reactivity to targets implied clonal inheritance and mechanisms for limiting the expression to one or few inhibitory KIRs on a given NK cell and its progeny (Cella et al., 1994; Ciccone et al., 1992; Colonna et al., 1993; Mandelboim et al., 1996; Moretta et al., 1990b). Along these lines, studies in mice revealed the probabilistic regulation of the functionally orthologous Ly49 receptors (Held et al., 1996), facilitated by a stochastic switch based on competitive binding of C/EBP and TBP transcription factors (TFs) to bidirectional promoters (Saleh et al., 2004) that results in stable, (predominantly mono-) allelic expression (Held et al., 1995; Tanamachi et al., 2001). Also human KIRs are predominantly transcribed from one allele and expression correlates inversely with DNA methylation at proximal promoter CpG sites (Chan et al., 2003; Santourlidis et al., 2002). The specific presence of antisense transcripts in cells lacking the respective KIRs suggests that their promoters also function bidirectionally (Davies et al., 2007; Li et al., 2008), with evidence of direct involvement of antisense RNAs in gene regulation (Cichocki et al., 2010). Observations that co-expression frequencies deviate from the stochastic product rule imply additional non-random regulation affecting NK cell repertoire composition (Andersson et al., 2009; Sternberg-Simon et al., 2013), including bias by the availability of ligands (Held et al., 1996; Tanamachi et al., 2001). Nevertheless, KIR receptor expression (and potentially that of other NK cell receptors) is regulated by *KIR* rather than *HLA* genotype arguing against a selection mechanism (Andersson et al., 2009; Shilling et al., 2002a).

Overall, stochastic receptor expression is the fundamental mechanism generating a diverse repertoire of NK cells with different abilities to sense pathological situations such as MHC class I downregulation (Figure 1). Although their regulation is less well characterized, combinatorial expression of a range of other receptors than KIRs or Ly49 receptors such as CD94/NKG2C and NKG2A, LILRB1 dissect NK cells into smaller subsets (Horowitz et al., 2013). This epigenetic diversification of the naive NK cell pool is an important parallel to the genetic diversification of

adaptive immune cells – both utilize stochastic combinatorial events to maximize the possibilities for successful pathogen recognition. While the diversity of antigen receptors that can be generated by genetic recombination remains unmatched, the variegated expression patterns of NK cell receptors create a remarkable diversity of up to 30,000 different NK cell phenotypes in humans (Horowitz et al., 2013) – with potentially different responsiveness and combinatorial specificities.

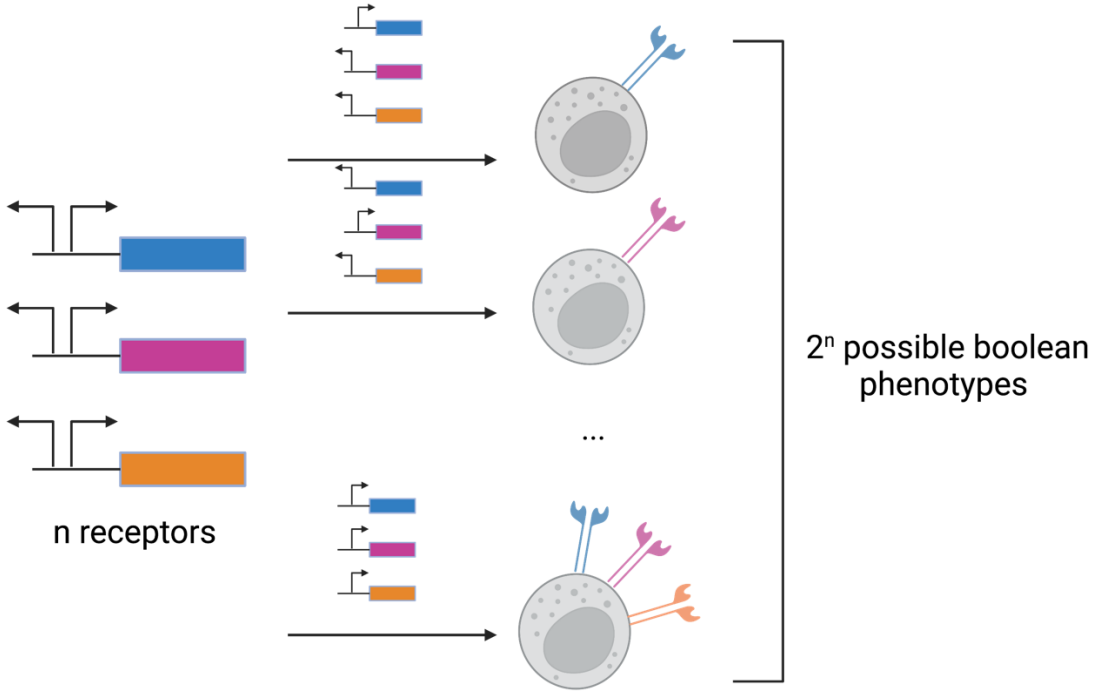

Figure 1 Stochastic diversification of the NK cell repertoire. Bidirectional promoters work as stable stochastic switches for the inhibitory KIR and Ly49 receptors in humans and mice, respectively. Assuming boolean expression, clonal inheritance of the expression states generates an NK cell repertoire with a theoretical combinatorial size of 2^n, where n is the number of different NK cell receptors. Created with BioRender.com.

1.1.3 Licensing maintains NK cell tolerance

Similar to the generation of self-reactive TCRs by genetic recombination, the stochastic expression of KIRs (and murine Ly49 receptors) generates subsets of NK cells that do not express self-MHC-specific inhibitory receptors. Interestingly, these are not deleted from the repertoire as they can be detected both in mice and humans (Anfossi et al., 2006; Fauriat et al., 2008; Fernandez et al., 2005), suggesting alternative mechanisms to maintain self-tolerance. The first indication of such a mechanism came from β2-microglobulin (β2m)-deficient mice that lack MHC class I surface expression and are unable to reject MHC class I negative T cell blasts or transplanted bone marrow (Bix et al., 1991; Höglund et al., 1991). This observation allowed the authors to rationalize decade-old observations of "hybrid resistance" (Cudkowicz and

Stimpfling, 1964) dependent on MHC class I (Ohlén et al., 1989) and mediated by NK cells (Murphy et al., 1987; Sentman et al., 1991). In accordance with these observations, NK cells from MHC class I-deficient mice showed reduced cytotoxic activity against missing-self targets (Liao et al., 1991), a phenotype that was mirrored by patients with genetic deficiencies of the amino acid transporters TAP1/2 involved in HLA peptide loading (Furukawa et al., 1999; de la Salle et al., 1994). A landmark study from Wayne Yokoyama's laboratory using wildtype (WT) and transgenic mouse strains with distinct MHC class I ligands finally demonstrated that the interaction of inhibitory MHC class I receptors, such as Ly49A and Ly49I, with their cognate ligands licenses NK cells to perform efficient effector functions upon activating receptor stimulation (Kim et al., 2005). Shortly after, studies followed which demonstrated that the same principle applies to human NK cells and KIRs (Anfossi et al., 2006; Kim et al., 2008).

Different mechanisms have been proposed to conceptually explain NK cell licensing, with the first opposing concepts most commonly referred to as the *arming* and *disarming* models (Brodin et al., 2009a; Raulet and Vance, 2006). In the arming model, self-MHC specific receptors induce functional maturation of immature NK cells, implying that signals transmitted via inhibitory receptors positively regulate maturation. The disarming model, in contrast, suggests that inhibitory receptors counteract activating signals and thereby oppose chronic stimulation which otherwise results in a hyporesponsive state (Raulet and Vance, 2006). Although still not fully resolved, a number of observations have favored the disarming model. Tolerance of NK cells against MHC class I-deficient target cells in a mixed bone marrow chimera setting consisting of WT and β2m-deficient cells pointed towards an actively tolerogenic process, in which low frequencies of MHC class I-deficient cells are sufficient to induce hyporesponsiveness (Johansson et al., 1997). This was supported by similarly tolerant NK cells from mice with mosaic expression of an MHC class I transgenes (Wu and Raulet, 1997). Further, transfer experiments demonstrated the reversibility and continuity of NK cell licensing, as licensed NK cells lost their ability to respond when transferred into MHC class I-deficient mice. Conversely, previously unlicensed NK cells from β2m-deficient mice gained responsiveness in WT mice, suggesting the hyporesponsiveness of uneducated NK cells is more easily reversible than T or B cell anergy (Joncker et al., 2010). Moreover, transgenic overexpression of ligands for the activating receptors Ly49H or NKG2D selectively pushed NK cells into hyporesponsiveness, showcasing how chronic activating signals can induce a state similar to unlicensed NK cells (Oppenheim et al., 2005; Sun and Lanier, 2008a; Tripathy et al., 2008; Wiemann et al., 2005). Similarly, human KIR2DS1^{+} NK cells are hyporesponsive in the presence of the endogenous HLA-C2 ligand (Fauriat et al., 2010a). Rather than being a digital on/off effect, the *rheostat* model proposes that NK cell li-

censing tunes functionality in a quantitative fashion (Brodin et al., 2009a). This concept is supported by a quantitative increase of NK cell responsiveness proportional to the number of available educating MHC class I ligands and the number of inhibitory receptors expressed by populations of NK cells (Brodin et al., 2009b; Joncker et al., 2009). Similarly, the correlation of inhibitory receptor-ligand affinity with NK cell function supports a quantitative model in which licensing is analog until a maximal threshold is reached (Jonsson et al., 2010).

Overall, NK cell licensing is an important control mechanism required to maintain self-tolerance of the diversified NK cell repertoire. In contrast to the clonal selection of the adaptive immune system, NK cell licensing is a continuous and reversible process, as potentially self-reactive NK cells are not deleted from the repertoire. Besides the many parallels of T and NK cells, this is a fundamental difference that likely reflects their starkly different receptor biology; even NK cells that lack inhibition via self-MHC rely on various defined receptors with conserved ligands to sense danger compared to the unpredictable specificity of rearranged antigen receptors. Therefore, it is tempting to speculate that NK cells do not pose as much of a self-reactivity threat as T cells. Hence, licensing might have evolved as a more economical tolerance strategy compared to clonal deletion. That unlicensed NK cells can still serve a crucial function is exemplified by immune responses against Cytomegalovirus (CMV) infection, where they escape hyporesponsiveness under the influence of pro-inflammatory cytokines (Orr et al., 2010; Sun and Lanier, 2008b).

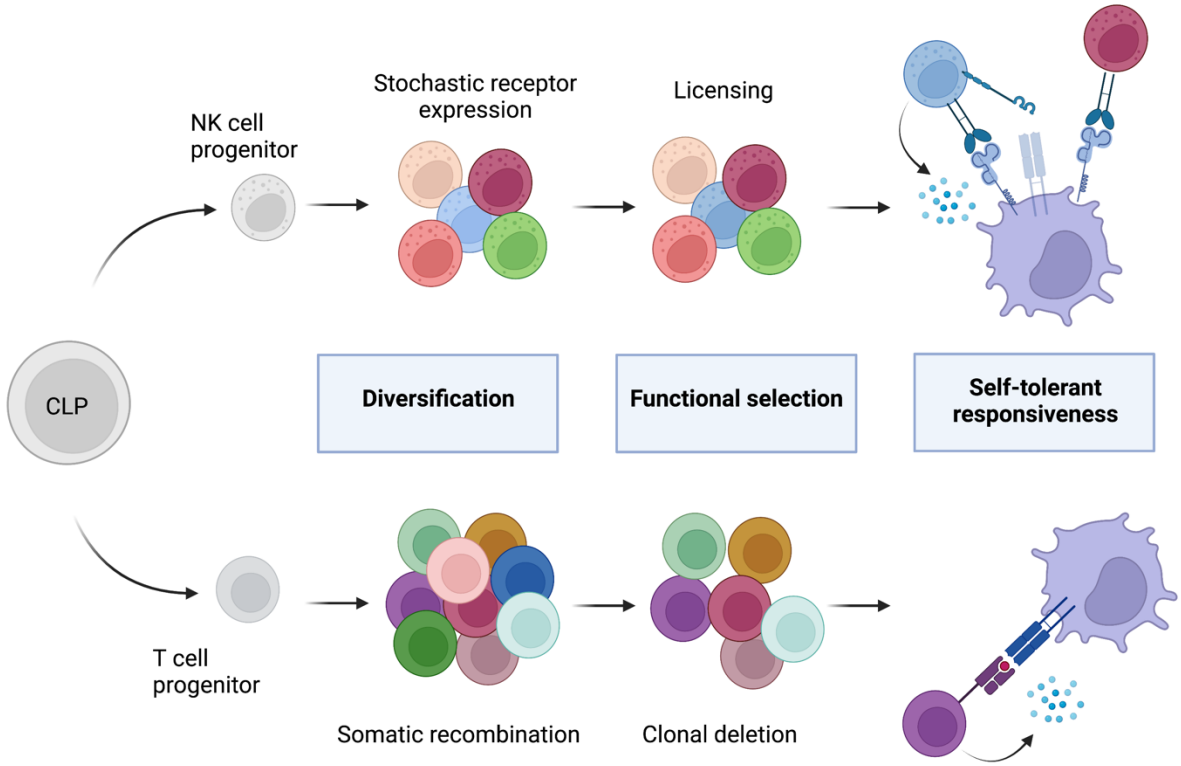

Figure 2 NK and T cell repertoires are shaped by different means. NK and T cells develop from a common lymphoid progenitor (CLP). They diversify their receptor repertoires by different mechanisms, namely stochastic expression of germline-encoded genes by NK cells, in contrast to somatic recombination utilizing RAG enzymes to generate unique TCRs. Both cell types undergo functional selection, with the important difference that T cells which do not match selection criteria die by apoptosis, whereas unlicensed NK cells remain in a hypofunctional state from which they can escape under inflammatory conditions. Both processes generate a self-tolerant repertoire of cells with anticipatory specificities. Created with BioRender.com.

1.1.4 Virus-specific NK cell receptors

An interesting aspect of some of the receptors expressed only by subsets of NK cells is their specificity towards viral ligands. The most prominent examples of specific pathogen recognition by NK cells have been described for CMV, which belongs to the herpesvirus subfamily of *Betaherpesvirinae* and utilizes a multitude of different immune evasion strategies to establish lifelong latent infection (Picarda and Benedict, 2018). Differential susceptibility of various laboratory mouse strains to murine CMV (MCMV) infection was mapped to the genetic locus *Cmv1* which contains the gene *Klra8* encoding the activating receptor Ly49H (Brown et al., 2001; Chalmer et al., 1977; Lee et al., 2001; Scalzo et al., 1990, 1995). Direct and specific recognition of the specific MCMV ligand m157 by Ly49H induces NK cell cytotoxicity and cytokine release (Arase et al., 2002; Smith et al., 2002). Therefore, the mechanism of MCMV resistance of mouse strains such as C57BL/6 was established to be mediated by Ly49H$^+$ NK cells, which are lacking

in Ly49H-deficient strains, such as BALB/c (Bubić et al., 2004; Daniels et al., 2001; Dokun et al., 2001). While the interaction between Ly49H and m157 is the most closely studied example, other receptors, namely Ly49P, Ly49D2, Ly49P1, and Ly49L from MA/My, PWK/Pas, NOD/Ltj, and BALB.K mice, respectively, are able to induce similar responses by recognizing the viral m04 protein (Desrosiers et al., 2005; Kielczewska et al., 2009; Pyzik et al., 2011). Also the NKR-P1B receptor family, containing the prototypical NK1.1 epitope in C57/BL6 mice, recognizes an MCMV ligand encoded by the *m12* gene (Aguilar et al., 2017). As all three of these ligands, m157, m04 and m12, engage also inhibitory receptors, such as Ly49I in 129/SvJ mice (Arase et al., 2002), Ly49A in BALB/c (Babić et al., 2010), and inhibitory isoforms of NK1.1 in various strains (Aguilar et al., 2017), it seems likely that these ligands initially evolved as immune evasion factors to which activating receptors developed as counter-mechanisms. Conversely, the activating receptors exert immune pressure onto the viral ligands, manifesting in a high degree of m157 polymorphisms. Structural variants from wild MCMV strains differ in their binding to Ly49 receptors, including complete lack of Ly49H activation, a phenotype that can be recapitulated by repeated viral passage in Ly49H-sufficient mice (Berry et al., 2013; Corbett et al., 2011; French et al., 2004; Voigt et al., 2003). Similarly, both *m04* as well as its viral co-factor are highly polymorphic (Železnjak et al., 2019). Moreover, both sides of the interaction between *m12* and the *NKR-P1* receptor family, exhibit a remarkable degree of functional polymorphisms (Aguilar et al., 2017). Together, all these examples illustrate the evolutionary arms race in which viral strategies to subvert immune recognition via ligands for inhibitory receptors are repeatedly followed by host re-utilization and adaptation of their recognition domains in activating receptors, rewiring their signaling to pre-existing activation pathways. Immunogenetic studies on the KIR and Ly49 families have closely described this process, highlighting the dynamic evolution of immune receptors and their ligands (Abi-Rached and Parham, 2005).

One of the consequences of this dynamic co-evolution is that viral descendants in different species employ different strategies for immune evasion, which are then again matched by different countermeasures. A prime example is the specific targeting of the inhibitory C-type lectin NK cell receptor CD94/NKG2A by HCMV. The endogenous ligand of CD94/NKG2A is the non-classical MHC class I molecule HLA-E, which in healthy cells presents mainly peptides derived from the leader sequences of classical MHC class I, thereby enabling NK cells to survey intact self-presentation (Braud et al., 1998; Brooks et al., 1999; Lee et al., 1998). Importantly, HCMV interferes with antigen-presentation via various proteins such as US11 or US2 that mediate MHC class I translocation and degradation to prevent detection by CD8 T cells (Berger et al., 2000; Wiertz et al., 1996a, 1996b). In this context, the HCMV gpUL40 provides an HLA-E-stabilizing peptide to preserve inhibition via NKG2A, thereby avoiding NK cell recognition (Cerboni et al., 2001;

Tomasec et al., 2000; Ulbrecht et al., 2000; Wang et al., 2002). However, HLA-E is also recognized by the activating-receptor CD94/NKG2C that signals via DAP12 to induce NK cell cytotoxicity and cytokine production. Similar to the MCMV ligands, the *UL40* gene is highly polymorphic, especially the region encoding the HLA-E-stabilizing peptide represents a mutational hotspot (Garrigue et al., 2007, 2008; Hammer et al., 2018b; Heatley et al., 2013). Consequently, we could show that differences in peptide affinity to NKG2C (Heatley et al., 2013) translate into vastly different responses of NKG2C$^+$ NK cells, inducing differential cytokine production, cytotoxicity, and proliferation (Hammer et al., 2018b). This peptide-specific recognition of HCMV is much more striking for NKG2C than for NKG2A (Hammer et al., 2018b). This effect is presumably due to an overall around 6-fold reduced affinity of NKG2C towards HLA-E (Heatley et al., 2013; Valés-Gómez et al., 1999), sensitizing the activating receptor complex to differences in the peptide ligand. Intriguingly, strongly activating peptides, as well as inactive peptides not able to engage neither NKG2C nor NKG2A appear only at low frequencies in clinical HCMV isolates (Hammer et al., 2018b). Instead, most HCMV isolates encode for peptides that efficiently engage the inhibitory NKG2A while inducing low or intermediate activation via NKG2C (Hammer et al., 2018b), suggesting balancing selection by both receptors might have shaped the gpUL40 peptide repertoire (Hammer et al., 2018a). Apart from NKG2C, activating KIRs have been discussed to be involved in the specific response against HCMV (Béziat et al., 2013; Della Chiesa et al., 2014; Liu et al., 2016) and there is evidence for recognition of HCMV infected fibroblasts by KIR2DS1 in the context of HLA-C2 (van der Ploeg et al., 2017).

Besides HCMV, specific recognition via activating KIRs has been observed in the context of various other pathogens such as human immunodeficiency virus (HIV) (Chapel et al., 2017; O'Connor et al., 2011, 2015) or flaviviruses including hepatitis C virus (HCV) (Naiyer et al., 2017). In these cases, pathogen-derived peptides mediate activating KIR engagement, suggesting this might also be the case for the recognition of HCMV via KIR2DS1, although the relevant peptide remains to be identified. Furthermore, binding of inhibitory KIRs can be modulated by pathogen-derived peptides, as described for HCV where the loss of inhibition via KIR2DL3 (Lunemann et al., 2016) might be part of the explanation for its genetic association with clinical outcome (Khakoo et al., 2004). Similarly, various HIV-derived peptides have been demonstrated to impact self-recognition by KIR2DL2 and KIR2DL3 (Alter et al., 2011; Fadda et al., 2012; van Teijlingen et al., 2014), with correlative evidence for selection pressure on viral sequences favoring inhibitory variants (Alter et al., 2011). This peptide specificity of activating, inhibitory KIRs and CD94/NKG2 receptor complexes highlights that self-surveillance is much more subtly regulated than by the mere presence of MHC class I – a striking resemblance with peptide scanning by TCRs.

Finally, the remarkable genetic diversity of NK cell receptors on a population level, especially of Ly49 and KIRs, is an important aspect of their biology. Variable gene content at the KIR locus constitutes different haplotypes (Shilling et al., 2002b; Uhrberg et al., 1997), and due to additional allelic polymorphisms, the KIR family ranks among the most polymorphic gene families, next to the HLA genes (Gonzalez-Galarza et al., 2020). The population-level diversity of these molecules so centrally involved in immune recognition suggests balancing selection maintains this variety, conferring the population with a breadth of different recognition repertoires across individuals that ensures the survival of the species as a whole (Single et al., 2007).

Overall, the specific recognition of viral ligands via diversified receptor families is a defining feature of NK cell responses across vertebrate species, which clearly distinguishes them from the broad responses launched via PRRs on myeloid cells. Their variegated expression patterns define NK cell subsets with specific ability to respond to different pathogens. This hard wiring of specificities has important consequences for NK cell effector responses and memory formation.

1.2 Memory formation in innate and adaptive effector responses

1.2.1 Innate lymphocyte effector programs are developmentally hardwired

In addition to their fundamentally different recognition strategies, the developmental polarization of NK cells towards type I immunity clearly distinguishes them from adaptive lymphocytes. Expression of the hallmark TFs T-bet and Eomes is induced during NK development and enables them to express the effector cytokine IFN-γ without prior priming (Gordon et al., 2012; Townsend et al., 2004). Mirroring T helper (T_H) cell subsets, other members of the innate lymphoid cell (ILC) family are programmed to develop into type II cytokine-producing ILC2 expressing high levels of GATA3 (Fallon et al., 2006; Moro et al., 2010; Neill et al., 2010), and type III cytokine-producing ILC3 under the control of RORγt (Eberl et al., 2004; Luci et al., 2009; Sanos et al., 2009; Satoh-Takayama et al., 2008; Vonarbourg et al., 2010). While plasticity of ILC subsets has been described, such as ILC3 converting towards type I immunity (Klose et al., 2013; Vonarbourg et al., 2010), this developmental imprinting of effector polarization stands in stark contrast to naïve T and B cells which require instruction from other immune cells to differentiate into functional and polarized subsets. As a result, ILCs and NK cells respond much more swiftly with the production of chemokines, effector cytokines, or cytotoxic responses and therefore serve important functions in the early defense and instruction of the mounting adaptive immune response (Stehle et al., 2018).

While their overall lineage polarization is developmentally determined, NK cells undergo homeostatic differentiation, which in addition to variable receptor expression diversifies their phenotypes and functional properties. In mice, NK cells mature from CD11b^{-} CD27^{+} over a double-positive stage to mature CD11b^{+} CD27^{-} NK cells, with KLRG1 additionally marking terminal maturation (Chiossone et al., 2009; Hayakawa and Smyth, 2006; Huntington et al., 2007a; Kim et al., 2002). Human NK cells gain maturity along a spectrum from CD56^{bright} NK cells over CD56^{dim} CD62L^{+} to mature cytotoxic CD56^{dim} CD16^{+} NK, and expression of CD57 is associated to further functional maturation (Abo et al., 1984; Björkström et al., 2010; Chan et al., 2007; Cooper et al., 2001; Juelke et al., 2010; Lanier et al., 1986; Lopez-Vergès et al., 2010; Romagnani et al., 2007). Importantly, maturation of NK cells is accompanied by changes in responsiveness to different stimuli, manifesting in pronounced cytokine-production by CD56^{bright} NK cells in response to pro-inflammatory cues such as IL-12 and IL-18, as well as enhanced proliferative capacity (Cooper et al., 2001; Juelke et al., 2010; Romagnani et al., 2007). Conversely, CD56^{dim} NK cells are more cytotoxic, respond more efficiently to activating receptor stimulation, participate in ADCC via CD16, and are the major subset involved in the missing-self response due to their expression of KIRs (Fauriat et al., 2010b; Lanier et al., 1986; Luetke-Eversloh et al., 2014a; Nagler et al., 1989). Maturation is supported by homeostatic signals such as IL-15 (Lee et al., 2011), which regulates proliferation and survival (Kennedy et al., 2000; Lodolce et al., 1998; Vosshenrich et al., 2005).

Different transcriptional and epigenetic networks shape the identities and functions of these innate lymphocyte subsets. Initial studies in mice have mainly focused on the differential activity of the master TFs Eomes and T-bet, with the early stages being especially dependent on Eomes, and T-bet supporting NK cell identity at the later stages (Gordon et al., 2012; Jenne et al., 2009; Wagner et al., 2020; Zhang et al., 2021). Recent work on humans has started to shed light on the more complex underlying networks, placing RUNX2, BACH2, and TCF7 at the center of the CD56^{bright} NK cell identity whereas PRDM1 (also known as BLIMP-1), ZEB2, ZBTB16 (also known as PLZF), and BCL11B are among the central factors shaping the epigenetic and transcriptional landscape of the mature CD56^{dim} subset (Collins et al., 2019; Holmes et al., 2021). Importantly, global subset distinctions seem to be mirrored between humans and mice, supporting epigenetic and transcriptional concordance between both species (Crinier et al., 2018). This also underlines the cross-species relevance of loss-of-function studies that demonstrate important effects of TFs such as TCF1 (encoded by *Tcf7*) (Jeevan-Raj et al., 2017), PRDM1 (Kallies et al., 2011) and ZEB2 (van Helden et al., 2015) on NK cell maturation. Intriguingly, functional distinctions of NK cell subsets as well as many of the underlying molecular regulators conferring these features are reminiscent of the division of labor between central and effector memory T cell subsets. Along

these lines, TCF1 is considered to be one of the main players involved in the self-renewal capacity of central memory T cells (Jeannet et al., 2010; Pais Ferreira et al., 2020; Zhao et al., 2010), whereas factors like PRDM1 or ZEB2 have been associated to terminally mature, and to short-lived effector and effector memory phenotypes (Dominguez et al., 2015; Ji et al., 2011; Kallies et al., 2009; Martins et al., 2006; Omilusik et al., 2015; Rutishauser et al., 2009; Shin et al., 2009). These parallels reveal the functional conservation of molecular programs between NK and T cells and raise important questions about how and when decisions for these alternative fates are made, how they contribute to effector and memory responses, and how they are maintained.

1.2.2 Specific NK cell memory induced by viral ligands

Despite their maturity at steady state, endowing innate lymphocytes with the ability for immediate responses, innate lymphocytes can be functionally tuned and long-term imprinted by pathogen exposure. Pathogen-specific recognition via receptors expressed only by subsets of cells translates this imprinting into the formation of NK cell memory for at least one specific pathogen, namely CMV. Specific recognition of the viral ligand m157 leads to the preferential activation and proliferation of Ly49H$^+$ NK cells, expanding their frequencies and absolute numbers during acute MCMV infection (Daniels et al., 2001; Dokun et al., 2001). Elegant studies transferring low numbers of congenically marked Ly49H$^+$ NK cells into DAP-12-deficient hosts demonstrated the robustness of their expansion from a relatively small pool of naïve progenitors. After control of the acute infection, a small but significant pool of memory cells persists with a terminally mature phenotype marked for example by expression of KLRG1 and Ly6C (Figure 3) (Sun et al., 2009a). Ly49H$^+$ memory (or also referred to as “adaptive”) NK cells are more readily activated and produce higher levels of IFN-γ upon stimulation via activating receptors, while being less responsive to bystander activation via pro-inflammatory cytokines or heterologous infection with *Listeria monocytogenes*, underlining their functional remodeling (Min-Oo and Lanier, 2014; Nabekura and Lanier, 2016; Sun et al., 2009a). Upon transfer, memory NK cells provide enhanced protection against MCMV infection, likely driven by their more pronounced effector functions, as their expansion kinetics are comparable to naïve Ly49H$^+$ NK cells (Sun et al., 2009a). Although not studied in as much depth, increased frequencies of Ly49L$^+$ NK cells after MCMV infection of BALB.K and their ability to provide protection upon transfer into neonates suggests that also receptors for m04 can induce a similar memory response (Pyzik et al., 2011). A different type of heightened responsiveness to pro-inflammatory cytokines can be induced in hepatic ILC1 by MCMV infection via m12 recognition by NK1.1 (Weizman et al., 2019).

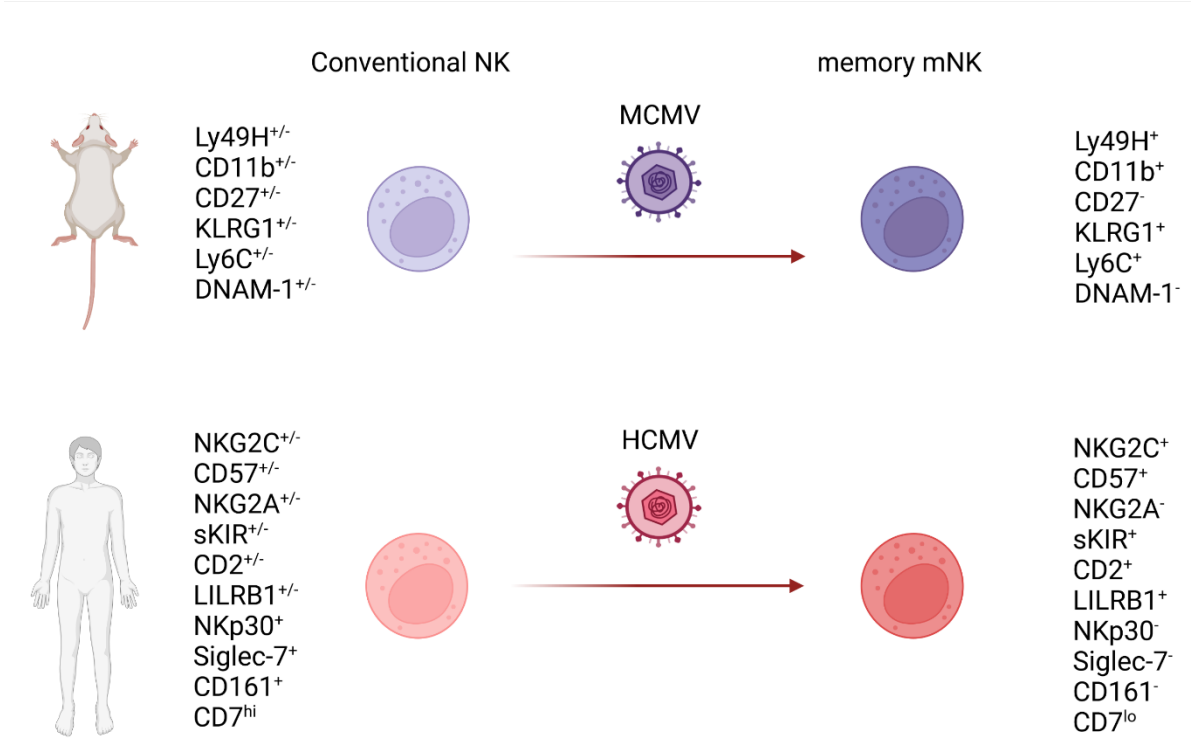

Figure 3 Memory NK cell phenotypes induced by CMV in mice and humans. Conventional NK cells have a more heterogeneous phenotype, whereas CMV-induced memory NK cells are marked by characteristic features. Adapted from (Hammer and Romagnani, 2017) created with BioRender.com

Similarly, expansions of NGK2C^{+} NK cells are specifically observed in HCMV-seropositive (HCMV^{+}) human individuals (Gumá et al., 2004) and persist long-term after acute infection of patients undergoing hematopoietic stem cell transplantation (Foley et al., 2012; Lopez-Vergès et al., 2011). Specific recognition of peptides derived from the HCMV gpUL40 protein via NKG2C expands NKG2C^{+} NK cells in an affinity-dependent manner (Hammer et al., 2018b). Accordingly, expansion of NKG2C^{+} NK cells is specific to HCMV, as effects initially attributed to other viral infections were always dependent on HCMV-seropositivity (Béziat et al., 2012; Björkström et al., 2011; Gumá et al., 2006; Hendricks et al., 2014). Phenotypically, human adaptive NK cells are terminally mature CD56^{dim} and mostly CD57^{+}, predominantly expressing self-MHC-specific KIRs (sKIR) (Figure 3). They lack expression of NKG2A, NKp30 or SIGLEC7, and typically express higher levels of CD2 and LILRB1 (Béziat et al., 2012, 2013; Gumá et al., 2004; Schlums et al., 2015; Zhang et al., 2013). Variable downregulation of the signaling adaptors FCER1G, SYK and EAT-2 is another distinguishing feature of adaptive NK cells (Schlums et al., 2015). Loss of expression of ZBTB16 and higher levels of BCL11B are considered to be among the major transcriptional features defining the adaptive NK cell identity (Holmes et al., 2021; Schlums et al., 2015). BCL11B was suggested to give them a certain degree of *"T-cellness"*, as exemplified by their variable expression of intracellular CD3 subunits or CD5 (Holmes et al., 2021).

It should be noted that apart from NKG2C, alternative recognition mechanisms have been considered to drive adaptive NK cell expansions. Similar adaptive NK cell responses in individuals with a homozygous deletion of *KLRC2* (the gene encoding NKG2C) suggest a potential involvement of activating KIRs (Béziat et al., 2013; Della Chiesa et al., 2014). However, no clear differences in the activating KIR profile of adaptive NK cells in relation to NKG2C genotype could be observed (Liu et al., 2016) and no specific ligand has been identified so far. Also activation of CD16 by anti-HCMV antibodies was discussed based on *in vitro* cultures of NK cells with HCMV-infected target cells in the presence of plasma from HCMV$^+$ individuals (Lee et al., 2015). Despite potential redundancies or alternative recognition mechanisms, the predominant expression of NKG2C on adaptive NK cells and its potent function in driving NK cell activation and expansion have established its central role in the adaptive NK cell response.

Importantly, NKG2C$^+$ adaptive NK cells are potent effectors as demethylation of the *IFNG* conserved non-coding sequence 1 (CNS1) enhancer region allows them to produce increased levels of IFN-γ, and also their production of TNF upon activating receptor stimulation is increased (Luetke-Eversloh et al., 2014b; Schlums et al., 2015). Conversely, downregulation of receptor components for cytokines such as IL-12 reduces adaptive NK cell responsiveness to these stimuli, which together with the lack or relative downregulation of natural cytotoxicity NKp30 and NKp46 achieves functional specialization of adaptive NK cells (Hammer et al., 2018c; Schlums et al., 2015).

Overall, CMV-specific NK cell responses are reminiscent of classical adaptive immunity in various aspects – the expansion and persistence of antigen-specific immune cells accompanied by functional maturation and their protection against re-infection upon adoptive transfer are all characteristic features of adaptive immune memory. A striking difference is that adaptive NK cells become more focused on antigen-recognition and lose their ability to pro-inflammatory cytokines, whereas T cells gain competence to respond to inflammatory cues after primary activation. Nevertheless, the similarities between adaptive NK cell responses and those of "truly adaptive" lymphocytes, especially T cells, go beyond macroscopic response patterns and extend to molecular aspects such as the signaling pathways and consequent epigenetic remodeling, allowing to draw important mechanistic parallels for the induction of different types of immune memory.

1.2.3 Cooperative signals induce immune activation and memory formation

The signal requirements for the induction of an efficient virus-specific response in NK and T cells are highly comparable in both cell types. It is well established that CD8 T cells require three signals to undergo effector differentiation and memory formation: Signal 1 is received via TCR recognition of a peptide-MHC complex, co-stimulation through receptors such as CD28 provides Signal 2, and Signal 3 refers to pro-inflammatory cytokines such as IL-12 or type I IFN which further enhance the response (Williams and Bevan, 2007). Similarly, adaptive NK cell responses are initiated by a comparable combination of signals (Figure 4). **Signal 1** is received via Ly49H or NKG2C, both of which associate with the signaling adaptor DAP12 (Lanier et al., 1998b; Smith et al., 1998), with DAP10 being additionally required for optimal signaling in mice (Orr et al., 2009). Upon receptor activation, phosphorylation of tyrosine residues within DAP12 immune receptor tyrosine-based activation motifs (ITAMs) by SRC family kinases results in the recruitment of signaling kinases such as SYK and ZAP70 to induce a cascade of events akin to TCR signaling (Lanier et al., 1998a). Transmembrane and cytoplasmic adaptors such as LAT and SLP-76 further propagate these signals resulting in PLCγ1 activation, which produces the secondary messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Downstream, several signaling mediators such as mitogen-activated protein (MAP) kinases, protein kinase c (PKC) and Calcineurin via IP_3-induced store-operated calcium entry. Besides, the Vav1-Rac pathway is central for the reorganization of the cytoskeleton (Billadeau et al., 1998; Caraux et al., 2006; Gaud et al., 2018; Vivier et al., 2004). Consequently, the first line of transcriptional and epigenetic outcomes of these signaling events are mainly induced by the activation and nuclear translocation of the key TFs downstream of these signaling pathways, namely NFAT, NFkB and AP1 (Lau et al., 2022).

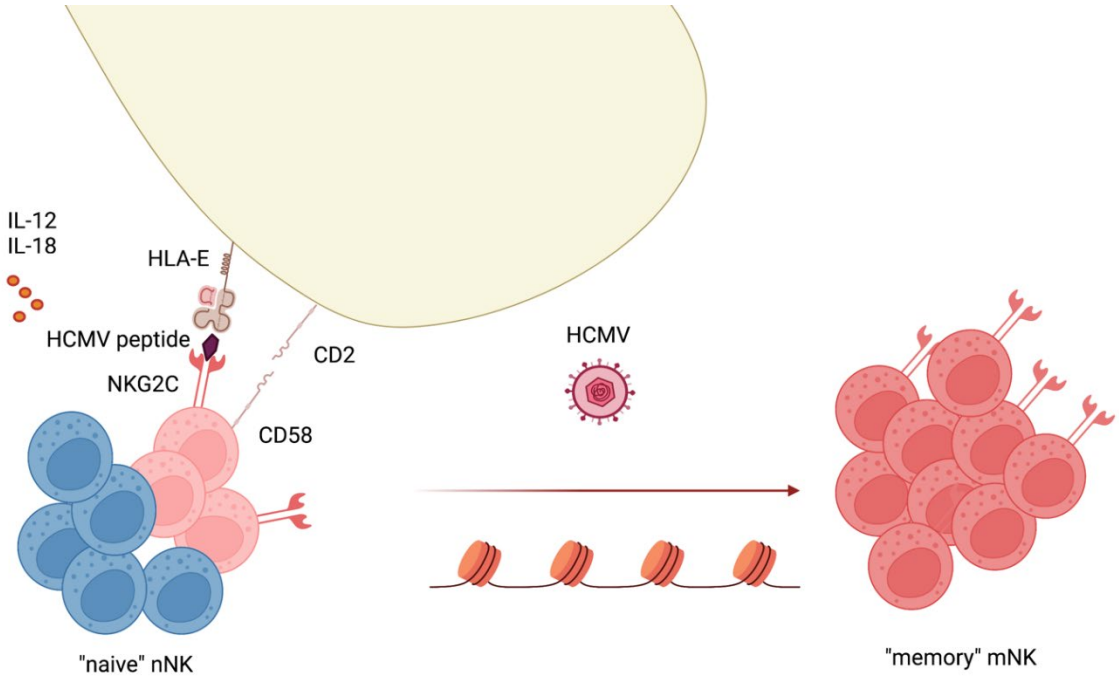

Figure 4 Cooperative signals induce NK cell memory. The combination of peptide recognition via NKG2C (Signal 1), co-stimulation via CD2 (Signal 2) and pro-inflammatory cytokines (Signal 3) induces the specific expansion, epigenetic remodeling, and differentiation of naïve into memory NKG2C$^+$ NK cells. Created with BioRender.com.

Co-stimulation via different receptors as **Signal 2** enhances and complements the events induced by antigen-recognition. For example, the Ly49H$^+$ NK cell response is especially dependent on signaling through DNAM-1 in parallel. In this context, its ligands CD155 and CD112 are rapidly upregulated on dendritic cells (DCs) and macrophages during MCMV infection, overall similarly contributing to the recognition of HCMV-infected DCs by human NK cells (Magri et al., 2011; Nabekura et al., 2014). While acute IFN-γ production is equally efficient in the absence of DNAM-1, expansion and memory formation are severely diminished, translating into impaired viral control in primary and especially secondary responses after adoptive transfer (Nabekura et al., 2014). Rescue experiments with DNAM-1 variants lacking recruitment domains for the signaling mediators Fyn and PKC demonstrated an absolute requirement for PKC activation during the expansion phase. Instead, lack of Fyn signaling manifested only in reduced memory formation. Paradoxically, DNAM-1 expression is progressively lost by Ly49H$^+$ NK cells during MCMV infection in an m157-dependent manner, resulting in a mostly DNAM-1$^-$ memory compartment. Since DNAM-1 expression is dynamically regulated, it remains unclear whether the predominance of DNAM-1$^-$ cells in the memory phase is due to changes in expression or preferential survival, although increased apoptosis of DNAM-1$^+$ cells at days 3 and 6 post-infection supports the latter hypothesis (Nabekura et al., 2014). Interestingly, DNAM-1 levels are higher

on educated NK cells expressing self-MHC-specific KIRs or Ly49s, suggesting inhibitory signaling maintains DNAM-1 surface expression, thereby enhancing co-stimulation (Anfossi et al., 2006; Enqvist et al., 2015; Wagner et al., 2017a). Besides DNAM-1, CD2 is another important co-stimulatory receptor that has been closely studied on human NK cells, where its high expression is one of the defining features of adaptive NK cells, including the adaptive NK cell pool NKG2C-deficient individuals (Liu et al., 2016). Accordingly, we could show that engagement of CD2 reinforces effector functions when combined with activation via NKG2C and is crucial for the expansion of NKG2C$^+$ NK cells *in vitro*, especially in response to low-affinity peptides (Hammer et al., 2018b). These effects are likely mediated by enhanced MAP kinase and PI3K/AKT/mTORC signaling (Liu et al., 2016). The importance of these different co-stimulatory signals for NK cell responses is supported by counteracting immune evasive strategies evolved by CMV. DNAM-1 is targeted by the MCMV and HCMV immune evasion proteins m20.1 and UL141, respectively (Lenac Rovis et al., 2016; Tomasec et al., 2005), whereas the HCMV protein UL148 reduces surface expression of the CD2 ligand CD58 to escape recognition by NK and T cells (Wang et al., 2018b). Interestingly, also NKG2D is in principle able to support Ly49H$^+$ NK cell expansion, but MCMV suppresses the upregulation of its ligands efficiently enough that this effect is only detectable for MCMV strains in which the relevant immune evasion gene *m152* has been deleted (Nabekura et al., 2017).

Finally, pro-inflammatory cytokines constitute **Signal 3** and are crucially required for the activation, expansion, and differentiation of memory NK cells. Especially IL-12 and IL-18 serve an important role in the early phases during the response (Orange and Biron, 1996). While NK cells lacking the IL-12 receptor develop normally and efficiently kill MHC class I-deficient or m157-expressing target cells, they fail to expand and do not form a sizeable Ly49H$^+$ memory population in response to MCMV infection (Sun et al., 2012). Consistent with IL-12 activation of STAT4, the effect of IL-12 receptor deficiency is largely phenocopied by NK cells from *Stat4*$^{-/-}$ mice. Similarly, IL-18 receptor-deficient NK cells have a competitive disadvantage in mixed bone marrow chimeras infected with MCMV, while their proliferation in response to supraphysiological levels of homeostatic cytokines as encountered upon transfer into immunodeficient *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ hosts is unaltered, arguing against an intrinsic proliferative defect (Madera and Sun, 2015). Neither survival nor their activity during a recall response is affected by IL-18 receptor deficiency, suggesting a stage-specific requirement for IL-18 during the primary response, in contrast to the complete inability of IL-12 receptor-deficient NK cells to form memory (Madera and Sun, 2015; Sun et al., 2012). As expected, deficiency of the main signaling adaptor MyD88, which mainly induces NFκB and AP1 activity (Adachi et al., 1998), recapitulated the effects of IL-

18 receptor deficiency (Madera and Sun, 2015), although this might also be partially due to deficiency in IL-33 signaling (Nabekura et al., 2015). A secondary effect has been attributed to type I IFN. Despite equal proliferation of NK cells from *Ifnar1*$^{-/-}$ mice, increased apoptosis severely stunts their ability to expand in numbers, with evidence for increased upregulation of NKG2D-ligands on *Ifnar1*$^{-/-}$ NK cells leading to killing by other NK cells (Madera et al., 2016). Deficiency of STAT1 phenocopies this effect, consistent with the role of STAT1 homo- and STAT1/STAT2 heterodimers in type I IFN signaling (Li et al., 1996). As also the other ISGF3 complex components STAT2 and IRF9 are independently required, canonical type I IFN signaling seems to be the basis of an auto-regulatory feedforward loop to support NK cell survival (Geary et al., 2018).

The importance of cytokines for functional remodeling and expansion of memory NK cells is further underlined by their ability to recapitulate some of memory-associated effects by themselves, without the engagement of Ly49H or NKG2C. Activation of NK cells with IL-12, IL-18, and IL-15 enhances IFN-γ production upon re-activation even weeks after the initial stimulus and is propagated across several rounds of proliferation, both in mice and humans (Cooper et al., 2009; Ni et al., 2016; Romee et al., 2012). Demethylation of the *IFNG* CNS1 region is one of the likely mechanisms driving the enhanced production of IFN-γ by cytokine-induced memory-like (CIML) NK cells, similar to what has been observed in HCMV-induced memory NK cells *ex vivo* (Luetke-Eversloh et al., 2014b; Ni et al., 2016). The relevance of cytokine-imprinting for *in vivo* infections has been demonstrated in an inducible fate-mapping model where the induction of Ly49H^{-} memory NK cells persisting after infection with MCMV is dependent on IL-12. These cells exhibit enhanced effector functions in response to activating receptor stimulation and express markers of terminal maturation such as KLRG1 and Ly6C at higher frequencies than naïve cells (Nabekura and Lanier, 2016). Nevertheless, Ly49H^{+} cells expand more efficiently than their Ly49H^{-} counterparts, accompanied by more pronounced phenotypic maturation and functional remodeling, revealing activating receptor signaling is required for full differentiation of memory NK cells during MCMV infection (Nabekura and Lanier, 2016). Accordingly, CNS1 demethylation is more consistent upon combined stimulation by pro-inflammatory cytokines and peptides with high affinity towards NKG2C compared to cytokines alone (Hammer et al., 2018b). That the differences between CMV-induced and CIML NK cells are not only of quantitative nature is illustrated by the high responsiveness of human CIML NK cells to pro-inflammatory cytokines (Cooper et al., 2009; Wagner et al., 2017b), in contrast to the more antigen-focused effector functions of CMV-induced memory NK cells (Hammer et al., 2018c; Min-Oo and Lanier, 2014; Nabekura and Lanier, 2016; Schlums et al., 2015). Also, phenotypically CIML NK cells do not acquire the characteristic changes induced by HCMV (Romee et al., 2012; Wagner et al., 2017b).

These qualitative differences underline that cytokine activation alone is not sufficient to fully recapitulate HCMV-induced NK cell memory.

Besides these activating signals received during infection, NK cells remain dependent on continuous supply of homeostatic cytokines. The common γ-chain cytokine IL-15 is the most important homeostatic signal for NK cells throughout their lifecycle, hence *IL-15*$^{-/-}$ and *IL-15-Ra*$^{-/-}$ mice are almost completely devoid of NK cells due to impaired homeostatic proliferation and survival (Cooper et al., 2002; Huntington et al., 2007b, 2009; Kennedy et al., 2000; Lodolce et al., 1998). However, this strict dependency on IL-15 can be overcome during MCMV infection, as pro-inflammatory signals compensate for the complete lack of γ-chain cytokine signaling in *Rag2*$^{-/-}$ × *Il2rg*$^{-/-}$ mice, inducing pronounced NK cell expansion (Sun et al., 2009b). Nevertheless, consistent with the upregulation of IL-15 and IL-15Rα on activated dendritic cells (DCs) (Ferlazzo et al., 2004; Koka et al., 2004; Lucas et al., 2007; Mattei et al., 2001; Mortier et al., 2008), also MCMV-induced NK cell responses are more efficient in the presence of functional γ-chain cytokine signaling (Wiedemann et al., 2020). IL-15 and IL-2 independently support NK cell survival and proliferation via STAT5, whereas IL-15 seems to be more important than IL-2 for survival through the contraction phase and formation of memory (Wiedemann et al., 2020).

Together, antigen recognition, co-stimulation and pro-inflammatory cytokines synergize to not only induce NK cell effector functions and proliferation, but also persistently imprint NK cells transcriptionally and epigenetically, thus forming a pool of innate memory cells paralleling adaptive memory.

1.2.4 Signal-regulated transcription factors induce NK cell memory

NK cells undergo pronounced transcriptional and epigenetic remodeling during infection with CMV, as has most closely been studied in a dynamic fashion in mouse models (Bezman et al., 2012; Lau et al., 2018; Wiedemann et al., 2021). Although MCMV-naïve NK cells are epigenetically already more similar to memory than to naïve CD8 T cells (Collins et al., 2019; Lau et al., 2018), consistent with their ability to perform effector functions without prior priming, both cell types follow similar differentiation trajectories during MCMV infection. As a result of the activation-induced chromatin remodeling, they approach each other epigenetically both as effectors and memory cells (Lau et al., 2018). The greatest global changes in NK cell chromatin accessibility coincide with the acute activation stage early after infection (day 1.5-day 7), defined by transient expression of a whole set of effector molecules and increased accessibility of the corresponding chromatin regions. As Ly49H^{+} NK cells return to a resting state, a fraction of the chromatin remodeling remains stable, clearly distinguishing memory from naïve Ly49H^{+} NK

cells (Bezman et al., 2012; Lau et al., 2018). Most of the memory-state defining characteristics are acquired until day 14, with only marginal differences appearing until day 35 (Lau et al., 2018). As mentioned in the previous section, the major TF activities induced downstream of signal 1, 2 and 3 are NFAT, NFκB, AP1, and STATs, which mediate both short-term transcriptional output as well as long-term epigenetic remodeling (Lau et al., 2022).

Groundbreaking work in T cells has demonstrated the subset-specific binding of **NFAT** TFs to cytokine regulatory regions of the *Il4* and *Ifng* genes (Agarwal et al., 2000). Equivalent binding of NFAT in nuclear extracts from T_H1 and T_H2 clones to the *Il4* enhancer fragment in electrophoretic mobility shift assays, in contrast to restricted endogenous binding to the *Ifng* and *Il4* enhancers, respectively, suggested that selective recruitment of NFAT at these effector sites is dependent on chromatin accessibility pioneered by other factors (Agarwal et al., 2000; Avni et al., 2002). NFAT TFs closely interact with AP1 family TFs, forming a ternary complex that is required to induce gene expression, as closely studied for *Il2* expression in T cells (Boise et al., 1993; Chen et al., 1998; Northrop et al., 1994). Nevertheless, the potential of NFAT TFs to initiate chromatin remodeling by themselves has been demonstrated for the *Csf2* enhancer, where a regulatory region becomes exposed in an NFAT-dependent manner, even when the co-localized AP1 motif is mutated, although this is not efficient to induce significant expression (Johnson et al., 2004). Besides their crucial function in inducing transcription of effector molecules, NFAT activity regulates expression of other TF such as RORγt, which are involved in shaping the chromatin of effector and memory lymphocytes (Yahia-Cherbal et al., 2019). Although critically required for NK cell activation and production of effector cytokines (Aramburu et al., 1995), the specific role of NFAT TFs in inducing NK cell epigenetic remodeling remains largely unknown.

Similarly, studies on the involvement of **NFκB** in establishing NK cell memory remain scarce. Work performed on other cell types suggests that NFκB mostly binds to accessible sites induced by other TF factors, including AP1 family members (Garber et al., 2012; Saccani et al., 2001). Nevertheless, NFκB can bind to previously inaccessible sites upon TNF stimulation with kinetics similar to pioneer TFs, but it cannot be excluded that this is dependent on the interaction with other TFs, leaving the question open whether NFκB has pioneering activity (Cieślik and Bekiranov, 2015). That such an activity might be restricted to a set of genes is suggested by the reduction of permissive chromatin marks at effector genes such as *Il17a* and *Il23r* in different models of NFκB signaling deficiency, whereas induction of key T_H17 genes such as *Rorc* and *Ahr* is unaffected (Molinero et al., 2012). Although previous studies had indicated T_H1 and T_H2 differentiation can only be induced when the NFκB pathway is intact (Corn et al., 2005; Das et al., 2001), this largely seems to be a survival effect, as exogenous supplementation of IL-2 enables

generation of T cells producing IFN-γ and IL-4 under the respective polarizing conditions (Molinero et al., 2012). Comparative analysis of NK cells stimulated with IL-12, IL-18 or both cytokines revealed largely cooperative actions between the TFs STAT4, NFκB and AP1, with significant motif enrichment for all three TFs in newly accessible chromatin (Wiedemann et al., 2021). While the activity of AP1 and NFκB cannot be deconvoluted based on these data – since these are induced together by IL-18 – the opening of a fraction of STAT4-bound sites by IL-18 stimulation alone was an interesting observation that points towards STAT4-independent facilitation of chromatin accessibility by these factors even at sites where STAT4 can bind. Despite this cooperative action and the enrichment of NFκB motifs in chromatin regions induced during acute activation, NFκB motifs are enriched in chromatin that is, in fact, *less* accessible in MCMV-induced memory NK cells *ex vivo* (Lau et al., 2018). While this is discussed as a potential mechanism for the lack of responsiveness of memory NK cells to pro-inflammatory cytokines and is consistent with the downregulation of the respective receptors (Lau et al., 2022), it also highlights gaps in the understanding of the transition from short-term transcriptional and epigenetic remodeling to long-term inflammatory imprinting.

More evidence for the direct involvement in establishing NK cell memory is available for **STATs**. Importantly, a large fraction of the remodeled chromatin sites are bound by STAT4 at the early time points after MCMV infection, underpinning the inability of NK cells from *Il12rb2*$^{-/-}$ or *Stat4*$^{-/-}$ mice to efficiently expand and form memory with a molecular mechanism (Lau et al., 2018; Sun et al., 2012). Consistently, *in vitro* stimulation with different combinations of IL-12, IL-18, IL-2, IL-15 and IFN-α identified the combination of IL-12 and IL-18 as crucial for inducing a chromatin accessibility landscape that resembles the signatures at day 2 after MCMV infection (Wiedemann et al., 2021). Addition of IL-2 and IL-15, IFN-α, or both slightly enhances the similarity between the NK cell stimulated *in vivo* vs. *in vitro*, but neither of these signals alone is sufficient to recapitulate the MCMV-induced changes. Binding of STAT4 and STAT5 to many of the regulated chromatin sites, oftentimes in a cooperative fashion, showcases their direct involvement in chromatin remodeling. While also STAT1 co-binds some of the remodeled enhancer regions, it is more enriched at promoters, manifesting in the deposition of permissive H3K4me3 histone marks (Wiedemann et al., 2021). This powerful ability of STAT TFs to induce chromatin remodeling is in line with previous observations in different cell types, especially different T_H subsets (Agarwal and Rao, 1998; Ciofani et al., 2012; Vahedi et al., 2012). Most relevant for NK cells, the global changes in chromatin occupancy of the histone acetyltransferase p300 associated with T_H1 differentiation are strikingly more dependent on STAT1 and STAT4 than on the master TF T-bet, although activation of a subset of putative *IFNG* enhancers requires all three factors (Vahedi et al., 2012).

Apart from STATs, also **AP1** family transcription factors have a well-established role in the chromatin remodeling after immune activation. A striking enrichment of AP1 motifs is one of the features that accessible chromatin in memory T and NK cells have in common, underlining the conserved function of the AP1 TF family in memory formation (Lau et al., 2018); a concept that was recently extended to various types of immune memory and training (Larsen et al., 2021). Originally defined as dimers of proteins from the FOS and JUN families (Curran and Franza, 1988), a relatively large number of homologous proteins is able to bind to AP1 motifs, all belonging to the basic leucine zipper domain (bZIP) family (Chinenov and Kerppola, 2001). Their pioneering function was first described in the context of glucocorticoid receptor binding (Biddie et al., 2011), and a similar effect after immune activation was observed upon macrophage stimulation (Ostuni et al., 2013). Indeed, more than two thirds of chromatin regions becoming accessible upon T cell activation are bound by FOS and/or JUNB, and chromatin remodeling is significantly blocked in cells electroporated with a dominant negative variant of FOS (Yukawa et al., 2020). While the originally described AP1 FOS:JUN heterodimer serves important functions during immediate transcriptional regulation of different T cell effector genes as closely studied for *Il2* (Chen et al., 1998), other bZIP family members, namely BATF and BATF3, are crucial drivers of effector and memory differentiation, as well as T helper cell polarization. Especially T_H17 differentiation has been closely studied in this context, as IL-17 production is strictly dependent on BATF and cannot be fully restored by expression of the T_H17-lineage defining RORγt in the absence of BATF (Betz et al., 2010; Schraml et al., 2009). Originally considered to be negative regulators of classical AP1 TF activity due to their lack of transactivating domains (Echlin et al., 2000), the specific leucin zipper domain of BATF family members endows them with the unique ability to form complexes with IRF4 and IRF8 (Glasmacher et al., 2012; Tussiwand et al., 2012). Such complexes assemble on AP1–IRF composite elements (AICEs) to induce expression of polarization-associated genes, such as *Il17a*, *IL23r*, *Il2rb1* or *Il10* (Glasmacher et al., 2012; Li et al., 2012; Tussiwand et al., 2012). Importantly, these complexes assemble not only under T_H17, but also under T_H2 polarizing and even under non-polarizing T_H0 conditions, suggesting a universal role in effector cell differentiation. Indeed, a landmark study by the Littman lab demonstrated that BATF and IRF4 renders T_H17-associated chromatin sites accessible even under non-polarizing conditions (Ciofani et al., 2012). The addition of polarizing cytokines results in enhancer activation as measured by p300 binding and induces gene expression in a STAT3- and partially RORγt-dependent manner. This pre-patterning of chromatin by BATF-IRF4 underlines their function as pioneering factors to nucleate larger complexes assembled together with polarization-associated TFs that reinforce an activating chromatin state (Ciofani et al., 2012). While effector programs of T_H1 and T_H2 cells

are only partially dependent on BATF and BATF3, as for example their key effector cytokines IFN-γ and IL-4 are unchanged in their absence (Tussiwand et al., 2012), a central role of BATF-IRF4 complexes for T_H2 remodeling was recently further supported by genome-wide binding and accessiblity studies (Henriksson et al., 2019). Induction of BATF and BATF3 under T_H1 conditions might suggest similar mechanisms apply despite independence of IFN-γ from both factors (Murphy et al., 2013), but a genome-wide analysis of the chromatin remodeling in T_H1 cells in the absence of BATF and BATF3 is still lacking. Similar observations in T_H9 (Jabeen et al., 2013), follicular T helper cells (Ise et al., 2011; Pham et al., 2019), and regulatory type 1 cells, where BATF acts in concert with IRF1 (Karwacz et al., 2017), further supports this is a general mechanism across T_H lineages and potentially other cell types. Along these lines, BATF is required for CD8 T cell effector differentiation, where it also forms a complex with IRF4 (Kurachi et al., 2014; Kuroda et al., 2011). This complex supports expression of many genes with well-described functions in effector differentiation including TCR components such as *Cd3d* and *Cd28*, cytokines signaling components such as multiple *Stats, Il12rb1, Il12rb2* and *Il18rap*, and TFs such as *Tbx21*, *Eomes*, *Prdm1* and *Id2*. Despite their crucial role for inducing effector differentiation, some AP1 target genes such as the effector-molecule encoding *Ifng* and *Prf1* were repressed by BATF, consistent with its originally described negative regulatory role (Echlin et al., 2000), potentially as a mechanism that connects effector differentiation with a "safety-switch" awaiting sufficient upstream signals to overcome this repression (Kurachi et al., 2014). While the function of BATF family members for NK cell effector differentiation remain unknown, $Ly49H^+$ NK cell expansion requires IRF8, which is induced by synergistic activation via pro-inflammatory cytokines (IL-12 and IL-18 but not type I IFN or IFN-γ) and Ly49H (Adams et al., 2018). One of the targets of IRF8 is the TF ZBTB32, which is induced by IL-12 and IL-18 via STAT4 binding and antagonizes PRDM1 to promote proliferation of memory NK cells (Adams et al., 2018; Beaulieu et al., 2014). Whether IRF8 forms a complex with BATF or other AP1-family TFs has not been addressed in this context. These functional pieces of evidence and the enrichment of AP1-motifs in memory-associated chromatin suggest complexes of AP1 and IRF family TFs play a similar role in effector differentiation and potentially memory induction in NK as they do in T cells.

On a biochemical level, binding of AP1 factors to methylated DNA was a first indication for their ability to counter-act epigenetic silencing (Gustems et al., 2014). A recent study further confirmed the role of BATF complexes as pioneering factors by parallel analysis of chromatin accessibility and BATF binding in effector T cells (Pham et al., 2019). 24 h after stimulation, 60 % of BATF binding sites were located in closed chromatin regions, of which many became accessible after 96 h. In addition, recruitment of the insulating factor CTCF to BATF-bound

chromatin regions and BATF-dependent genome-wide differences in chromatin looping established that BATF is involved in rearranging chromosomal architecture. To achieve this, BATF cooperated with ETS1 in particular in the presence of IL-6. Since ETS1 expression and binding to chromatin were regulated by BATF, the authors suggested a dual role of BATF in controlling ETS1 levels and creation of permissive binding sites (Pham et al., 2019). A model in which signal-regulated TFs (SRTFs) like STATs or AP1 prime sites for binding of LDTFs like ETS1 had been suggested earlier (Bevington et al., 2016; Kaikkonen et al., 2013; Ostuni et al., 2013) and has also been brought forward for NK cells (Sciumè et al., 2020). Along these lines, chromatin remodeling by STAT4 and STAT5 induced by activation with IL-12 and IL-2 creates novel permissive binding sites for T-bet, including at sites without canonical T-bet motifs. Similarly, RUNX family TFs are not only required for NK cell development, but continue to be crucial for efficient expansion of Ly49H$^+$ in response to MCMV (Rapp et al., 2017), consistent with the important function of RUNX3 in CD8 memory T cell differentiation (Wang et al., 2018a). Conversely, LDTFs shape NK cell chromatin organization during development and differentiation, so that the majority of enhancers that recruit p300 upon NK cell activation are already accessible at steady state and strongly enriched for binding motifs for developmentally required TF families such as T-bet, IRF, ETS and RUNX (Sciumè et al., 2020). In this way, LDTFs establish the latent transcriptional potential and build the basis for the layered architecture of dynamically regulated TF networks (Garber et al., 2012). It seems likely that this close interaction between SRTFs and LDTFs might be one of the underlying mechanisms restricting the pioneering activity during activation as also *de novo* induction of chromatin accessibility is highly selective. New studies integrating measurements of chromatin architecture with TF chromatin occupancy might shed light on the mechanisms underlying this regulation.

In addition to differences in chromatin accessibility and histone decoration, dynamic changes in genome-wide DNA methylation are a related outcome of CD8 T cell effector differentiation and clearly distinguish CD8 T cell memory subsets (Abdelsamed et al., 2017; Scharer et al., 2013; Youngblood et al., 2017). Similarly, human adaptive NK cells have a distinct methylation pattern (Schlums et al., 2015). The templated replication of this covalent DNA modification by DNMT1 (Hermann et al., 2004; Leonhardt et al., 1992) is one of the likely mechanisms enabling long-term maintenance of the epigenetic imprinting through cell division. Accordingly, genome-wide methylation patterns of CD8 T cell memory subsets are stably maintained during homeostatic proliferation *in vitro* (Abdelsamed et al., 2017). Although the precise order of events remains to be identified, enrichment of binding motifs for the TFs discussed above in differentially methylated regions underlines the close interplay between their binding, transcriptional regulation and modification of epigenetic marks.

Overall, several lines of evidence showcase striking conservation of the molecular players inducing effector and memory programs in NK and T cells. Their cooperativity and oftentimes synergistic interactions shape these cells in similar ways, suggesting memory formation and maintenance might be more similar than expected also on a cellular level.

1.2.5 Clonal dynamics and functional diversification

The drastic clonal expansion of individual antigen-specific cells is the fundamental basis of the adaptive immune system, generating a critical mass of effector cells that share the same antigen-specificity and store the information imprinted by the instructive signals they have received together with their cognate antigen. Once the acute infection is under control, the large pool of effector cells enters a contraction phase and only few cells survive to become memory cells that can persist for many years (Ahmed and Gray, 1996).

At least in mice, NK cell responses to MCMV follow similar dynamics. Ly49H$^+$ NK cells, which constitute approximately 50 % of the compartment in C57BL/6 mice, increase 2-3-fold in the spleen and 10-fold in the liver until day 7 after MCMV infection (Dokun et al., 2001; Sun et al., 2009a). An even higher intrinsic expansion potential of m157-specific NK cells was initially demonstrated by transfer into DAP12-deficient mice at lower frequencies where they proliferated 100-fold in spleen and 1000-fold in liver (Sun et al., 2009a). A recent study following fluorescently barcoded Ly49H$^+$ NK cells transferred into immunodeficient *Rag2*$^{-/-}$ *Il2rg*$^{-/-}$ mice increased this estimate by another order of magnitude, yielding upwards of 10,000 progeny from one fluorescently barcoded cell (Grassmann et al., 2019). Transferred Ly49H$^+$ cells were followed up to 70 days after infection, declining rather slowly compared to CD8 T cells with a contraction kinetic that is more similar to CD4 T cells (Sun et al., 2009a). A series of studies transplanting barcoded hematopoietic stem and progenitor cells (HSPCs) into macaques suggest clonal expansion of rhesus CMV (RhCMV)-specific NK cells might also occur in primates (Truitt et al., 2019; Wu et al., 2014, 2018). Large and persistent clones were observed especially within the CD56$^-$ CD16$^+$ compartment, which likely corresponds to CD56^{dim} NK cells in humans. Increased clonal size in RhCMV$^+$ animals implied an involvement of RhCMV, although also the NK cell compartment in RhCMV$^-$ macaques contains sizeable clones especially early after transplantation, pointing towards a potential engraftment bias. As experimental infection with a laboratory RhCMV strain did not induce convincing clonal expansion, this experimental system still needs further optimization before drawing clear conclusion. Nevertheless, an intriguing segregation of clones by KIR profiles and expression of *KLRC2* transcripts by expanding cells in RhCMV$^+$ monkeys suggest at least some of these clones might indeed represent macacine adaptive NK cells

(Truitt et al., 2019; Wu et al., 2018). Indeed, as the RhCMV protein Rh67 is a functional homologue to the HCMV UL40 protein containing an MHC-E-stabilizing peptide (Hansen et al., 2016; Richards et al., 2011), it is tempting to speculate that the recognition mechanism might be conserved. In humans, proliferation of NKG2C$^+$ NK cells coincides with acute HCMV infection or reactivation after stem cell transplantation (Foley et al., 2012; Lopez-Vergès et al., 2011). Although contraction of NKG2C$^+$ NK cell frequencies has been observed after control of the acute infection in some transplantation patients (Lopez-Vergès et al., 2011), this was variable between individuals and not replicated in a study of patients reactivating HCMV after kidney transplantation, where NKG2C$^+$ NK cell frequencies were stable for years or even continued to increase (Ataya et al., 2021).

So what are the drivers of clonal expansion? As the frequency of T cells responsive to a given epitope is low, estimated to be in the range of 1 to 10 per million for CD4 and 1 to 100 per million for CD8 T cells (Jenkins et al., 2010), the antigen receptor is considered to be the main determinant of clonal success. Studies analyzing the evolution of TCR repertoires during *Listeria monocytogenes* infection or after protein immunization have established the role of TCR affinity in the selective recruitment of CD8 T cells (Busch and Pamer, 1999; Savage et al., 1999). Important mechanistic insights could be gained by following T cell responses driven by the transgenic ovalbumin-peptide-specific OT-1 TCR, revealing that the initial proliferation of epitope-specific T cells is robustly induced over a wide range of affinities and instead the magnitude of expansion and the timing of the onset of contraction are determined by TCR signal strength (Zehn et al., 2009). Apart from differences in antigen-receptor affinity, the remarkable variation of clonal burst sizes of T cells expressing the same transgenic TCR highlights that additional mechanisms, including stochastic events, determine clonal success and extent of expansion, and only population averaging results in robust and predictable expansion patterns (Buchholz et al., 2013; Gerlach et al., 2013). Other signals like co-stimulation and cytokines similarly influence the number of divisions T cells go through; additive integration of signals 1-3 at the initial stimulation sets a heritable "division destiny" that is proportional to the sum of signals and highly concordant for progeny of a clonal family (Marchingo et al., 2014, 2016). The impact of these signals is not only concentration- and affinity-dependent but also modulated by presumably stochastic variation in receptor expression levels between naïve cells as demonstrated for CD28, underlining that cell-intrinsic differences between founder cells besides TCR affinity influence clonal fate (Marchingo et al., 2016).

Despite being driven by invariant receptors, the differential ability of individual NK cells to engage in antigen recognition is one of the factors determining their expansion potential in response to CMV. Compared to the naïve pool, memory NK cells express higher per-cell levels of

the main CMV-specific activating receptors Ly49H and NKG2C in mice and humans, respectively (Gumá et al., 2004; Sun et al., 2009a). A recent study elegantly shows that this phenotype – at least in mice – is the result of an avidity selection process (Adams et al., 2019). Ly49H^{hi} cells have more frequent and efficient contacts with target cells and expand more robustly than Ly49H^{lo} cells by proliferating more extensively and succumbing less frequently to apoptosis. Importantly, Ly49H expression differences are maintained through infection and Ly49H^{+} cells produce larger clonal families in the aforementioned barcoded transfer setting, suggesting that quantitatively inherited Ly49H expression is one of the drivers of clonal success (Adams et al., 2019; Grassmann et al., 2019). Intriguingly, also the per-cell expression of NKG2C increases significantly in transplantation patients that reactivate HCMV, indicating a similar selection occurs in humans (Adams et al., 2019). Along these lines, the frequency of NKG2C^{+} NK cells correlates with the per-cell expression levels in HCMV^{+} donors, and a similar correlation was observed for Ly49H in MCMV-infected mice, suggesting the expression levels of the cells recruited into the response might bias the extent of the NK cell expansion (Adams et al., 2019; Muntasell et al., 2013). In humans, deletion of *KLRC2* (the gene encoding NKG2C) is relatively common, with an allelic frequency of ~4 % in Spanish, Japanese and Dutch cohorts (Miyashita et al., 2004; Moraru et al., 2012). Higher per-cell expression and frequencies of NKG2C^{+} NK cells in individuals with two functional alleles, in particular in the context of HCMV seropositivity, suggest that gene dosage affects these parameters (Muntasell et al., 2013). Indeed, NK cells from *KLRC2*$^{+/+}$ donors show more pronounced calcium influx upon receptor engagement and proliferate more efficiently in response to NKG2C engagement than those from *KLRC2*$^{+/-}$ individuals, indicating a functional impact of *KLRC2* zygosity that predisposes for efficient expansion. It is tempting to speculate that allelic expression might be one of the mechanisms for heterogeneity in NKG2C expression levels on which avidity selection might act, although the correlation between per cell expression and frequency of NKG2C^{+} NK cells stands also in *KLRC2*$^{+/-}$ individuals (Muntasell et al., 2013), suggesting additional mechanisms contribute to diversity in NKG2C levels. Besides the main CMV antigen-specific receptors, other heterogeneously co-expressed receptor bias the expansion potential of naïve NK cell subsets. Important examples are self-MHC-specific Ly49 receptors, which modulate NK cell effector function, expansion, and their ability to protect against MCMV infection. Initially, licensing was suggested to have a negative effect on Ly49H^{+} NK cell expansion in C57BL/6 mice by overriding the intrinsically increased responsiveness as long as self-MHC is expressed at high enough levels (Orr et al., 2010). However, a series of studies performed in C57L and MA/My mice have indicated better protection by NK cells educated via Ly49G$_2$ (Prince et al., 2013; Sungur et al., 2013; Wei et al., 2014; Xie et al., 2010). As these

mouse strains lack Ly49H but express other receptors involved in MCMV recognition (see section 1.1.4), the effect of NK cell education might be co-dependent on the mode of recognition. In humans, there is more equivocal evidence for a positive role of self-MHC-specific KIRs, which are dominantly expressed on adaptive NK cells (Béziat et al., 2013). The absence of KIR profile skewing of adaptive NK cells in HCMV^{+} TAP-deficient patients, who have strongly reduced HLA class I expression, further highlights the need for efficient ligand engagement in this process (Béziat et al., 2015). While KIR expression has been demonstrated to be clonally inheritable (see section 1.1.2), such evidence is lacking for the other receptors differentially expressed by human adaptive NK cells. Hence, it is unclear which aspects of their specific phenotype are resulting from selection or a defined differentiation program. Due to the well described co-stimulatory function of CD2 and the inhibitory effect of NKG2A (Hammer et al., 2018b; Liu et al., 2016; Valés-Gómez et al., 1999), it would be interesting to assess whether qualitative and, especially for CD2, also quantitative differences in their expression influence the propensity of NK cells to expand in response to HCMV. Further the differential expression of other receptors LILRB1, SIGLEC7 or NKp30 (Gumá et al., 2004) by adaptive NK cells might suggest they are also involved in shaping the response, although their functions during HCMV infection remain elusive.

Apart from receptors modulating NK cell responses, the competitive survival advantage of NK cells with a history of *Rag2* expression is an example of a receptor-independent trait pre-disposing a subset of cells for more efficient expansion during MCMV infection (Karo et al., 2014). As *Rag2*$^{-/-}$ NK cells showed increased genomic instability and impaired recovery to low doses of ionizing radiation, *Rag2* expression seems to confer cellular fitness by enhancing expression of DNA damage sensors and repair enzymes. This effect was dependent on the enzymatic activity of *Rag2*, as an inactive variant phenocopied the effects, and applied similarly to OT-I T cells, underlining its TCR-recombination independent function in lymphocytes. Another factor that determines NK cell expansion potential in mice is cellular age, older NK cells marked by an inducible fate-map reporter expanded more vigorously than their non-labeled younger competitors (Adams et al., 2021). This effect probably only lasts within a certain timeframe – the observation period was up until ~ 1 month – as a cellular history of extensive homeostatic proliferation marked by KLRG1 expression is associated with reduced expansion potential (Kamimura and Lanier, 2015).

The recruitment of immune cells is only the first step of an antigen-specific immune response and different models have been suggested to explain the dynamics and decision-making of memory formation by CD8 T cells, which shall be discussed here to draw possible parallels with NK cell responses (Adams et al., 2020; Buchholz et al., 2016). A linear model in which memory

T cells differentiate from effectors at the onset of contraction had been suggested based on adoptive transfer experiments (Ahmed and Gray, 1996; Bruno et al., 1995; Swain, 1994) and fate-mapping of memory cells with Cre driven by effector genes such as *Gzmb* (Jacob and Baltimore, 1999). However, seminal findings on the differential ability of CD8 T cell subsets to become memory cells have defined CD127^{+} KLRG1^{-} memory precursor cells (MPECs) and CD127^{-} KLRG1^{+} short-lived effector cells (SLECs) well before the beginning of the contraction phase, suggesting divergent cell-fate decisions rather than a linear progression (Huster et al., 2004; Joshi et al., 2007; Kaech et al., 2003; Sarkar et al., 2008). Studies following the fate of single T cells have demonstrated that this decision is not made by individual naïve T cells, but rather that differentiation into effector and memory subsets can occur from the same cell (Gerlach et al., 2010; Stemberger et al., 2007). Inflammatory signals like IL-12 and IFN-α regulate this process, with selectively sustained expression of the high-affinity IL-2 receptor CD25 on SLECs being one of the mechanisms for their increased expansion (Starbeck-Miller et al., 2014). Conversely, experimentally reducing inflammatory signals during dendritic-cell vaccination or *Listeria monocytogenes* infection accelerates and enhances the generation of memory cells (Badovinac et al., 2004, 2005). Also combined initial signal strength and duration bias the outcome on a population level, so that stronger signals give rise to larger populations of effector cells (Gett et al., 2003; Zehn et al., 2014). Based on this integration of initial and continuous signals the models of *decreasing potential* and *progressive differentiation* both postulate a hierarchy in which MPECs differentiate into SLECs, as was also supported by mathematical models fitting memory precursor and effector cell kinetics (Buchholz et al., 2013), but ascribe different weights to the influence of instructive signals during expansion versus initial signal strength (Buchholz et al., 2016). Whereas the decreasing potential model proposes that the initial activation is mainly required to kick off proliferation and ascribes the decision about effector fates to continuous signals, the progressive differentiation model claims that mainly signal strength during priming determines proliferation and differentiation, with a secondary role of later signals. Since there is experimental evidence supporting both, the relative contribution is likely context-dependent and can be integrated into a *composite model* (Adams et al., 2020). Moreover, recent work suggests that the hierarchy between MPECs and SLECs is not absolute, at least not when relying on individual markers to define these subsets. Fate-mapping studies using *Klrg1*^{Cre} mice have demonstrated that KLRG1^{+} cells significantly contribute to the KLRG1^{-} memory pool (Herndler-Brandstetter et al., 2018). These “exKLRG1” cells mostly originate from CD127^{+} KLRG1^{+} cells, indicating MPECs and SLECs span a spectrum of cell fate bias. This degree of dynamic regulation and reversibility is supported by effector-associated *de novo* DNA methylation in MPECs, followed by dedifferentiation into a memory state (Youngblood et al., 2017).

A recent study suggests a similar commitment occurs for Ly49H$^+$ NK cells at the acute phase of MCMV infection, distinguishing cells with a memory from those with a short-lived effector fate (Riggan et al., 2022). Ly49H$^+$ that lack expression of Ly6C at the peak of infection have a cell-intrinsic, competitive survival advantage compared to Ly6C$^+$ cells, with increased expression of anti-apoptotic BCL2 being one possible mechanism for their preferential maintenance. The significant enrichment of the FLI1 TF motif in the accessible chromatin of Ly6C$^-$ effectors identified this TF as a candidate regulator of their preferential survival. *Fli1* was dynamically regulated during infection, likely under the control of common γ-chain signaling and STAT5. Surprisingly, cells in which *Fli1* was ablated had a competitive survival advantage upon MCMV infection, which was particularly pronounced within the Ly6C$^-$ subset and potentially driven by decreased expression levels of pro-apoptotic BIM. Despite some inconsistencies such as difficulties in convincingly showing that a cluster of cells with a transcriptional signature akin to CD8 memory T cells corresponds to the Ly6C$^-$ subset, and a lack of data demonstrating differential regulation of *Fli1* between the Ly6C$^+$ and Ly6C$^-$ subsets, the study provides the first evidence that distinct NK cell subsets during the effector phase might be committed towards different fates, suggesting a similar division of labor as for CD8 T cell responses. Tuning of *Fli1* expression levels by IL-2/IL-15 *in vitro* further indicates that extrinsic signals might be integrated into these fate decisions.

Diversity continues to exist in the memory compartment, as initially described in human blood, where based on the expression of homing molecules CD45RA$^-$ CCR7$^+$ central memory T cells (T_{CM}), CD45RA$^-$ CCR7$^-$ effector memory T cells (T_{EM}) and CD45RA$^+$ CCR7$^-$ T_{EMRA} can be distinguished (Sallusto et al., 1999). CCR7 and CD62L confer T_{CM} with homing capacity to lymphoid organs. Together with the preferential expression of IL-2 by T_{CM}, contrasted by increased production of effector cytokines like IFN-γ or IL-4 by T_{EM}, this highlights that labor continues to be divided in the T cell memory compartment. More pronounced proliferation of T_{CM} to weaker TCR stimulation and the ability to give rise to effector-cytokine producing progeny upon re-stimulation, suggested T_{CM} might be the source of a new wave of effectors in a secondary response (Geginat et al., 2003; Sallusto et al., 1999). Indeed, serial transfer of single T_{CM} cells demonstrated their ability to reconstitute a diverse memory compartment after secondary and even tertiary challenge (Graef et al., 2014). Recent work using a new fate-map reporter marking proliferative history suggests this re-expansion is mediated by a subpopulation of cells within the T_{CM} compartment undergoing little proliferation during the primary response (Bresser et al., 2022). Studies following the fate of individual OT-1 T cell clones have demonstrated that their differentiation fate is tightly linked to proliferative burst sizes of clonal families, suggesting not only the ability to survive and become memory cells *per se*, but also that subset differentiation fate is set during the effector phase (Buchholz et al., 2013; Gerlach et al., 2013). Along these lines,

a small subset of central memory precursors (CMPs) expressing high levels of *Tcf7* was recently identified within the CD127$^+$ MPEC population, which can be enriched by surface expression of CD62L and preferentially differentiates into T_{CM} (Grassmann et al., 2020; Johnnidis et al., 2021; Pais Ferreira et al., 2020). Conversely, expression levels of CX_3CR1 on effector cells have been associated with a differential ability to give rise to memory cells with potent effector capacity (Gerlach et al., 2016). Whereas CX_3CR1^{hi} cells are mostly terminal effectors, the majority of CX_3CR1^- and around half of CX_3CR1^{int} cells acquire CD62L expression and become T_{CM}. At the same time, CX_3CR1 expression is largely maintained, and later marks virtually all T_{EM} cells, as well as a fraction of highly functional T_{CM} (Böttcher et al., 2015; Gerlach et al., 2016). Since only KLRG1$^+$ or exKLRG1 cells maintained CX_3CR1 expression, a model was proposed in which the CD127$^-$ KLRG1$^+$ effector pool loses expression of KLRG1 to become exKLRG1 cells that generate $CX_3CR1^{hi/int/lo}$ T_{CM} and T_{EM}, whereas the CD127$^+$ KLRG1$^-$ effector pool preferentially produces CX_3CR1^{lo} T_{CM} (Herndler-Brandstetter et al., 2018).

Whether also memory NK cells undergo a similar phenotypic and functional diversification into different memory subsets remains elusive. A significant association between expression of CD27 and clonal family size in adoptively transferred immunodeficient hosts suggests that comparable coupling between differentiation and proliferative burst size might apply early after infection (Grassmann et al., 2019). As expected from the well-established CD27 downregulation during homeostatic NK cell differentiation (see section 1.2.1), CD27$^+$ NK cells mostly lose expression upon MCMV infection (Flommersfeld et al., 2021). However, individual clonal families retain CD27 expression, which mostly coincides with the expression of CD160 and lack of CD62L. Conversely, CD62L$^-$ cells consistently generate CD62L$^-$ progeny partially co-expressing CD27, whereas CD62L$^+$ cells give rise to CD62L$^+$ and CD62L$^-$ populations. CD62L expression on naïve cells, although negatively associated with CD27, does not affect their ability to expand to primary and secondary challenge, suggesting the negative correlation between CD27 expression and clonal burst size is an independent phenomenon (Flommersfeld et al., 2021; Grassmann et al., 2019). Interestingly, the naïve CD62L$^-$ subset shows transcriptional similarities to ILC1, which are partially preserved at the peak of MCMV infection. A higher capacity of the CD27$^+$ CD62L$^-$ population to produce GM-CSF and TNF compared to CD27$^+$ CD62L$^+$ cells, as well as higher levels of IFN-γ in response to activating receptor stimulation *in vitro* and 24 h after MCMV infection *in vivo* point towards functional differences between the naïve subsets (Flommersfeld et al., 2021). These findings suggest that pre-existing heterogeneity within the naïve compartment is clonally preserved in the effector phase, with potential consequences for the memory pool.

Overall, NK cells have the potential to undergo clonal expansion in response to MCMV infection, at least after adoptive transfer into immunodeficient hosts. Despite similarities in the epigenetic mechanisms, this potential clearly separates adaptive NK cell responses from other forms of innate immune memory such as the population level adaptation of the myeloid compartment commonly referred to as *trained immunity* (Hole et al., 2019; Kaufmann et al., 2018; Mitroulis et al., 2018; Saeed et al., 2014). Therefore, the large body of knowledge on the mechanics of clonal expansion within the T cell compartment is a useful resource for the starting point of studies that will decipher the mechanisms driving NK cell clonality and clonal divergence, which are only beginning to be uncovered. Conversely, studies on NK cell clonality might generate insights on the drivers of clonal success that go beyond antigen-specificity and might similarly apply to adaptive lymphocytes.

1.3 Maintenance of immune memory

1.3.1 T cell memory homeostasis

Initially considered to be dependent on the presence of low amounts of antigen, landmark studies in the 90s have demonstrated that CD8 T cells can persist for long periods of time when transferred into antigen-free hosts and mount efficient recall responses upon rechallenge (Hou et al., 1994; Lau et al., 1994). Accordingly, functional CD8 memory T cells in humans were demonstrated to persist for decades by measuring the response to vaccinia virus antigens (Demkowicz and Ennis, 1993; Hammarlund et al., 2003). Similarly, CD4 T cells specific to vaccination antigens presumably only encountered during childhood can be detected in a resting state in the bone marrow of adult individuals (Okhrimenko et al., 2014). Their enrichment in bone marrow compared to peripheral blood points towards the bone marrow as the preferential site not only for plasma cell (Manz et al., 1997), but also for CD4 memory maintenance (Okhrimenko et al., 2014; Tokoyoda et al., 2009). The bone marrow has also been suggested as a privileged site for the maintenance of CD8 memory T cells (Siracusa et al., 2017). Estimates of CD8 memory T cell half-life based on their presence after smallpox vaccination were in the range of 8-15 years (Hammarlund et al., 2003). A recent study was able to refine these estimates by utilizing *in vivo* deuterium labeling in the context of yellow fever virus (YFV) vaccination (Akondy et al., 2017). Combining cell proliferation and death rate (since these measurements only assessed blood, this might also include migration into tissues), the decline in antigen-specific T cell numbers suggested an average half-life of 94 days in the first two years. However, the vast majority of YFV-specific T cells labeled during the effector phase retained the deuterium label as assessed at 1-2 years after vaccination, underlining the presence of a subset of long-lived

memory T cells. Stem-cell potential had previously been described for specialized subsets within a murine memory compartment that lacks expression of the classical memory marker CD44 and acquires expression of the self-renewal associated TFs *Tcf7* and *Lef1* under the influence of Wnt signaling. These cells have even higher proliferative capacity than T_{CM} and can differentiate into T_{CM} and T_{EM}, which is why they are referred to as stem cell-like memory T cells (T_{SCM}) (Gattinoni et al., 2009; Zhang et al., 2005). An analogous population could be identified in humans, which largely has a phenotype of naïve T cells and expresses increased levels of CD95 and IL-2Rβ (Gattinoni et al., 2011). Indeed, the long-lived YFV-specific population shares this naïve-like phenotype, despite its immediate ability to produce effector cytokines (Akondy et al., 2017; Fuertes Marraco et al., 2015). Although they lack GZMB and PRF1 expression at steady state, the promoter regions of these effector genes remain stably demethylated many years after vaccination, and the T_{SCM} chromatin accessibility landscape is more similar to effector than to naïve T cells, showcasing their *bona fide* memory state (Akondy et al., 2017). This is consistent with the dynamic regulation of DNA methylation and expression of genes associated with naïve T cells discussed before (Youngblood et al., 2017). Importantly, T_{SCM} are not only induced by YFV, tetramer stainings identified various viral antigen specificities within this population, in particular to the persistent herpesviruses Epstein-Barr virus (EBV) and HCMV (Fuertes Marraco et al., 2015; Gattinoni et al., 2011). Despite their increased capacity to proliferate in response to homeostatic cytokines like IL-15 (Fuertes Marraco et al., 2015; Gattinoni et al., 2011), the label uptake rate in the memory phase (4-9 months after vaccination) suggested extremely slow homeostatic proliferation of CD8 memory T cells, with an estimated doubling time of 476 days (Akondy et al., 2017), which is approximately 10 times longer than previous estimates from mouse studies (Choo et al., 2010). This discrepancy between mice and humans is interesting, as it either showcases drastic differences in the turnover of their memory compartments, or otherwise supports findings made in our institute that suggest proliferation rates in mice have been overestimated (Sercan Alp et al., 2015; Siracusa et al., 2017), which has raised some controversies in the field (Di Rosa, 2016). Apart from circulating memory cells, tissue-resident memory cells (T_{RM}) with immediate effector functions and self-renewal capacity have been discovered and play an important role for protection at mucosal surfaces (Gebhardt et al., 2011; Mackay et al., 2012; Masopust et al., 2001).

Finally, different signals have been described to be essential for the maintenance of CD8 memory T cells. Survival and slow stochastic proliferation are considered to be driven by the homeostatic cytokines IL-7 and IL-15 (Becker et al., 2002; Judge et al., 2002; Kennedy et al., 2000; Schluns et al., 2000). Accordingly, high expression of the IL-7Rα and IL-2Rβ might enable T_{SCM} to be

maintained for such long periods of time (Akondy et al., 2017; Fuertes Marraco et al., 2015; Gattinoni et al., 2011). Colocalization of CD8 memory T cells with IL-7^{+} stromal cells supports the notion of the bone marrow as a resident memory site accessible to systemic antigens (Sercan Alp et al., 2015), whereas a study in non-human primates suggest T_{SCM} are most enriched in lymph nodes (Lugli et al., 2013).

1.3.2 Memory inflation induced by CMV

Memory responses to CMV are particular in that they induce the drastic expansion of highly functional memory T cells with a terminally mature phenotype (Appay et al., 2002; Klenerman and Oxenius, 2016). HCMV-specific T cell responses can be enormous in size, comprising on average 10 % of the CD8 T cell pool, oftentimes dominated by individual clones (Sylwester et al., 2005; Weekes et al., 1999). Although there is no significant difference between frequencies of HCMV-specific T cells in children and young adults, there is a strong correlation with age in older adults (Komatsu et al., 2003, 2006). The progressive accumulation of MCMV-specific T cells has been studied more closely in mice, where this process has been termed *memory inflation* (Holtappels et al., 2000; Karrer et al., 2003). These inflationary T cells lack expression of IL-7Rα and IL-2Rβ, hence they are poorly maintained in lymphopenic hosts (Snyder et al., 2008). Despite sporadic antigen-driven proliferation, transferred inflationary T cells also disappear in infected hosts, underlining their inability to self-maintain. Nevertheless, inflationary T cells are mainly derived from the primary infection and not from newly recruited naïve cells during the latent phase (Loewendorf et al., 2011; Snyder et al., 2008). Memory inflation is dependent on the continuous presence of antigens expressed during latency or sporadic reactivation events, with different contributions of hematopoietic and non-hematopoietic viral reservoirs (Seckert et al., 2011; Smith et al., 2014; Torti et al., 2011). Based on observations of proliferating T_{CM} in lymph nodes, it has been suggested that recurrently stimulated T_{CM} feed this pool of inflationary T cells (Torti et al., 2011). Along these lines, a recent study elegantly showed that the frequency of CMPs within a clonal family during acute infection is a strong predictor for its magnitude of memory inflation (Grassmann et al., 2020). Given the aforementioned observations on HCMV-specificities with the T_{SCM} compartment (Fuertes Marraco et al., 2015; Gattinoni et al., 2011), these might be placed even higher in the hierarchy or potentially constitute a separate pool, as only limited clonal overlap has been reported in HCMV-specific T_{CM} and T_{EM} (Remmerswaal et al., 2015). Overall, these dynamics of CD8 memory T cells highlight how persistent viruses continue to shape the memory compartment. The relatively high frequency of terminally differentiated, HLA-E-restricted T cells recognizing peptides from HCMV gpUL40 (Mazzarino

et al., 2005; Pietra et al., 2003; Romagnani et al., 2002), might suggest repeated antigen-exposure could also shape adaptive NK cell responses recognizing the same ligand.

1.3.3 Stability of NK cell memory

Generally, NK cells are considered to be short-lived, with deuterium labeling indicating a half-life of around two weeks (Zhang et al., 2007). Therefore, persistently increased frequencies of memory NK cells in the range of months or up to a year in a transplantation were a striking observation (Foley et al., 2012; Sun et al., 2009a). However, the cellular and molecular mechanisms of adaptive NK cell homeostasis remain incompletely understood. The long-term stability of donor-specific subpopulations with variable combinations of signaling adaptor downregulation was the first convincing piece of evidence for the long-term maintenance of individually expanded populations (Schlums et al., 2015). Following up on this observation, two studies elegantly addressed this question by analyzing patient cohorts with progressive hematopoietic deficiencies to follow NK cell progeny. The first study followed patients with paroxysmal nocturnal hemoglobinemia, a disease caused by somatic mutations in the *PIGA* gene, which encodes for an enzyme required for the synthesis of glycosylphophatidylinositol (GPI) anchors (Brodsky, 2014). Loss of functional *PIGA* in hematopoietic stem cells enables to follow immune cell turnover, as hematopoietic output after the time point of *PIGA* mutation lacks GPI anchors. As a result, short-lived cells like neutrophils are rapidly replaced by GPI^- cells, whereas long-lived T cells remain GPI^+ as they are independent of hematopoietic output (Corat et al., 2017). Intriguingly, within the NK cell compartment, adaptive NK cells were enriched for GPI^+ cells, whereas conventional NK cells, especially the more immature $CD56^{bright}$ and $NKG2A^+$ $CD56^{dim}$ subsets seemed to have a higher turnover as judged by the frequencies of GPI^- cells. The second study took a similar angle by analyzing patients with somatic mutations in the *GATA2* gene, which cause severe hematopoietic defects leading to monocytopenia, B, NK and CD4 T cell lymphocytopenia (Dickinson et al., 2014; Spinner et al., 2014). Curiously, the depletion of NK cells is selective for the $CD56^{bright}$ compartment in some patients, sparing at least a subset of $CD56^{dim}$ NK cells (Dickinson et al., 2014; Mace et al., 2013). Stratification of patients by HCMV-status and phenotypic analysis with a focus on adaptive-associated markers revealed a striking enrichment of adaptive NK cells persisting in *GATA2*-mutated patients, in some cases $NKG2C^+$ NK cells completely dominated the NK cell pool (Schlums et al., 2017). Together, these two studies provide at least circumstantial evidence for a relative independence of adaptive NK cells on hematopoietic output and suggest alternative mechanisms for their long-term maintenance, that remain to be identified.

2 Open questions and aims

The specific proliferation and stable expansion of NK cell subsets in mice and humans infected with CMV is an interesting exception to the paradigm of adaptive immunity and immune memory as exclusive hallmarks of T and B cells. Although this pathogen-specificity is reminiscent of classical adaptive immunity, recognition via conserved innate receptors expressed is an important difference to the recognition via unique antigen-receptors, likely affecting the size of the pool recruited into the response. The stably skewed phenotype of human adaptive NK cells has sparked discussions on a potential oligoclonal origin. Along these lines, animal transfer studies of barcoded NK cells or progenitors have demonstrated the potential of NK cells to undergo clonal expansion in immunodeficient or depleted hosts, raising conceptual parallels to adaptive immune cells. However, how these findings apply to natural infection in healthy individuals, where much larger populations of cells compete with each other and clonal diversity is not biased by transfer efficiency, is still completely unclear. Moreover, whether adaptive NK cell expansions induced by HCMV emerge from oligoclonal "founder" populations and persist to form long-lasting memory remains elusive.

Therefore, the aims of my PhD were:

1. To characterize the transcriptional and epigenetic landscape of human NK cells from HCMV$^+$ and HCMV$^-$ individuals to define differences between conventional ("naïve") and adaptive NKG2C$^+$ NK cells and the impact HCMV infection has on the NK cell compartment as a whole.
2. To define how HCMV-derived signals coordinate the recruitment and differentiation of naïve to memory NK cells.
3. To characterize the clonal composition and stability of memory NK cells.

Synthesizing the findings from these three goals aims at generating a model that explains the origin of adaptive NK cells and how their developmental history shapes their phenotypes.

3 Materials and Methods

3.1 Data code and availability

Raw sequencing data as bam-files, as well as processed data as fragment files, gene count table and antibody count tables are publicly available under the accession number GSE197037. Single-nucleotide variants and indels were removed from bam files as described in the detailed methods section. To enable reproducibility of the clonotype analysis based on mitochondrial mutations, the mgatk results were uploaded for each experiment. Accession numbers are listed in the key resources table.

All analysis code has been deposited at Github: https://github.com/timorueckert/Clonal_NK

3.2 Experimental model and subject details

3.2.1 Human studies

All analyses were carried out in compliance with the relevant ethical regulations and all donors gave informed consent. Primary NK cells were isolated from freshly drawn peripheral blood of healthy donors (Table 1) or from buffy coats obtained from DRK Blutspendedienst Nord-Ost, Dresden, Germany. The Charité ethics committee approved the study (EA4/196/18 and EA4/059/17).

Table 1 Donor characteristics. Blood NK cells from 9 healthy donors were analyzed; HCMV serostatus was determined by anti-HCMV IgG ELISA.

Donor ID	HCMV Serostatus	Sex	Age at inclusion	scATAC	scRNA	mtDNA
P1	+	m	45	x	x	x
P2	+	m	27	x	x	x
P3	+	w	29	x	x	x
P4	+	w	27	x	x	x
P5	+	m	28		x	
N1	-	w	30	x	x	
N2	-	m	29	x	x	x
N3	-	m	33	x		x
N4	-	w	29	x		x

3.2.2 Cell lines

RMA-S/HLA-E cells (Borrego et al., 1998) were maintained in complete medium (RPMI-1640 containing glutamine and supplemented with 10 % (v/v) fetal bovine serum (FBS), 20 µM β-mercaptoethanol and 100 U/ml penicillin-streptomycin; all Thermo Fisher) in the presence of 400 µg/ml Hygromycin B (InvivoGen).

3.3 Method details

3.3.1 Cell isolation

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation (Ficoll Paque Plus, GE Healthcare) and either processed immediately or cryopreserved in FBS containing 10 % dimethylsulfoxide. Different strategies for purification of NK cells were used, as described for the individual experiments.

3.3.2 CMV serology

For buffy coats, CMV serology was performed at DRK Dresden, Germany. Serological status of fresh blood donors was analyzed by CMV IgG ELISA (IBL International) following the manufacturer's instructions. Briefly, 10 µl plasma was diluted with 1 mL IgG Sample Diluent, incubated for 1 h at 37 °C in pre-coated wells, washed three times with washing buffer and incubated with Peroxidase-conjugate for 30 min at room temperature. After another three washes, 100 µl TMB Substrate Solution were added and incubated for 15 min at room temperature in the dark. After addition of 100 µl Stop Solution, absorbance was measured at 450/620 nm. All samples were measured in duplicates and were either clearly above or below the cut-off control.

3.3.3 Flow cytometry

Cell suspensions were stained with combinations of fluorochrome-conjugated antibodies (Key resources Table) following established guidelines (Cossarizza et al., 2021). Dead cells were excluded using Fixable Viability Dye eFluor780 (ThermoFisher), or Zombie Aqua Fixable Viability Kit (BioLegend). Data were acquired on an LSR Fortessa (BD Biosciences). Cell sorting was performed on a FACSAria II (BD Biosciences). FlowJo v10 was used for analysis of flow cytometry data.

Table 2 Fluorochrome-conjugated antibodies.

Antigen	Fluorochrome	Manufacturer	RRID	Dilution
CD56	PE/Dazzle™ 594	Biolegend	AB_2563564	1:200
CD137	PE/Cyanine7	Biolegend	AB_2207741	1:50
CD57	BV605	Biolegend	AB_2728426	1:25
CD7	BV786	BD Biosciences	AB_2740589	1:25
CD56	BUV737	BD Biosciences	AB_2871176	1:50
CD3	BUV805	BD Biosciences	N/A	1:50
Streptavidin	BUV395	BD Biosciences	AB_2869553	1:100
CD16	BUV496	BD Biosciences	AB_2870224	1:50
CD337 (NKp30)	BV421	BD Biosciences	AB_2738171	1:25
CD159a (NKG2A)	Biotin	Miltenyi Biotec	AB_2783969	1:50
CD159a (NKG2A)	PE-Vio770	Miltenyi Biotec	AB_2655388	1:50
CD159c (NKG2C)	PE	Miltenyi Biotec	AB_2751866	1:100
CD337 (NKp30)	eFluor 450	ThermoFisher	AB_2574058	1:25
CD3	APC-eFluor 780	ThermoFisher	AB_10717514	1:50
CD14	APC-eFluor 780	ThermoFisher	AB_1834358	1:50
CD19	APC-eFluor 780	ThermoFisher	AB_1582230	1:50
FcεRIγ	FITC	Merck Millipore	N/A	1:50

3.3.4 KIR ligand genotyping

DNA was isolated from 100 µl peripheral blood using spin-column based purification implemented in the DNeasy Blood & Tissue Kit (Qiagen), concentrations were measured using a Nanodrop 2000c Spectrophotometer (ThermoFisher). KIR ligand genotyping was performed using the Olerup SSP KIR HLA Ligand typing kit (CareDx) following the manufacturer's instructions. Briefly, DNA concentrations were adjusted to 30 ng/µl and added to PCR master mix. Master mix was added to wells containing different combinations of sequence-specific primers and incubated in a thermal cycler: 94 °C for 2 min, 10 cycles at 94 °C for 10 s, 65 °C for 60 s followed by 20 cycles at 94 °C for 10 s, 61 °C for 50 s and 72 °C for 30 s. Reactions were analyzed on a 2 % (w/v) agarose gel pre-stained with GelRed (Biotium) in 0.5 x TBE buffer. Gels were documented on an UV transilluminator and interpreted using the manufacturer supplied reference tables. All lanes displayed the required control bands.

3.3.5 Cell preparation for scATAC-seq

For single-cell assay of transposase-accessible chromatin sequencing (scATAC-seq), NK cells were enriched from PBMCs isolated from freshly drawn peripheral blood by magnetic depletion using microbeads against CD3, CD14 and CD19. The enriched fraction was stained with fluorochrome-conjugated antibodies against CD3, CD14, CD19, CD7, NKG2A and NKG2C (Table 2) and Fc receptors blocked using Human TruStain FcX (Biolegend) for 15 min at 4 °C. Dead cells were excluded using Fixable Viability Dye eFluor780 (ThermoFisher). In a second step, cells were stained with nucleotide-barcode labeled antibodies (Table 3) for 30 min at 4 °C. As described in the scATAC with Select Antigen Profiling by sequencing (ASAP-seq) and mitochondrial scATACseq (mtscATAC) protocols, cells were fixed with 1 % para-formaldehyde for 10 min. Fixation was quenched by adding Glycin to 0.125 M final concentration, and cells were washed twice with PBS/BSA. NK cells were sorted on a FACS Aria II (BD Biosciences) as viable, single cells being $CD3^-$ $CD14^-$ $CD19^-$ $CD7^+$, separating $NKG2C^+$ and $NKG2C^-$ cells. We utilized CD7 to sort for NK cells, as it is widely expressed on all NK cell subsets (Milush et al., 2009), and enabled us to stain markers informative for NK cell differentiation such as CD56 and CD16 with nucleotide-barcode labelled antibodies. After sorting, $NKG2C^{+/-}$ NK cells were pooled and lysed for 3 min on ice using modified lysis buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.1 % NP40, 1 % BSA). Afterwards, nuclei were washed with washing buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl, 1 % BSA) and resuspended in diluted nuclei buffer (10x genomics). Nuclei were counted and further processed as described below.

Table 3 Nucelotide-labeled antibodies.

Antigen	Barcode ID	Manufacturer	RRID	Dilution
CD56	A0084	Biolegend	AB_2734445	1:200
CD16	A0083	Biolegend	AB_2734255	1:500
Biotin	A0436	Biolegend	AB_2801086	1:100
CD62L	A0147	Biolegend	AB_2750365	1:100
CD57	A0168	Biolegend	AB_2810588	1:100
CD2	A0367	Biolegend	AB_2783172	1:1000
CD337 (NKp30)	A0801	Biolegend	AB_2800852	1:100
CD161	A0149	Biolegend	AB_2749998	1:100
CD158	A0420	Biolegend	AB_2800901	1:100
CD158b	A0592	Biolegend	AB_2800818	1:100
CD158e1	A0599	Biolegend	AB_2800819	1:100
CD127	A0390	Biolegend	AB_2734366	1:400
CD328	A0902	Biolegend	AB_2832662	1:1000

CD94	A0867	Biolegend	AB_2814142	1:100
CD85j (ILT2)	A0896	Biolegend	AB_2814225	1:100
Phycoerythrin	A0911	Biolegend	AB_2820078	1:100
KLRG1	A0250	Biolegend	AB_2800648	1:100
CD117	A0061	Biolegend	AB_2734287	1:100
HLA-DR, DP, DQ	A1018	Biolegend	AB_2832712	1:400
CD137	A0355	Biolegend	AB_2783173	1:100
CD223	A0152	Biolegend	AB_2749999	1:100
CD184	A0366	Biolegend	AB_2800790	1:100
Hashtag 1	A0251	Biolegend	AB_2750015	1:200-1:400
Hashtag 2	A0252	Biolegend	AB_2750017	1:200-1:400
Hashtag 4	A0254	Biolegend	AB_2750018	1:200-1:400
Hashtag 5	A0255	Biolegend	AB_2750019	1:200-1:400
Hashtag 6	A0256	Biolegend	AB_2750020	1:200-1:400
Hashtag 7	A0257	Biolegend	AB_2750021	1:200-1:400
Hashtag 8	A0258	Biolegend	AB_2750022	1:200-1:400
Hashtag 9	A0259	Biolegend	AB_2750023	1:200-1:400
Hashtag 10	A0260	Biolegend	AB_2750024	1:200-1:400
Hashtag 12	A0262	Biolegend	AB_2750025	1:200-1:400
Hashtag 13	A0263	Biolegend	AB_2750026	1:200-1:400
Hashtag 14	A0264	Biolegend	AB_2750027	1:200-1:400
Hashtag 15	A0265	Biolegend	AB_2750028	1:200-1:400

3.3.6 scATAC-seq

scATAC-seq libraries were prepared using the Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 following the manufacturer's instructions with the modifications described in the ASAP-seq and mtscATAC-seq protocols to retrieve TotalSeq antibody derived tags (ADTs), hashtag oligos (HTOs), as well as mitochondrial DNA (Lareau et al., 2021; Ludwig et al., 2019; Mimitou et al., 2021). Briefly, nuclei were transposed at 37 °C for 1 h before generation of GEMs with the addition of bridge oligos to capture ADTs and HTOs. Barcoding was performed at 72 °C for 5 min, 72 °C for 5 min, 12 cycles at 98 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min and samples held at 15 °C. GEMs were broken down by addition of 125 µl recovery agent and DNA extracted with DynaBeads MyOne Silane (Thermo Fisher Scientific). DNA was eluted with 43 µl and 3 µl saved for ADT/HTO library generation. After additional purification using SPRIselect reagent, libraries were constructed by amplification with unique sample-index containing primers: 98 °C for 45 s, 11 cycles at 98 °C for 20 s, 67 °C for 30 s, 72 °C for 20 s, final elongation at 72 °C for 1 min and

samples held at 4 °C. Libraries were purified by double sided size selection using SPRIselect reagent. The saved eluate from silane bead elution and the supernatant of the first SPRIselect purification were combined for amplification of ADT/HTO libraries using the KAPA HiFi ready mix (Roche) with sample-specific index primers (Illumina small RNA RPIx/Truseq D7xx) and purified using SPRIselect reagent.

Library size and quality were analyzed using a Fragment Analyzer (Advanced Analytical) before fragmentation and after final purification. Final library concentrations were measured on a Qubit 2.0 Fluorometer (ThermoFisher). Libraries were sequenced on a NextSeq500 or NovaSeq6000 sequencer (Illumina) using longer read1/2 configurations than suggested by 10x Genomics to improve mitochondrial genotyping (NextSeq500: R1 72 cycles, R2 72 cycles, I1 8 cycles, I2 16 cycles; NovaSeq 6000: R1 88 cycles, R2 88 cycles, I1 8 cycles, I2 16 cycles).

3.3.7 Cell preparation for scRNA-seq

NK cells were enriched, stained and sorted as described above for scATAC-seq (without fixation). NKG2C^{+} and NKG2C^{-} cells were separately labelled with different nucleotide-labelled hashtag antibodies (Biolegend). NKG2C$^{+/-}$ populations were pooled at a 1:1 ratio and the cell concentration adjusted to 1000 cells/µl for further processing.

3.3.8 scRNA-seq

scRNA-seq libraries were prepared using the Chromium Single Cell 3' Reagent Kits v2/v3.1 Chemistry (10x Genomics) following the manufacturer's instructions with the modifications described in the protocols for Cellular Indexing of Transcriptomes and Epitopes by sequencing (CITE-seq) to recover of ADTs and HTOs (Stoeckius et al., 2017). Briefly, single-cell Gel Bead-in-Emulsions (GEMs) were generated on a Chromium Controller (10x Genomics) with a target cell recovery of 5,000-10,000 cells. GEM-RT was performed in a thermal cycler: 53 °C for 45 min, 85 °C for 5 min and held at 4 °C. GEMs were broken down by addition of 125 µl recovery agent and cDNA extracted with DynaBeads MyOne Silane (Thermo Fisher Scientific). During cDNA amplification, 1 µl of 0.2 µM ADT and HTO additive primers were added. cDNA and ADTs/HTOs were amplified: 98 °C 3min, 11-13 cycles of 98 °C for 15 s, 67 °C/63 °C (v2/v3.1) for 15 s, 72 °C for 1 min, final elongation at 72 °C for 1 min and held at 4 °C. Amplified cDNA was purified with SPRIselect reagent (Beckman Coulter) prior to enzymatic fragmentation. The supernatant was further purified using SPRIselect reagent to retain ADT libraries. cDNA was fragmented at 32 °C for 5 min, ends repairedand A-tailed at 65 °C for 30 min and held at 4 °C. After

double-sided size selection using SPRIselect reagent, sequencing adaptors were ligated at 20 °C for 15 min, libraries purified again using SPRIselect reagent and amplified using sample-specific index primers: 98 °C for 45 s, 10-14 cycles of 98 °C for 20 s, 54 °C for 30 s, 72 °C for 20 s, final elongation at 72 °C for 1 min and held at 4 °C. Finally, libraries underwent another double-sided size selection using SPRIselect reagent and were stored at -20 °C until sequencing. ADT and HTO libraries were amplified using the KAPA HiFi ready mix (Roche) with sample-specific index primers (Illumina small RNA RPIx/Truseq D7xx) (Stoeckius et al., 2017) and purified using SPRIselect reagent.

Library size and quality were analyzed using a Fragment Analyzer (Advanced Analytical) before fragmentation and after final purification. Final library concentrations were measured on a Qubit 2.0 Fluorometer (ThermoFisher). Libraries were sequenced on a NextSeq500 or NovaSeq6000 sequencer (Illumina) using the read configurations recommended by 10x Genomics (v2: R1 26 cycles, R2 98 cycles, I1 8 cycles; v3: R1 28 cycles, R2 91 cycles, I1 8 cycles).

3.3.9 Stimulation of NK cells from CMV$^-$ donors

RMA-S/HLA-E target cells were prepared by seeding them in 24-96 well plates at a cell concentration of 2 x 10^6 cells/mL in OptiMEM (ThermoFisher) and irradiated with 3000 cGy. Afterwards, cells were pulsed either with the negative control peptide VMAPQSLLL or the activating peptide VMAPRTLFL (Peptides&Elephants) at a concentration of 300 µM for 6-12 h.

For scATAC-seq, NK cells were isolated from freshly drawn blood by magnetic depletion of CD3$^+$, followed by enrichment of CD56$^+$ cells. For read-out by flow cytometry, cells were isolated from buffy coats (DRK), CD56$^+$ cells enriched using CD56 microbeads and cryopreserved in FBS containing 10% DMSO. Viable CD56$^+$CD3$^-$ cells were sorted from the cryopreserved CD56-enriched fractions. Purified NK cells were co-cultured for 12 h with peptide-pulsed RMA-S/HLA-E cells in complete medium supplemented with 10 ng/mL IL-15 (Miltenyi Biotec), the respective peptides at 300 µM, in the presence or absence of 10 ng/mL IL-12 (Miltenyi Biotec) and 100 ng/mL IL-18 (MBL International). Cells were stained with fluorochrome-labelled antibodies and either analyzed by flow cytometry or additionally stained with nucleotide-barcode labelled antibodies, including hashtag antibodies marking experimental conditions and donors, before sorting for viable CD7$^+$ NKG2C$^+$ cells. Sorted cells were pooled at equal numbers per condition and processed for scATAC-seq as described above.

3.4 Data analysis

3.4.1 Removal of sensitive genetic variant information

Raw bam files for all experiments were processed with BAMboozle (Ziegenhain and Sandberg, 2021) (see a list of all critical software in Table 4) to remove potentially identifying donor-related single nucleotide polymorphisms and indels, by replacing them with the sequences present in the corresponding reference genomes. To enable reproducibility of the clonotype analysis based on mitochondrial mutations, the mitochondrial genotyping results were deposited with the raw and processed data for each experiment.

Table 4 Software utilized in this study.

Software	Version	Source
Python	3.7.9	Python Software Foundation
cellranger-atac	1.2.0	10x Genomics
cellranger	3.0.2	10x Genomics
ASAP to kite	2	(Mimitou et al., 2021)
kallisto	0.46.0	(Bray et al., 2016)
bustools	0.39.2	(Melsted et al., 2021)
bamboozle	0.5.0	(Ziegenhain and Sandberg, 2021)
CITE-seq count	1.4.1	(Roelli et al., 2019)
mgatk	0.5.9	(Lareau et al., 2021)
R	4.1.0	The R Foundation
Seurat	4.0.6	(Hao et al., 2021)
Signac	1.3.0	(Stuart et al., 2021)
Harmony	0.1.0	(Korsunsky et al., 2019)
chromVAR	1.14.0	(Schep et al., 2017)
tidyheatmap	0.0.0.9000	https://jbengler.github.io/tidyheatmap/
pheatmap	1.0.12	CRAN
eulerr	6.1.0	CRAN
Homer	4.11	(Heinz et al., 2010)
FlowJo	10.7.1	BD Life Sciences
GraphPad Prism	8.4.3	GraphPad Software

3.4.2 scATAC-seq

3.4.2.1 Pre-processing

Base call files were demultiplexed using cellranger-atac v1.2.0 mkfastq and bcl2fastq v2.20.0.422 into scATAC and ADT/HTO libraries. scATAC reads were mapped with cellranger-atac count to the GRCh38 reference genome, hardmasked for regions which would otherwise interfere with mapping to the mitochondrial genome (Lareau et al., 2021), generating count tables and fragments files as output for further analysis. ADT and HTO reads were preprocessed using ASAP to kite (Mimitou et al., 2021), followed by pseudoalignment with kallisto (Bray et al., 2016) to a mismatch map generated with kite and counting with bustools (Melsted et al., 2021).

3.4.2.2 Integrated analysis ex vivo

Peak sets called from different experiments were reduced to a joint peak set as a basis for combined analysis of individual experiments using Signac (Stuart et al., 2021). Cells were filtered for outliers based on nucleosome signal (<1-1.2), transcription start site (TSS) enrichment (>2.5-4) and the frequency of reads in peaks (>55-70 %) and blacklisted regions (<0.0001). ADT and HTO counts were imported, centered-log ratio (CLR) normalized and joined with the scATAC-seq data. Normalized hashtag reads were utilized for exclusion of doublets and to demultiplex donors. Chromatin accessibility counts were normalized by term frequency-inverse document frequency (TF-IDF), data from different experiments merged, and dimensionality reduced by singular value decomposition (SVD). The resultant latent semantic index (LSI) was integrated across experiments using harmony (Korsunsky et al., 2019) and used as input into further dimensionality reduction by uniform manifold approximation and projection (UMAP) and neighborhood graph-based clustering. A small population of non-NK cells as judged by their lack of CD56 and CD16 expression concomitant with high levels of CD127 and CD117 were excluded. The merged and integrated dataset was used for peak calling using MACS2 (Zhang et al., 2008) to enable more sensitive calling of cluster-specific open chromatin regions. This new assay was again processed by LSI, followed by anchor-based integration of donors (Stuart et al., 2019). The UMAP embeddings and clustering for final analysis were based on this integrated LSI. Unbiased clustering initially yielded three major clusters that were annotated based on surface protein expression, with early $CD56^{dim}$ NK cells separated from $CD56^{bright}$ NK cells by further subclustering. Gene activities per cell were calculated by summing up counts within the gene body and up to 2000 bases upstream of the TSS.

For the analysis of the global signatures, differential accessibility analysis of individual chromatin regions was performed using logistic regression with the number of peaks as latent variable (Ntranos et al., 2019), while gene activities were assessed using Wilcoxon rank sum test. For both, the log_2FC threshold was set at 0.1 and significant hits from the direct comparison of $CD56^{bright}$ to $CD56^{dim}$ added for visualization, to include the $CD56^{dim}$ signature that was mostly shared with adaptive NK cells. Averaged values were visualized in heatmaps using tidyheatmap, an implementation of pheatmap.

To compare $HCMV^+$ and $HCMV^-$ donors, these were separated from the integrated dataset followed by UMAP embedding and clustering as described above. To directly compare the number of differentially accessible regions between $NKG2C^+$ and $NKG2C^-$ cells, these populations were defined based on anti-PE ADT counts marking NKG2C surface expression and then downsampled to equal numbers (4300 $NKG2C^{+/-}$ cells) for both donor groups. Further, $NKG2C^+$ NK cells from $HCMV^+$ donors were extracted, followed by UMAP embedding and their cluster identities assessed based on the signatures from the integrated analysis of the full dataset.

3.4.2.3 Motif analysis

Position frequency matrices for TF motif analysis were downloaded from the JASPAR2020 human core database and amended by Stat-TF motifs from the murine database (Fornes et al., 2020). Motif activities per cell were calculated using chromVAR (Schep et al., 2017) as implemented in Signac. Enrichment of motifs in differentially accessible peaks between clusters were assessed using the hypergeometric test implemented in Signac. De novo motif analysis on the adaptive NK cell specific peaks was performed with homer v4.11 findMotifsGenome.pl using the standard parameters (Heinz et al., 2010).

3.4.2.4 Mitochondrial genotyping and clonotypes

Mitochondrial genotyping was performed with mgatk (Lareau et al., 2021) in *tenx* mode using the barcodes identified as cells by cellranger. Only cells with at least 5x coverage of the mitochondrial genome were included in the analysis, achieving a median coverage of 11-20x across experiments. As sensitivity and positive predictive value are relatively independent of coverage for mutations with high heteroplasmy (Lareau et al., 2021), those could be confidently detected at this coverage. Using a combination of highly abundant, homoplasmic mitochondrial mutations characteristic of each donor, we noted donor demultiplexing was slightly incomplete in

some cases, so we utilized these mutations as additional donor barcodes to further improve demultiplexing before the donor-specific analysis. Mitochondrial mutations called by mgatk were filtered for high-confident variants detected in at least three cells, with strand-concordance >65 % and a variance-to-mean ratio >0.01. Clonotypes were identified by clustering on a neighborhood graph constructed on mitochondrial mutation frequency using the Euclidean distance metric, since we noted that the majority of adaptive clonotypes were defined by mutations with high allele frequencies and thereby achieved better clonotype resolution than with the cosine distance metric originally suggested for mtscATAC-seq (Lareau et al., 2021). Clustering parameters were empirically optimized per donor so that clonotypes were defined by ~1 mutation and only those clonotypes included which had at least one significantly enriched mutation. Clonotypes only defined by differential frequency of highly abundant mutations were excluded from the analysis. Association of clonotypes to clusters defined by their chromatin accessibility profiles was analyzed by χ^2 test and comparing the resultant false-discovery rates to a randomly permuted matrix (Lareau et al., 2021). Analysis of clonotype association to open chromatin regions was performed using χ^2 test on the binarized accessibility matrix and compared to a randomly permuted matrix.

3.4.2.5 Donor-specific and -unique signatures

Individual HCMV$^+$ donors were analyzed based on the peak set called for the integrated dataset. For donors P2 and P4, both time points were included in this analysis and integrated using harmony (Korsunsky et al., 2019). Dimensionality reduction and clustering was performed for each individual donor as described above. To analyze overlaps and differences between the signatures defining clusters across donors, differential accessibility analysis was performed per donor as described for the integrated dataset, and overlaps visualized using eulerr. To assess the actual signatures, adaptive subclusters defined in each donor were merged and averaged accessibility per cluster visualized with tidyheatmap. Differential gene scores and motif activities between the two emerging adaptive subcluster groups was analyzed as described before by directly comparing them to each other within the merged object. To assess similarity of their signatures to those of CD56^{bright}/early CD56^{dim} and CD56^{dim} NK cells, fold changes of the adaptive subcluster group-defining differentially accessible regions (DARs) were plotted for both comparisons and analyzed for linear correlation.

3.4.2.6 Cluster heterogeneity: distance in LSI embedding

Cluster heterogeneity was assessed based on the median Euclidian distance of 200 randomly sampled cells per cluster to their 10 nearest neighbors in LSI space (component 2-30). Sampling was repeated 100 times and results plotted as boxplots of the distribution of median distances of all simulations. The k parameter was varied from 5-30 to test for robustness. This analysis approach was developed by Caleb Lareau.

3.4.2.7 Longitudinal analysis

The two time points for donor P2, P3 and P4 were separated from the merged, harmony-integrated objects and the signatures defining adaptive subclusters analyzed separately for each time point using hierarchical clustering and visualization with tidyheatmap. Allele frequency of clonotype-defining mutations was visualized for each time point. To analyze the stability of clonotypes over time, only clonotypes which had an association to chromatin-accessibility defined clusters with a false discovery rate (FDR) < 0.05 were included; these were further curated by assessment of their association to adaptive subclusters in UMAP embeddings. Stability of clonotypes significantly associated to adaptive subclusters was analyzed by testing association of clonotypes to time points with Fisher's exact test. The stability of the total clonotype distribution was further assessed by calculating their $\log_2$ fold changes between time points and comparing the distribution to a randomly permuted clonotype-time point relationship by Kolmogorov-Smirnov test.

3.4.2.8 scATAC after activation

Quality control, joining of ADT, HTO and scATAC counts, demultiplexing, peak calling on the full dataset with MACS2, normalization and dimensionality reduction by LSI were performed as described for the ex vivo analysis. The two analyzed donors were integrated with harmony and the corrected LSI used for UMAP embedding and clustering. Upon inspection of the distribution of cells from the different conditions and donors, the majority of cells were clearly separated based on whether they had received peptide or cytokine stimulation, but we also noted two smaller clusters, each enriched for cells from one donor respectively, in which cells from different conditions were mixed, likely driven by underlying donor-specific signatures that were not corrected by harmony integration. To analyze stimulus-induced changes in chromatin accessibility, we focused the analysis on the majority of cells that clustered by conditions and irrespective of donor origin. The cell barcodes used for the final analysis were deposited together with

the raw and processed data. After clustering these cells at higher resolution, we annotated clusters according to a clear enrichment of cells derived from different conditions. Analysis of differential motif activity and chromatin accessibility was performed as described for the *ex vivo* analysis. To assess the similarity of the *in vitro* induced signatures to those defining NK cell subsets *ex vivo*, the MACS2-called peak set of the *ex vivo* analysis was quantified for the cells stimulated in vitro, and the two datasets integrated using anchor-based integration, setting the *ex vivo* dataset as reference and the *in vitro* dataset as query (Stuart et al., 2019). Integration anchors were then utilized to transfer cluster labels from the *ex vivo* to the *in vitro* dataset and the per-cell prediction scores plotted for each cluster. To identify which individual chromatin regions overlap between adaptive NK cells *ex vivo* and the cells stimulated with cytokines and peptide, fold changes for the regions differentially accessible overlapping between the comparisons of $CD56^{dim}$ to adaptive NK cells *ex vivo* and control to LFL+IL-12+IL-18 *in vitro* were analyzed for linear correlation.

3.4.3 scRNA-seq

3.4.3.1 Pre-processing

Base call files were demultiplexed using cellranger v3.0.2 mkfastq and bcl2fastq v2.20.0.422 into scRNA and ADT/HTO libraries. scRNA reads were mapped to the hg19 reference genome with cellranger count, generating gene per cell count tables as output for further analysis. ADT and HTO reads were either quantified with CITE-Seq-Count (Roelli et al., 2019) or pseudoaligned with kallisto (Bray et al., 2016) to a mismatch map generated with kite and counted with bustools (Melsted et al., 2021).

3.4.3.2 Main analysis

scRNA-seq counts generated with cellranger were imported into Seurat (Hao et al., 2021) and filtered for outliers with regards to frequency of mitochondrial transcripts (v2: <5-6 %; v3: <10 %), total number of transcripts (v2: <6000; v3: <10000) and number of genes (v2: >500-800, <2000-2500; v3: >1000, <5000) per cell. Contaminating erythrocytes and B cells were excluded based on the expression of *HBA1/2* and *IGJ*, respectively. ADT/HTO counts were imported, CLR normalized and joined with the scRNA-seq data. Normalized hashtag reads were utilized for exclusion of doublets and to demultiplex $NKG2C^{+/-}$ populations and donors. Initially, donors were analyzed individually. Counts were normalized with scran (Lun et al., 2016) and used for principal component analysis (PCA) on the 2000 most variable genes. The number of principal

components used for UMAP embedding and clustering was chosen based on elbow plots for each individual. After initial high-resolution clustering, a small population of non-NK cells as judged by their lack of CD56 expression concomitant with high levels of *IL7R*, *GATA3*, *IL2RA* and *CD40LG* were excluded. PCA was re-calculated on the remaining cells and used as input for UMAP embedding and clustering as before. $HCMV^+$ and $HCMV^-$ donors were then integrated separately based on the variable features overlapping between $HCMV^+$ donors, using anchor-based integration. The integrated data was scaled, regressing out the number of transcripts per cell. The $HCMV^{+/-}$ objects were merged, the integrated data re-scaled, also here regressing out the number of transcripts per cell. PCA was performed on the shared variable features and used as input for UMAP embedding and clustering. The normalized, non-integrated RNA assay was used for all differential expression analyses. For differential expression analysis between $NKG2C^{+/-}$ populations in $HCMV^+$ and $HCMV^-$ donors, cells were downsampled to equal numbers per population and donor group (6000 cells each) and only genes included that were detected with a minimum count of 100 in both objects. Further, $NKG2C^+$ NK cells from $HCMV^+$ donors were extracted using hashtag counts, followed by UMAP embedding. Signatures from the integrated analysis were assessed to confirm the cluster identities.

3.4.3.3 Integration of scRNA and scATAC

Fully processed and donor-integrated datasets of both modalities were integrated by anchor-based integration as implemented in Seurat (Hao et al., 2021; Stuart et al., 2019) using the integrated RNA assay as reference and the gene scores calculated from scATAC data as query. Integration anchors were utilized to impute RNA expression of clusters defined by chromatin accessibility and inspected for consistency of key marker genes. Imputed RNA expression was assessed for correlation with chromatin accessibility to identify links between individual chromatin regions and gene expression.

4 Results

4.1 Mapping human NK cell subsets onto transcriptional and epigenetic landscapes

To resolve the relationship of human NK cell subsets and the impact of HCMV on these populations, we generated single cell profiles of chromatin accessibility, gene expression and cell surface proteins of NK cells from peripheral blood of HCMV$^+$ donors with NKG2C$^+$ NK cell expansions and HCMV$^-$ controls (Figure 5A, Table 1) by ASAP-seq (Mimitou et al., 2021) and CITE-seq (Stoeckius et al., 2017). NKG2C$^{+/-}$ NK cells (sorted as CD3$^-$ CD14$^-$ CD19$^-$ CD7$^+$) from all donors were equally enriched to a 1:1 ratio to correct for different frequencies of NKG2C$^+$ cells between HCMV$^+$ and HCMV$^-$ individuals (Figure 5B).

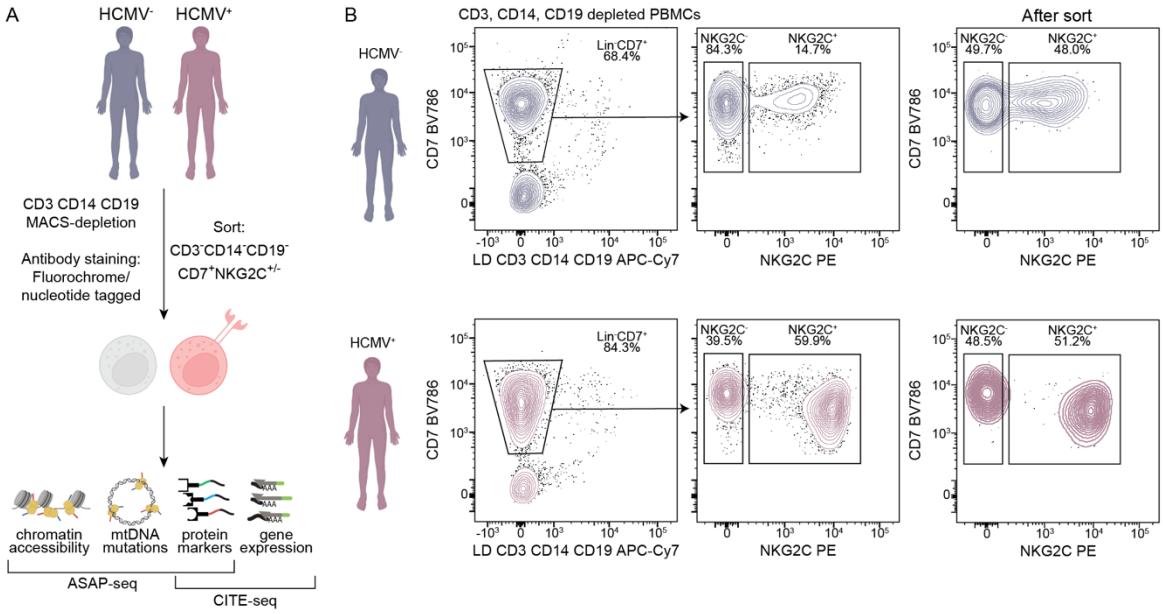

Figure 5 Experimental and sorting strategy. (A) NKG2C$^+$ and NKG2C$^-$ NK cells were isolated from 4-5 HCMV$^+$ and 2 HCMV$^-$ healthy blood donors, stained with nucleotide-barcode labeled antibodies, mixed at a 1:1 ratio and analyzed by ASAP-seq (n=6) and CITE-seq (n=7). **(B)** Sorting strategy for one representative HCMV$^-$ and HCMV$^+$ donor, respectively. Created with BioRender.com.

After filtering for high-quality cells (see sections 3.4.2.1 and 3.4.3.1), 39,106 cells and 49,530 cells were analyzed for their epigenetic and transcriptional profiles, respectively. We detected expression of 19,975 genes and accessibility of 147,299 chromatin regions. Utilizing cell surface protein information, we annotated cell clusters defined by their transcriptomes and chromatin accessibility with long-standing NK cell subset definitions, while at the same time testing the validity of these population definitions in an unbiased manner (Figure 6A-D). Namely, we observed 4 main clusters in both modalities that could be designated as CD56^{bright}, early CD56^{dim}, mature CD56^{dim} and adaptive NK cells (Figure 6A,C) with extensively distinct genomic signatures (Figure 6E-G), in line with previous data (Crinier et al., 2018; Collins et al., 2019; Yang et al., 2019;

Smith et al., 2020; Holmes et al., 2021). CD56^{bright} (CD16$^{-/lo}$ CD57^{-} CD62L^{+} NKG2A^{+}) cells (Figure 6A-D) were characterized by high accessibility around loci of characteristic transcription factors (TFs) *TCF7, RUNX2, BACH2, ZEB1* and *MAML3*, and expression of signature transcripts, including *GZMK, XCL1* and *IL7R* (Figure 7A-B). In contrast, CD56^{dim} (CD16^{+} CD57$^{+/-}$ CD62L$^{+/-}$ NKG2A$^{+/-}$) NK cells, which were characterized by chromatin accessibility in the proximity of *ZBTB16*, *BCL11B, GLI3* and *ZEB2,* as well as expression of *GZMB*, *PRF1* and *CX3CR1*. Between CD56^{bright} and CD56^{dim} NK cells, a population of seemingly intermediate, early CD56^{dim} (CD16^{+} CD57^{-} CD62L^{+} NKG2A^{+}) NK cells shared signatures of both subsets (Figure 6E-G), such as high gene scores for *TCF7, ZEB1* and of *ZBTB16* (Figure 7B). An additional cluster of proliferating cells expressing *MKI67* and *STMN1* was only resolved by transcriptional profiling (Figure 7C).

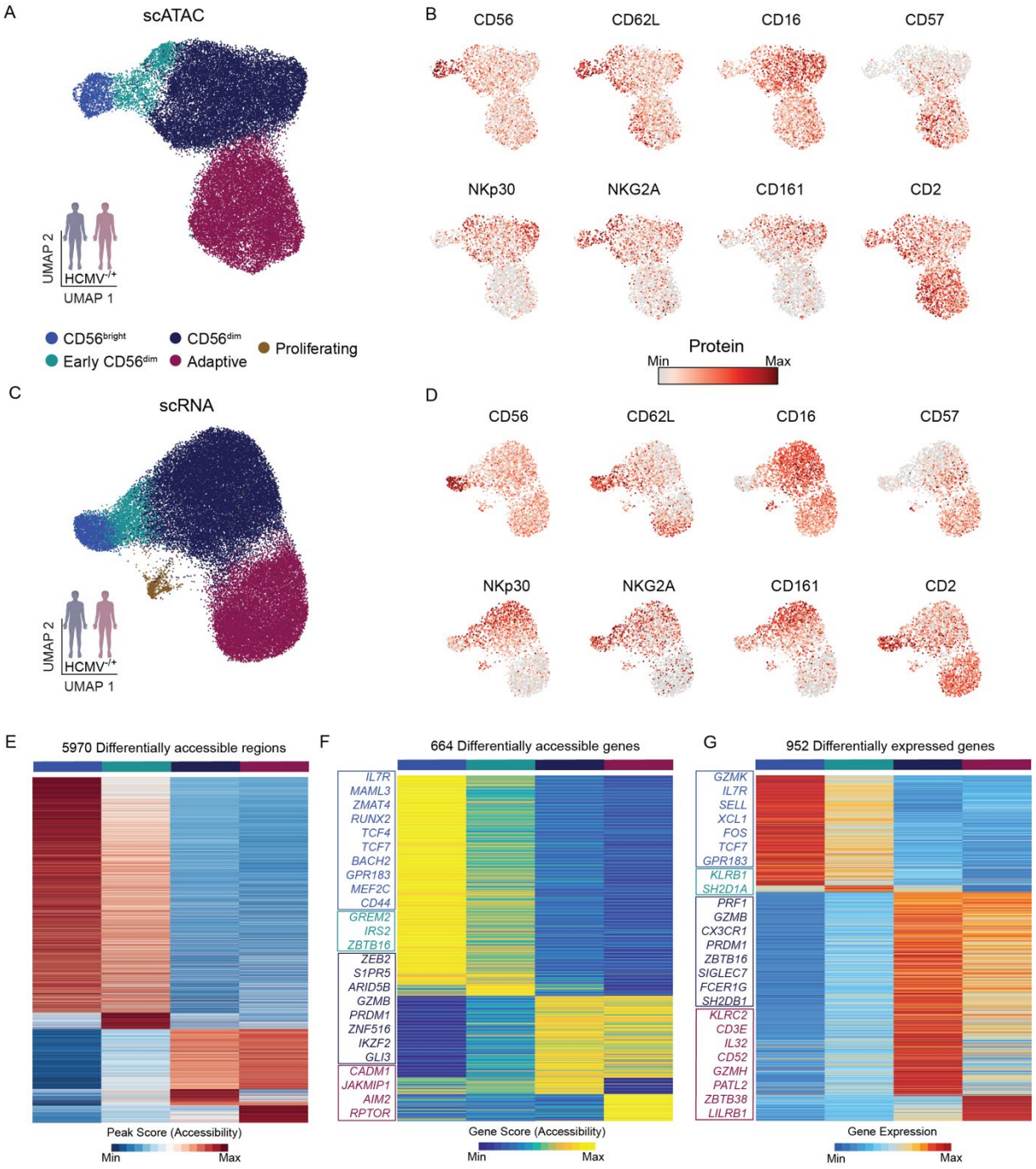

Figure 6 Mapping NK cell subsets onto transcriptional and epigenetic landscapes. (A+C) Integrated UMAP embedding of NK cells from donors analyzed by scATAC-seq (A) (n=6) and scRNA-seq (C) (n=7). **(B+D)** Surface protein expression of the indicated markers as measured by ASAP-seq (C) or CITE-seq (E). **(E-G)** Row-scaled differentially accessible regions (E), genes (F) and differentially expressed genes (G) per cluster. Exemplary cluster-associated genes are listed in the respective colors. Created with BioRender.com.

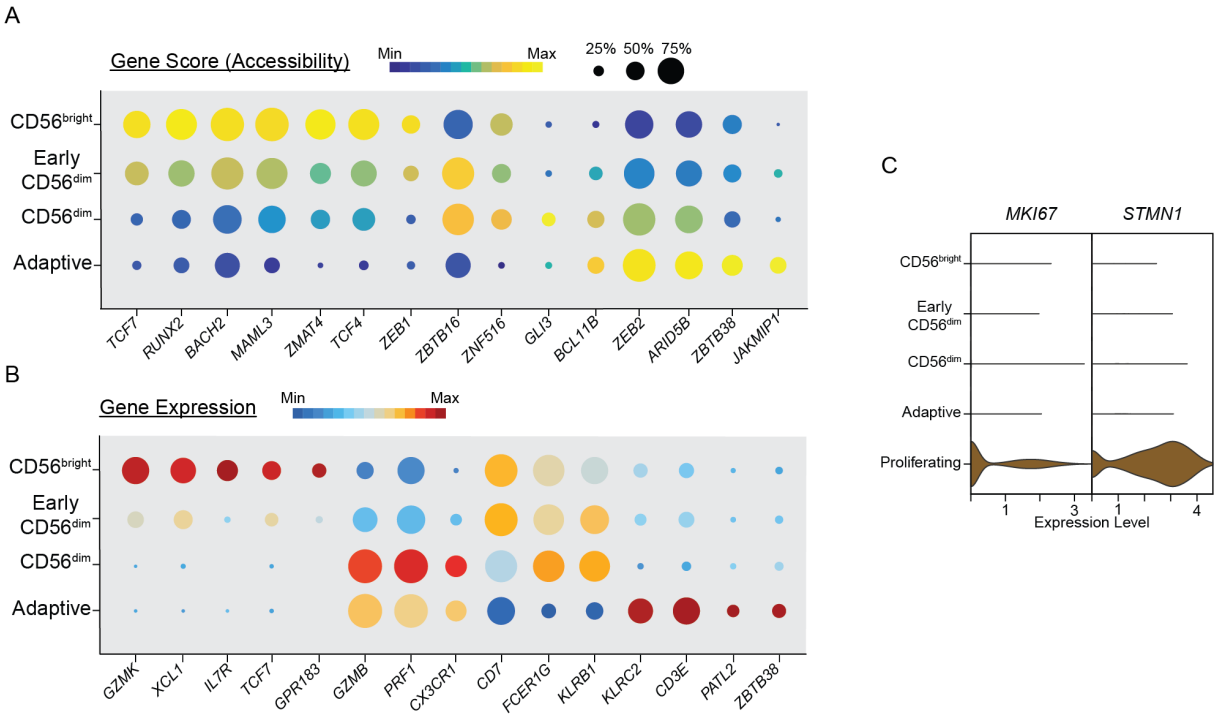

Figure 7 Epigenetic and transcriptional signatures of human NK cell subsets. (A+B) Column-scaled accessibility scores (A) and expression (B) per cluster and detection frequencies for the indicated genes. **(C)** Expression of proliferation genes *MKI67* and *STMN1*.

Label transfer between modalities further allowed to directly link gene expression and chromatin accessibility, generating a fully integrated resource that enables correlation-based prediction of subset-specific gene regulatory elements for core regulators of NK cell identity, such as *TCF7*, *RUNX2* and *ZEB2* (Figure 8A-D). The validity of these predictions was supported by specific association to motifs for TF such as RUNX in the CD56^{bright} associated TCF7 enhancers, whereas accessibility in early CD56^{dim} NK cells seemed to correlate with ZEB1 motifs (Figure 8B), consistent with the gene scores of these TFs in the respective subsets (Figure 7A). Overall, we provide the first multimodal map of human NK cells, combining single cell measurements of chromatin accessibility, gene expression and surface phenotypes.

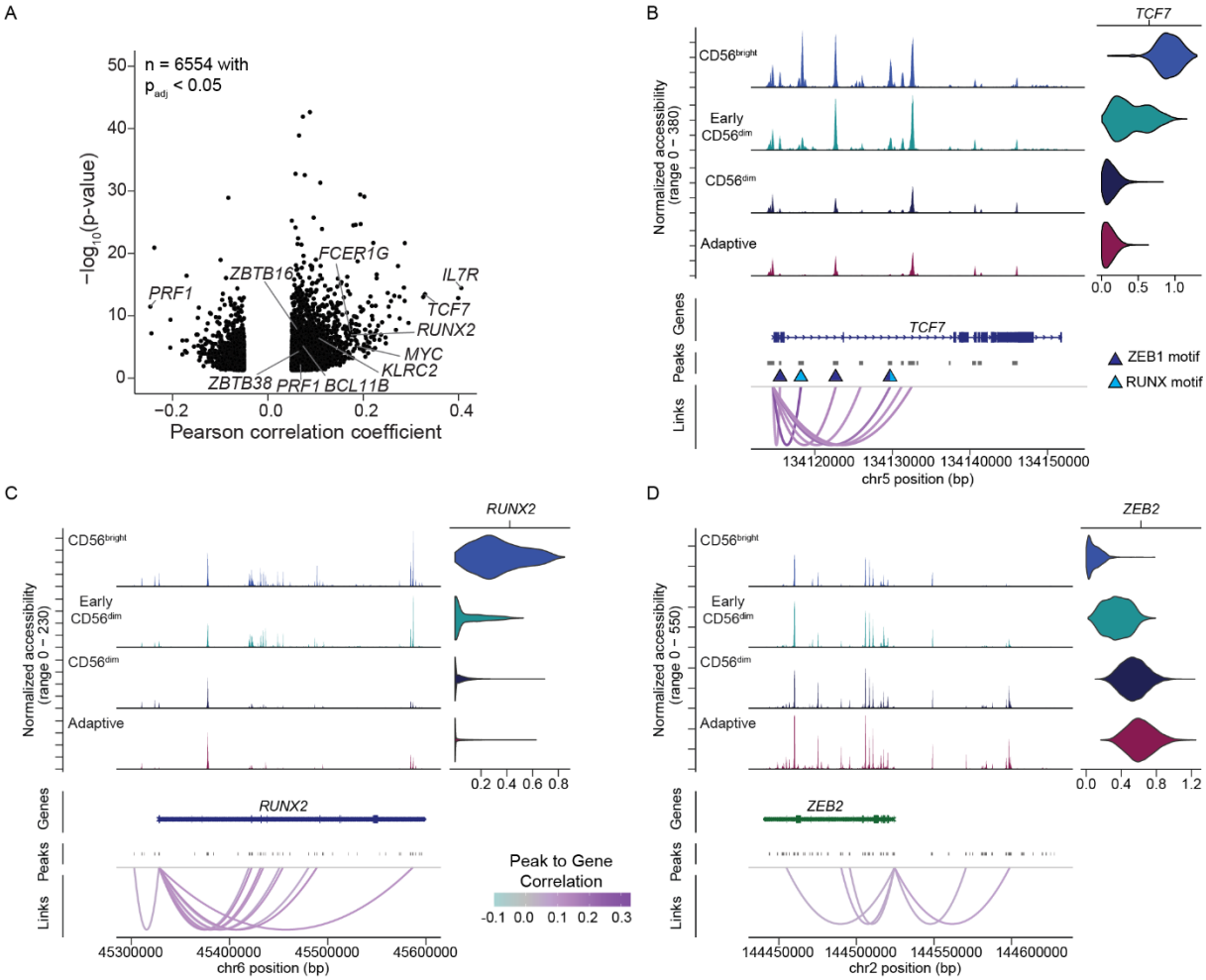

Figure 8 Label transfer links gene expression to subset-specific cis-regulatory elements. (A) Pearson correlation between open chromatin regions and gene expression. **(B-D)** Gene accessibility, imputed expression (violins), and their Pearson correlation (indicated as links) for *TCF7, RUNX2* and *ZEB2*.

4.2 Distinct signatures dissect NKG2C⁺ NK cells into naïve and adaptive

Our multi-omic analysis of human NK cells clearly revealed the presence of a separate and genomically distinct adaptive NK cell cluster (Figure 6A-G), characterized by high transcript levels of *KLRC2*, *CD3E* and *PATL2* together with marked downregulation of *CD7, KLRB1* and *FCER1G* (Figure 7B), as well as distinct surface marker expression of CD57 and CD2 coinciding with low NKp30, CD161 and NKG2A (Figure 6B,D). While adaptive NK cells shared large parts of their genomic effector signature with conventional CD56^{dim} NK cells, they displayed a marked reduction of *ZBTB16* and *ZNF516* and further increased gene scores for *ZEB2* and the metabolic regulator *ARID5B* (Cichocki et al., 2018), as compared to CD56^{dim} NK cells (Figure 7A). Moreover, they were characterized by unique expression and accessibility of *ZBTB38* and *JAKMIP1* (Figure 7A-B). Importantly, the integrated analysis demonstrated remodeling at chromatin regions around the *KLRCX* locus, with several of those being positively correlated with increased *KLRC2* expression and one region having a negative correlation with *KLRC1* expression (Figure 9). Their

functional role was supported by the presence of several TF binding motifs, with the most strongly correlating putative *KLRC1* enhancer containing a TCF7 motif, consistent with expression and accessibility in CD56^{bright} NK cells, while AP1 motifs were notable in the putative *KLRC2* enhancers specifically accessible in adaptive NK cells, which together may regulate the core adaptive NK cell phenotype characterized by high surface expression of NKG2C combined with lack of NKG2A (Gumá et al., 2004).

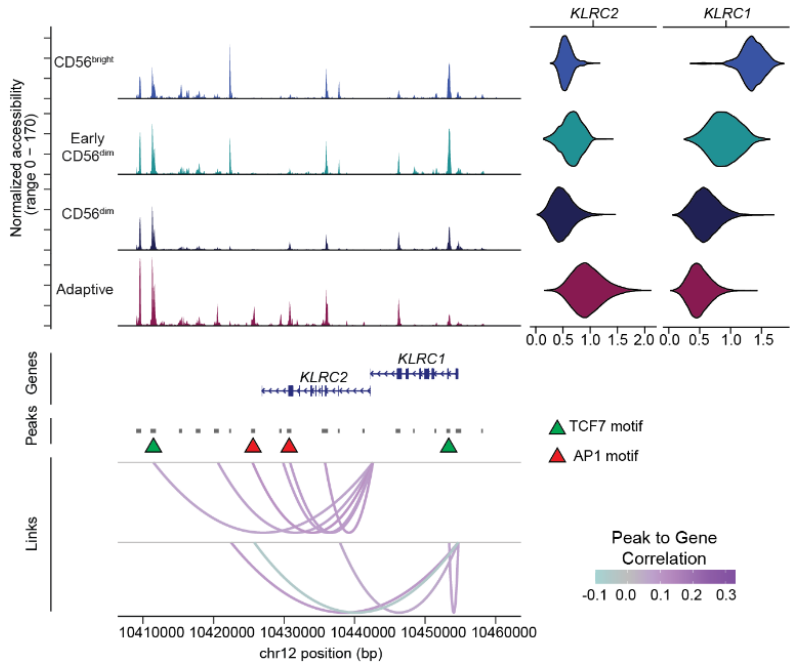

Figure 9 Adaptive NK cells are characterized by a remodeled *KLRCX* locus. Gene accessibility, imputed expression (violins), and their Pearson correlation (indicated as links) for *KLRC1* and *KLRC2*.

While expansion of adaptive NKG2C^{+} NK cells is associated with HCMV infection, NKG2C^{+} NK cells are present also in HCMV^{-} individuals, albeit at lower frequencies. To directly compare the transcriptional and epigenetic landscape of NKG2C$^{+/-}$ NK cells and their distribution within NK cell clusters in HCMV^{+} and HCMV^{-} individuals, we divided the datasets by HCMV-serostatus (Figure 10). As expected, the adaptive cluster was selectively present in HCMV^{+} individuals and mostly comprised of NKG2C^{+} NK cells (Figure 10A,C,E,G). Comparative analysis of chromatin accessibility and gene expression after down-sampling to equal cell numbers for both donor groups highlighted the specific impact of HCMV on NKG2C^{+} cells. A total of 232 genes were differentially accessible between the NKG2C^{+} and NKG2C^{-} populations in HCMV^{+} individuals, including key adaptive genes such as *JAKMIP1, ZBTB38, ZBTB16* or *ZNF516* (Figure 10I), along with 269 differentially expressed genes, including adaptive NK cell markers *CD3E*, *IL32*, *FCER1G* and *KLRB1* (Figure 10K). Conversely, NKG2C^{+} NK cells from HCMV^{-} donors were randomly dispersed among NKG2C^{-} NK cells (Figure 10E-H), and differences in chromatin accessibility and gene expression, apart from *KLRC2* (encoding NKG2C), were minor (Figure 10M,O), highlighting their largely similar epigenomes and transcriptomes. Genes differentially accessible or expressed between NKG2C$^{+/-}$ cells in HCMV^{+} individuals, including *ZBTB16* and *FCER1G*, followed

a coordinated pattern separating conventional from adaptive NK cells, whereas this was not observed in HCMV⁻ individuals (Figure 10,N,L,P), underlining that these differences reflect the adaptive remodeling induced in NKG2C⁺ cells by HCMV.

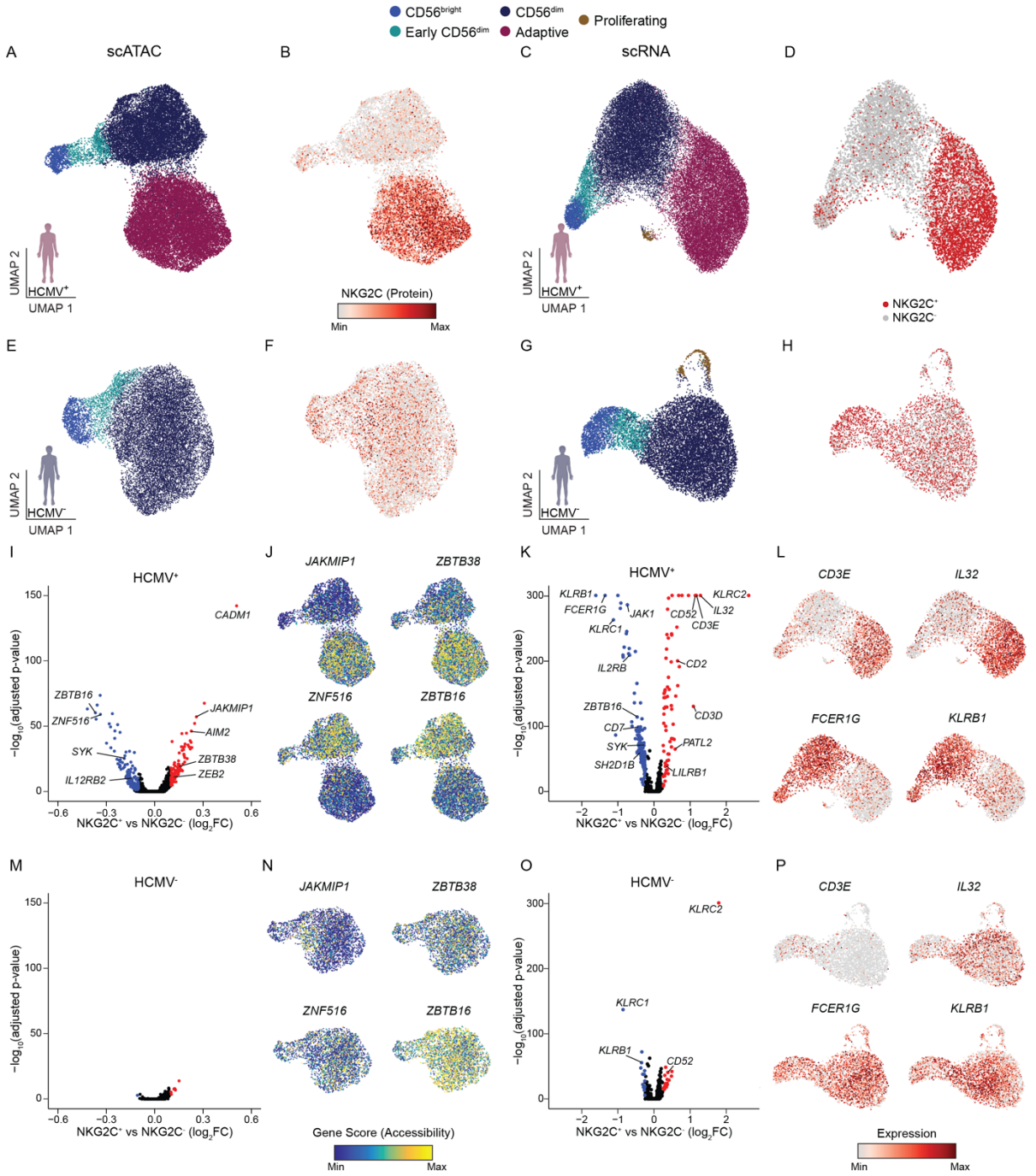

Figure 10 Epigenetic and transcriptional signatures of naïve and adaptive NKG2C⁺ NK cells. (A+E) UMAP embedding of NK cells from HCMV⁺ (A, n = 4) and HCMV⁻ (E, n = 4) donors analyzed by scATAC-seq. **(B+F)** NKG2C surface expression as measured by ASAP-seq. **(C+G)** UMAP embedding of NK cells from HCMV⁺ (C, n = 5) and HCMV⁻ (G, n = 2) donors analyzed by scRNA-seq. **(D+H)** Distribution of barcoded NKG2C⁺ and NKG2C⁻ populations. **(I+M)** Differentially accessible genes between NKG2C⁺ and NKG2C⁻ NK cells for HCMV⁺ (I) and HCMV⁻ (M) donors as determined by logistic regression. **(J+N)** Representative accessibility scores of genes defining adaptive NK cells for HCMV⁺ (J) and HCMV⁻ (N) donors. **(K+O)** Differentially expressed genes between NKG2C⁺ and NKG2C⁻ NK cells for HCMV⁺ (K) and HCMV⁻ (O) donors by Wilcoxon rank sum test. **(L+P)** Representative expression of genes defining adaptive NK cells for HCMV⁺ (L) and HCMV⁻ (P) donors. Created with BioRender.com.

Interestingly, even HCMV^{+} individuals displayed a small fraction of NKG2C^{+} NK cells that were present in all three subsets of the conventional NK cell compartment (Figure 10A-D). Indeed, separate analysis of NKG2C^{+} NK cells alone from HCMV^{+} individuals revealed the co-existence of adaptive NKG2C^{+} NK cells with a minority of "naive" NKG2C^{+} NK cells (Figure 11A,D) that lacked adaptive remodeling (Figure 11B,E) and spanned the whole spectrum of conventional NK cells, recapitulating the signatures identified from the full integrated analysis (Figure 11C,F). We could further confirm by flow cytometry that the frequency of naive NKG2C^{+} NK cells was comparable between HCMV$^{+/-}$ individuals and that they were similarly distributed between the CD56^{bright} and CD56^{dim} compartment (Figure 11G-I).

Together, this comparative analysis of HCMV^{+} and HCMV^{-} donors highlighted the pronounced remodeling HCMV imposes on the NKG2C^{+} NK cell pool that coexist with a minority of naive NKG2C^{+} NK cells during the latent phase of HCMV infection.

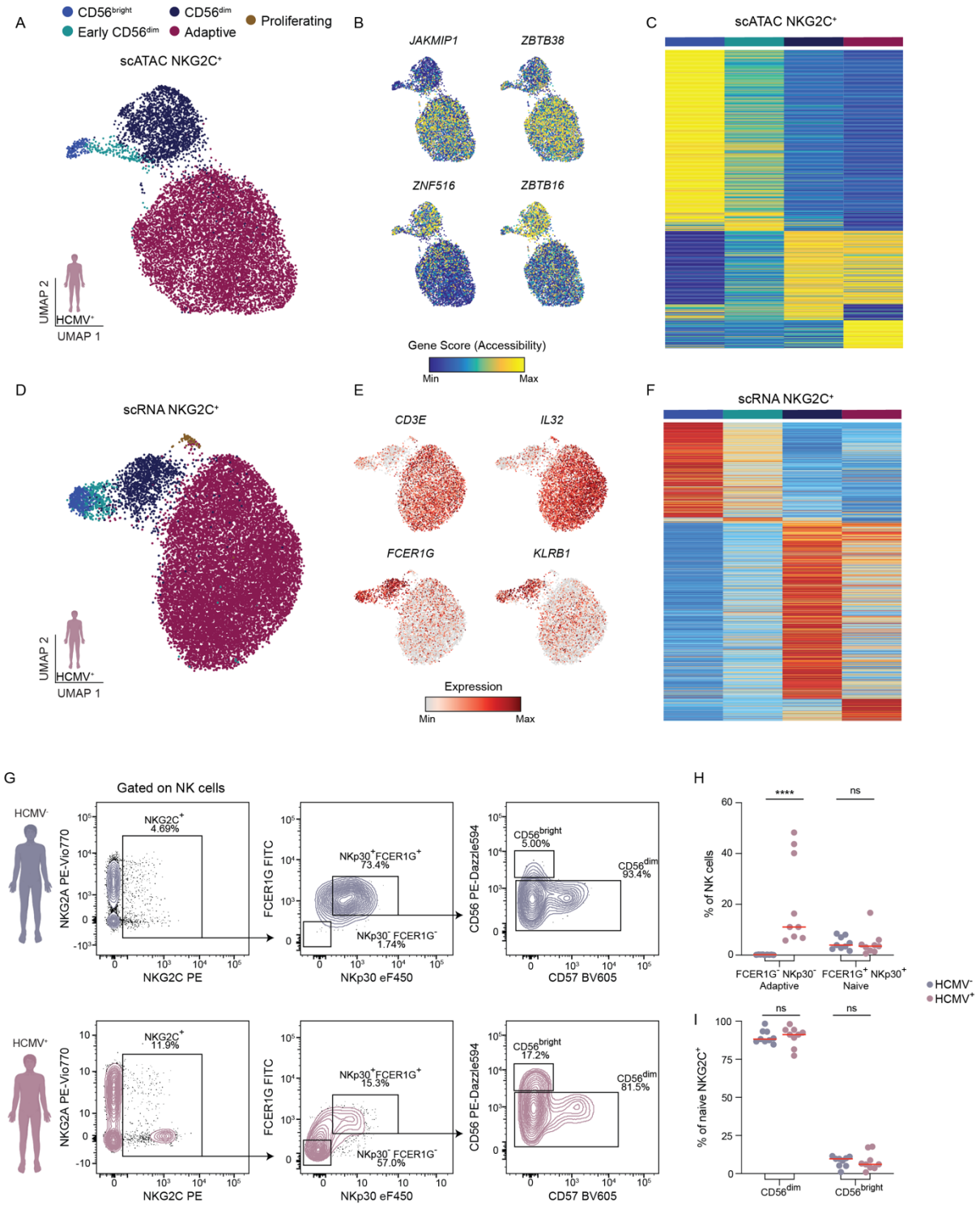

Figure 1l Naïve NKG2C⁺ NK cells are present in HCMV⁻ and HCMV⁺ individuals. **(A+D)** UMAP embedding of scATAC-seq (A) and scRNA-seq (D) of NKG2C⁺ NK cells from HCMV⁺ individuals. **(B+E)** Accessibility (B) and expression (E) of key genes regulated in adaptive NK cells. **(C+F)** Row-scaled accessibility (C) and expression (F) of NK cell subset-specific signatures from fully integrated datasets in NKG2C⁺ NK cell clusters from HCMV⁺ donors. **(G-I)** Representative expression (G) and frequencies (H) of FCER1G⁺ NKp30⁺ ("Naive") and FCER1G⁻ NKp30⁻ ("Adaptive") NKG2C⁺ NK cells and distribution of naïve NKG2C⁺ NK cells into CD56^{bright} and CD56^{dim} subsets (I) in HCMV⁻ (n = 9) and HCMV⁺ (n = 9) donors. Kruskal-Wallis test. **** $p < 0.0001$, ns non-significant. Created with BioRender.com.

4.3 HCMV infection leaves an inflammatory memory footprint enriched for AP1 motifs

While gene expression profiles and accessibility of regulatory elements in the proximity of *trans*-acting factors provide a view on the current state of a cell, analysis of TF motif enrichment in *cis*-regulatory elements has the ability to capture molecular events that have left footprints in the generation of this state. Global analysis using chromVAR (Schep et al., 2017) identified a strong signature in CD56^{bright} NK cells with enhanced activity of motifs for TFs such as TCF7, RUNX2 or ZEB1 (Figure 12A-E), consistent with an important role of these TFs in driving the epigenetic and transcriptional identity of this subset (Figure 7A-B) (Collins et al., 2019; Holmes et al., 2021), as well as increased motif activity for NFκB-related TFs (REL) and STAT4 (Figure 12A-E), reflecting the enhanced responsiveness of CD56^{bright} NK cells to activation by pro-inflammatory cytokines (Cooper et al., 2001). Accordingly, an NFκB- and STAT4-motif containing peak in the proximity of the *IFNG* gene was specifically accessible in CD56^{bright}, while closed in the more mature subsets (Figure 12A-E F). Similarly, T-box family motifs, to which the NK-cell lineage-defining TFs Eomes and T-bet belong, were most active in CD56^{bright} NK cells (Figure 12A,C), but were also significantly enriched in accessible chromatin regions specific to CD56^{dim} NK cells (Figure 12D), consistent with their lineage-defining role in NK cells (Gordon et al., 2012). While CD56^{dim} NK cells were mostly characterized by a lack of the CD56^{bright}-specific TF motif activity, both CD56^{dim} and adaptive NK cells were further distinguished from CD56^{bright} NK cells by enhanced CTCF motif activity (Figure 12B,E), as previously described (Holmes et al., 2021).

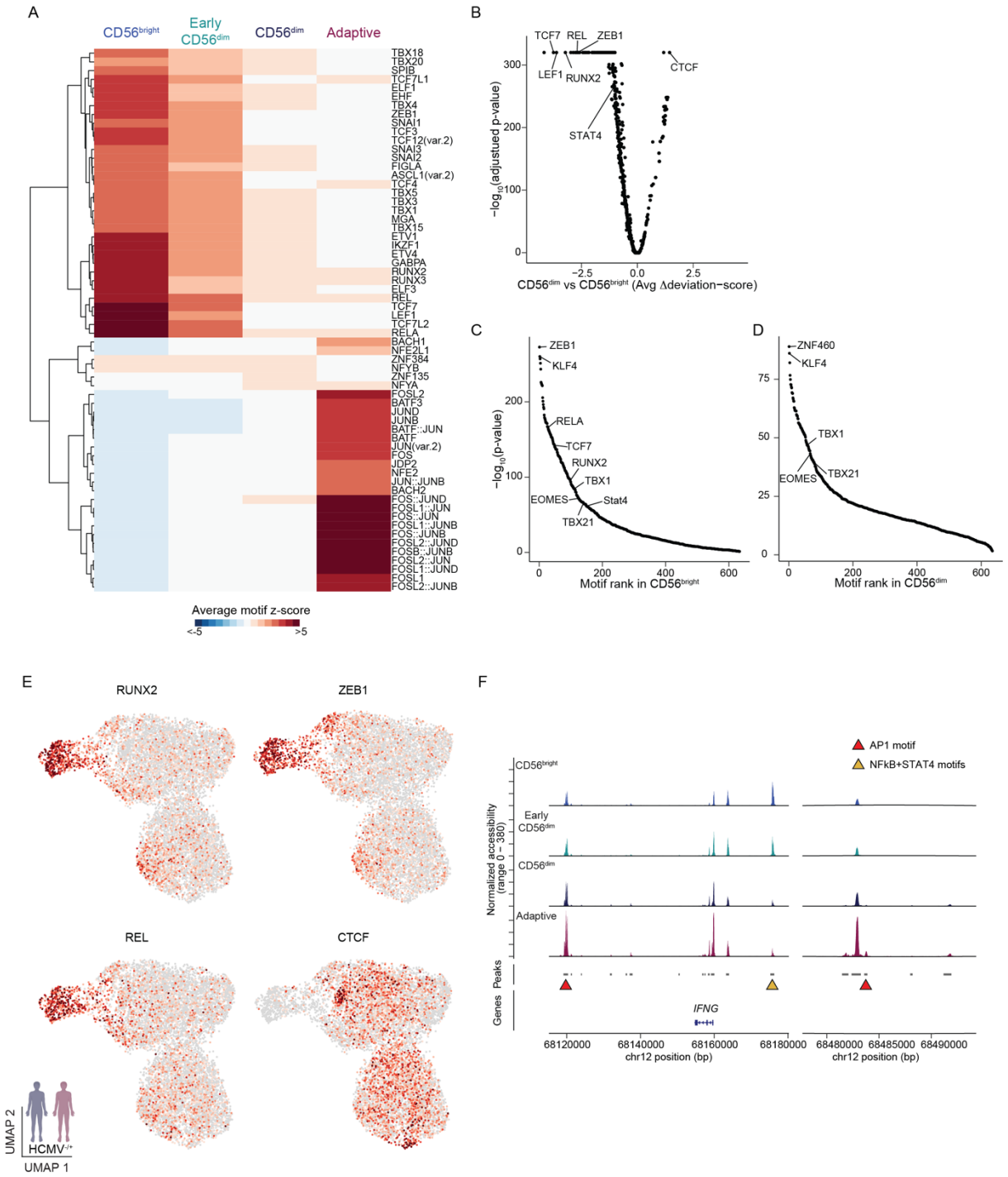

Figure 12 Motif enrichment reflects subset-specific TF imprinting. (A) Heat map of differentially active motifs represented as average chromVAR deviation-scores per cluster. **(B)** Differentially active motifs between CD56^{dim} and CD56^{bright} NK cells by Wilcoxon rank sum test. **(C-D)** Motif enrichment in chromatin regions specifically accessible in CD56^{bright} (B) and CD56^{dim} NK cells as determined by hypergeometric test. **(E)** Activity of the respective TF motifs projected onto UMAP embedding. **(F)** Accessibility of *IFNG*-associated open-chromatin regions containing AP1 or STAT4 and NFκB motifs. Created with BioRender.com.

Importantly, adaptive NK cells showed a strikingly separated signature in comparison to both conventional NK cell subsets, characterized by enhanced activity of AP1 motifs, with the strongest difference observed for FOS::JUNB (Figure 12A, Figure 13A-B) and AP1 motifs were the most significantly enriched in adaptive-NK cell specific accessible regions (Figure 13C-D). *De novo*

motif prediction on these peaks also yielded a canonical AP1 motif as the most significant hit, further corroborating these results (Figure 13E). The same analysis also yielded an IRF-associated motif, pointing towards potential cooperation between these TF families, as described for T cells (Glasmacher et al., 2012; Li et al., 2012). Recently, AP1 motifs have been described to be at the core of a unifying inflammatory memory signature in different immune cell types, as well as murine epithelial stem cells, characterized by specific accessibility in the proximity of the *Aim2* and *Cadm1* loci (Larsen et al., 2021). Notably, their human orthologs showed also highly specific opening of AP1-motif containing regions in adaptive NK cells (Figure 13D,F), and were among the genes with the most pronounced difference in accessibility between $NKG2C^+$ and $NKG2C^-$ cells in $HCMV^+$ individuals (Figure 10I). The existence of a memory unifying signature was further supported by an adaptive NK-cell specific open chromatin region with an AP1 motif in the proximity of *RPTOR* (Figure 13D,F), the key component of the metabolic regulatory complex mTORC1 required for the metabolic reprogramming of T cells (Yang et al., 2013) and trained macrophages (Cheng et al., 2014). Finally, among the more accessible peaks with AP1 motifs were several enhancers of the *IFNG* gene (Figure 12F), providing a possible mechanism for the increased IFN-γ production by adaptive NK cells to activating receptor stimulation (Foley et al., 2012; Luetke-Eversloh et al., 2014b). Overall, these findings demonstrate how differential TF activity shapes NK cell epigenetic remodeling during differentiation and identify AP1 TFs as main drivers shaping adaptive NK cell chromatin accessibility, an intriguing parallel to the inflammatory imprinting utilized to establish immune memory in different cell types (Larsen et al., 2021; Ostuni et al., 2013; Yukawa et al., 2020).

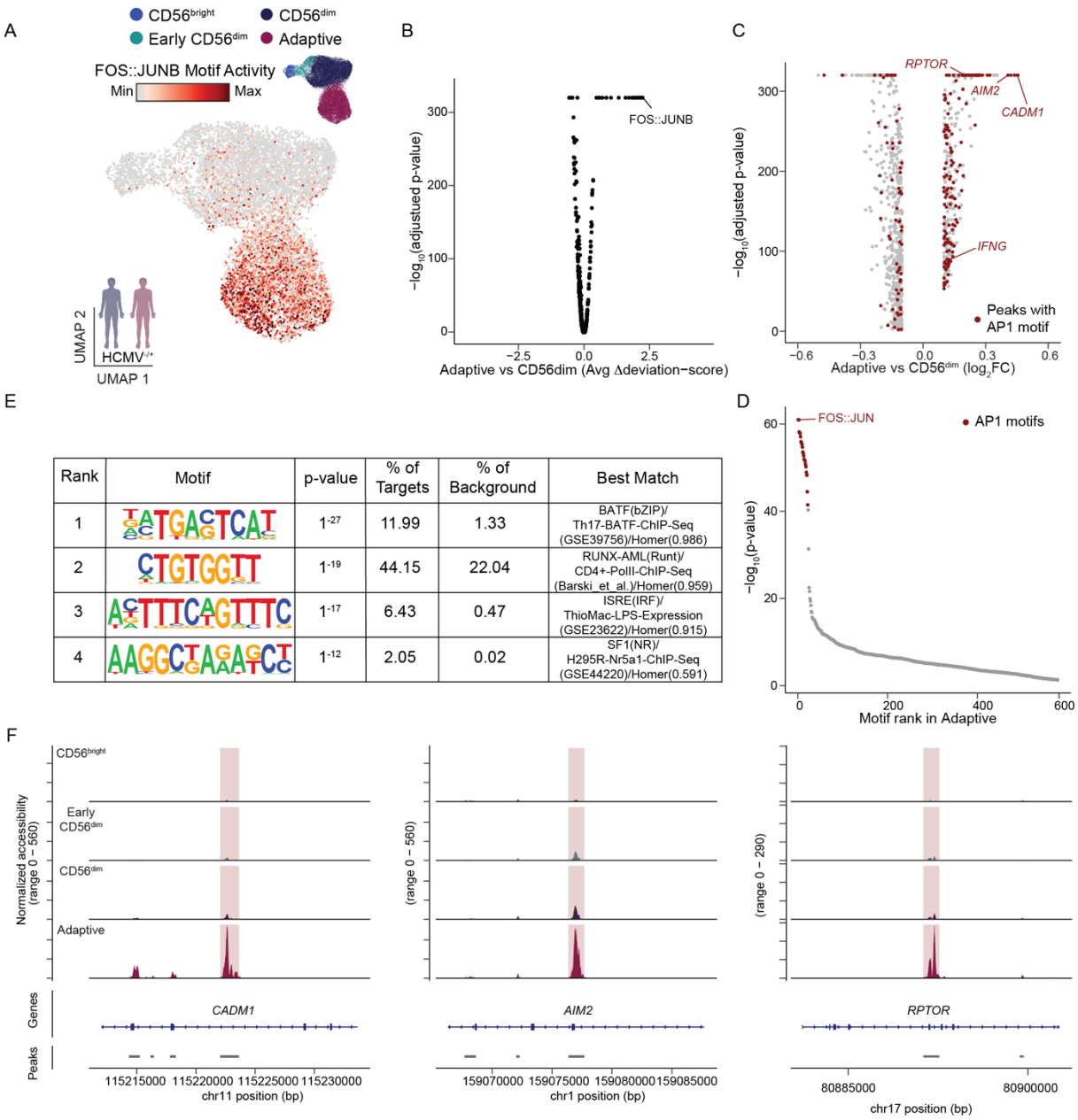

Figure 13 HCMV infection leaves a persistent chromatin footprint enriched for AP1 motifs. (A) FOS::JUNB motif activity projected on UMAP embedding. **(B)** Differentially active motifs between adaptive and CD56^{dim} NK cells by Wilcoxon rank sum test. **(C)** Differentially accessible regions between adaptive and CD56^{dim} NK cells by Wilcoxon rank sum test; AP1-motif containing regions are marked in red and selected regions annotated by gene proximity. **(D)** Motif enrichment in chromatin regions specifically accessible in adaptive NK cells as determined by hypergeometric test. **(E)** *De novo* motif analysis results on chromatin regions specifically accessible in adaptive NK cells. **(F)** Per cluster accessibility of AP1-motif containing chromatin regions in the proximity of *CADM1*, *AIM2* and *RPTOR*. Created with BioRender.com.

4.4 Synergistic imprinting by HCMV peptides and pro-inflammatory cytokines

We have previously shown that activation by IL-12 and IL-18 in combination with the engagement of NKG2C by HCMV-derived peptides induces the specific expansion of NKG2C^{+} NK cells with some of the transcriptional characteristics also observed *ex vivo*, as well as demethylation

of the *IFNG* locus (Hammer et al., 2018b). To assess whether these signals drive global epigenetic reprogramming, including AP1 TF activity, and mimic HCMV-induced activation of naïve NKG2C$^+$ NK cells, we co-cultured NK cells from HCMV$^-$ donors for 12 h with IL-15 and RMA-S/HLA-E target cells pulsed with the NKG2C activating VMAPRTLFL (LFL) or non-activating VMAPQSLLL peptide, in the presence or absence of the pro-inflammatory cytokines IL-12 and IL-18. We marked the different conditions with individual nucleotide-barcoded hashtags, sorted for NKG2C$^+$ cells and performed ASAP-seq (Figure 14A).

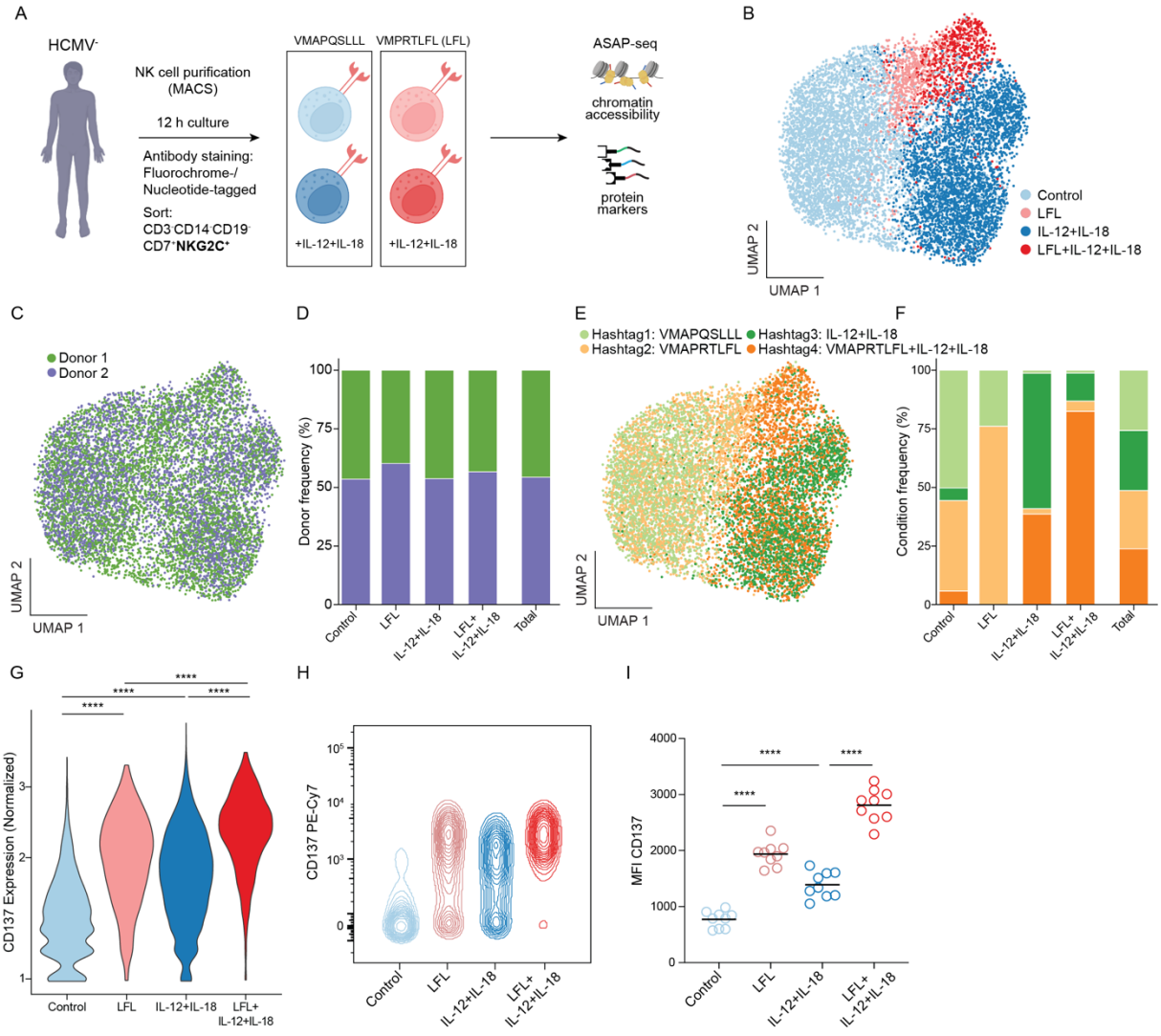

Figure 14 HCMV peptides and pro-inflammatory cytokines induce distinct response patterns. (A) NK cells from 2 HCMV$^-$ individuals were co-cultured for 12 h with RMA-S/HLA-E target cells pulsed with the indicated peptides and 10 ng/mL IL-15, in the presence (dark shades) or absence (light shades) of IL-12 and IL-18. Different conditions were marked with nucleotide-labeled hashtags and analyzed by ASAP-seq. **(B)** UMAP embedding, clusters were annotated based on clear enrichment of cells from the indicated conditions. **(C-D)** Distribution of cells colored by donor origin and quantification per cluster. **(E-F)** Distribution of cells colored by culture condition and quantification per cluster, utilized for cluster annotation. **(G)** Surface expression of CD137 per cluster; Wilcoxon rank sum test. **(H-I)** Representative (H) and mean (I) CD137 expression on NKG2C$^+$ NKG2A$^-$ NK cells from HCMV$^-$ donors (n = 9) cultured under the indicated conditions; One-way ANOVA. Created with BioRender.com.

Analysis of high-quality cells clustering by condition and irrespective of donors (Methods) enabled us to identify the epigenetic signatures acquired by activated NKG2C$^+$ NK cells in a stimulus-dependent fashion, defining 4 main clusters clearly enriched for cells cultured under the respective conditions, i.e. non-activated ("control"), activated by LFL peptide ("LFL"), pro-inflammatory cytokines ("IL-12+IL-18") or a combination thereof ("LFL+IL-12+IL-18") (Figure 14B-F, Methods). Activation status of cells recruited in stimuli-associated clusters was clearly exemplified by surface up-regulation of CD137 (4-1BB), with maximal expression observed on cells receiving both peptide and cytokine stimulation (Figure 14G). Differences in expression levels of CD137 across conditions were confirmed by flow cytometry (Figure 14H-I), validating the proteogenomic-based cluster annotations.

While IL-12+IL-18 stimulation alone promoted the activation of NKG2A$^+$ NKG2C$^+$ naive NK cells, hardly any NKG2A$^+$ NKG2C$^+$ cell was recruited into response patterns induced by peptide stimulation (Figure 15A), in line with the dominant inhibitory function of NKG2A engagement (Valés-Gómez et al., 1999). As, in contrast to adaptive NKG2C$^+$ NK cells (Gumá et al., 2004), a large fraction of naive NKG2C$^+$ NK cells co-express NKG2A (Figure 15B-C), these data suggest that peptide recognition might be decisive in biasing the pool of naive NKG2C$^+$ cells recruited during HCMV infection towards NKG2A$^-$ NKG2C$^+$ cells.

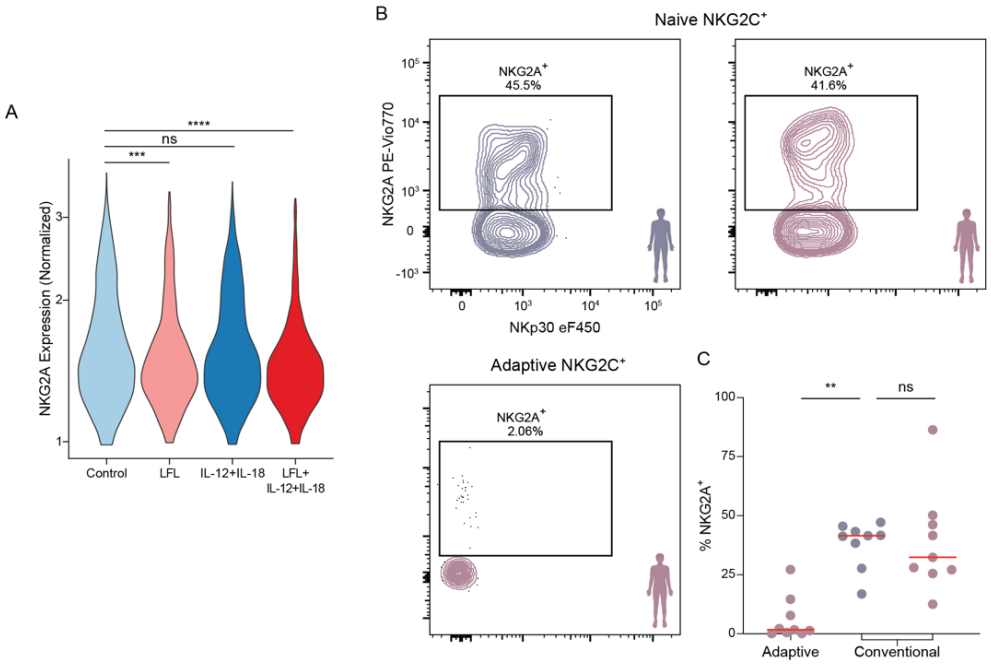

Figure 15 NKG2A biases recruitment of NKG2C$^+$ NK cells by HCMV peptides. (A) Surface expression of NKG2A per cluster; Wilcoxon rank sum test. (B-C) Representative (B) and quantification (C) of NKG2A expression in naïve and adaptive NKG2C$^+$ NK cells from HCMV$^-$ (n = 9) and HCMV$^+$ (n = 9) donors; see Figure 11G for gating strategy; Wilcoxon rank sum test. Created with BioRender.com.

Activation via pro-inflammatory cytokines or by peptide-recognition via NKG2C induced pronounced chromatin remodeling, manifesting in 1232 and 936 differentially accessible regions (DARs) respectively, as compared to the control condition (Figure 16A). Analysis of the overlaps between the individual conditions revealed both distinct and shared effects of activation by cytokines and peptide, with most regions undergoing remodeling upon synergistic activation by the two combined stimuli, with 2693 DARs in total (Figure 16A). Analysis of motif activity revealed different TF families contributing to these effects (Figure 16B). Besides EGR, MYC and NFκB, LFL peptide activation induced remarkable activity of AP1- and IRF-family TFs. Consistent with reports in mice (Wiedemann et al., 2021), pro-inflammatory cytokines IL-12+IL-18 were highly specific in their induction of POU TF-family activity, along with NFκB. However, clear synergism between the two stimuli, cytokines and peptide, became apparent on the TF motif activity level. In particular, AP1 activity was significantly enhanced as compared to either single stimulus (Figure 16B-C). Importantly, we noted that the stimuli induced several features associated with adaptive NK cells *ex vivo*. This included opening of chromatin in the *ZEB2* and *ARID5B* loci, which was mainly dependent on pro-inflammatory cytokines or similarly enabled by each individual stimulus, respectively, as well closing of the *ZBTB16* locus (Figure 16D-G). However, most of these changes were more pronounced upon synergistic activation, as was also the case for chromatin accessibility in the *AIM2* encoding region (Figure 16D-G).

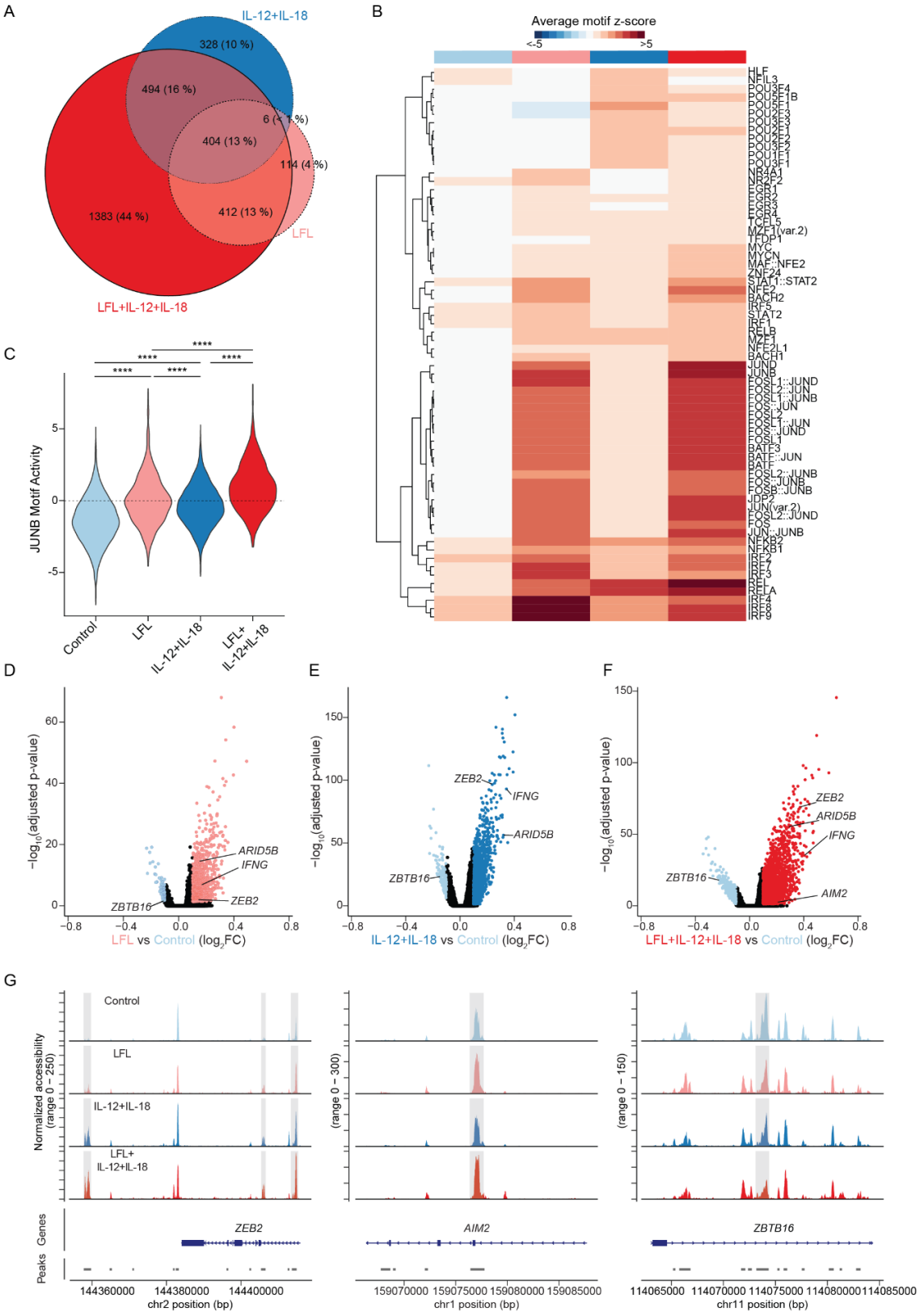

Figure 16 HCMV peptides and pro-inflammatory cytokines synergistically drive AP1 activity. (A) Euler diagram illustrating overlap of differentially accessible regions between clusters. **(B)** Motif activity of differentially active motifs represented as average chromVAR deviation-scores per cluster. **(C)** JUNB motif activity per cluster; Wilcoxon rank sum test. **(D-F)** Differentially accessible regions between LFL and control (D), IL-12+IL-18 and control (E), and LFL+IL-12+IL-18 and control (F) by logistic regression. **(G-I)** Accessibility of regions near key adaptive NK cell-related genes regulated after stimulation. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

To globally assess whether and which of the activation-induced signatures best reconcile the adaptive signature, we performed anchor-based integration of the *in vitro* stimulated cells with our *ex vivo* dataset (see section 3.4.2.8) (Stuart et al., 2019), thereby enabling a classification of the *in vitro* induced signatures based on chromatin profiles observed *ex vivo*. Both, activation by peptide and cytokines induced a certain extent of adaptive NK cell remodeling, but by far the highest adaptive prediction score was observed for cells activated by both stimuli, in concert with the reduction of the prediction score for conventional CD56^{dim} NK cells (Figure 17A-C). The close relatedness of the adaptive *ex vivo* signature to the chromatin remodeling induced by synergistic *in vitro* activation was further supported by a strong correlation of overlapping DARs when comparing CD56^{dim} to adaptive NK cells and vs. control to LFL+IL-12+IL-18 activated cells (Figure 17C). Among these conserved DARs, we identified core features of adaptive NK cells such as reduced accessibility at the *ZBTB16* locus and increased accessibility of *AIM2* or *IFNG* loci (Figure 17C). Importantly, these modulated chromatin regions showed a strong enrichment of AP1 motifs, whereas NFκB, NFAT and STAT4 motifs were much less enriched (Figure 17D). Together, these findings directly connect pro-inflammatory cytokines and NKG2C activating peptide, both provided during HCMV infection, to the characteristic phenotype and epigenetic remodeling of adaptive NK cells as observed *ex vivo*. Their synergism manifested on several layers from surface upregulation of CD137, over TF motif activity, to global chromatin remodeling, thereby recapitulating features of adaptive NK cell signatures of open chromatin enriched for AP1 motifs.

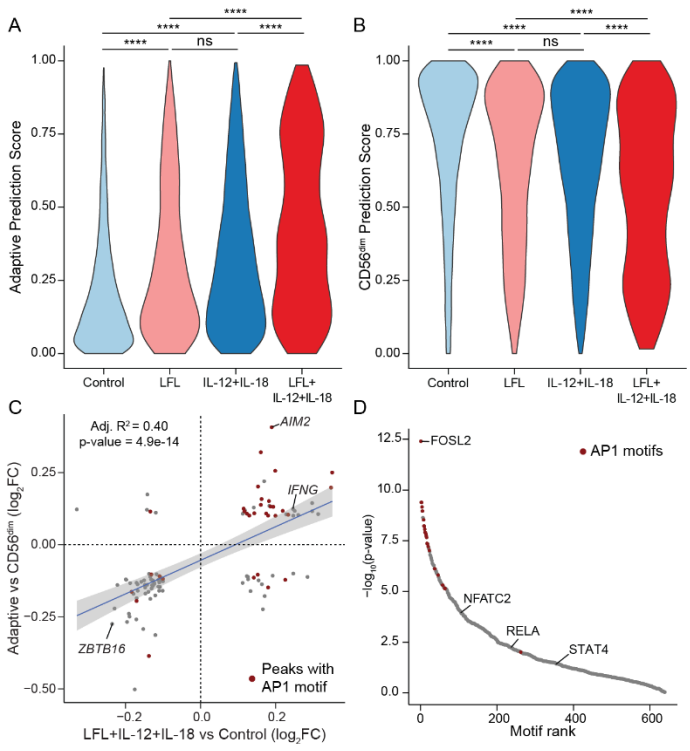

Figure 17 HCMV peptides and pro-inflammatory cytokines synergistically promote adaptive chromatin remodeling in naïve NKG2C^{+} NK cells. (A-B) Prediction scores for classification as adaptive (A) and CD56^{dim} (B) NK cells based on integration with the *ex vivo* dataset as reference; Wilcoxon rank sum test. **(C)** Linear correlation between differentially accessible regions both induced by in vitro activation and observed ex vivo between adaptive and CD56^{dim} NK cells; p-value from F-test. **(D)** Motif enrichment in open chromatin regions displayed in (C) as determined by hypergeometric test.

4.5 Convergent and divergent epigenetic features of adaptive NK cells

The integrated analysis of different HCMV$^+$ donors revealed consistent epigenetic, transcriptional and phenotypic features of the adaptive NK cell signature *ex vivo*. However, integration of donors masked donor-specific heterogeneity within the adaptive NK cell compartment. Indeed, separate analysis of the epigenetic landscape of individual donors revealed clearly defined adaptive subclusters (Figure 18A). These populations were characterized by an adaptive phenotype, largely lacking surface expression of NKp30 and NGK2A, while being mostly positive for NKG2C and self-MHC-specific KIRs (Figure 18B, Table 5). Interestingly, subcluster 4 in donor P1 likely represents an NKG2C$^-$ adaptive NK cell expansion (Figure 18B), as previously described by others (Béziat et al., 2013; Liu et al., 2016; Schlums et al., 2015).

Table 5 KIR ligand genotyping results. KIR ligand genotypes of four HCMV+ donors were determined by sequence-specific priming PCR (see Methods).

Donor ID	HLA-A	HLA-B	HLA-C	Educating KIRs
P1	Bw4$^-$	Bw4$^+$Ile80	C1/C2	KIR2DL1, KIR2DL2/3, KIR3DL1
P2	Bw4$^+$	Bw4$^+$Ile80	C2/C2	KIR2DL1, KIR3DL1
P3	Bw4$^+$	Bw4$^+$Thr80	C1/C2	KIR2DL1, KIR2DL2/3, KIR3DL1
P4	Bw4$^-$	Bw4$^-$	C1/C2	KIR2DL1, KIR2DL2/3

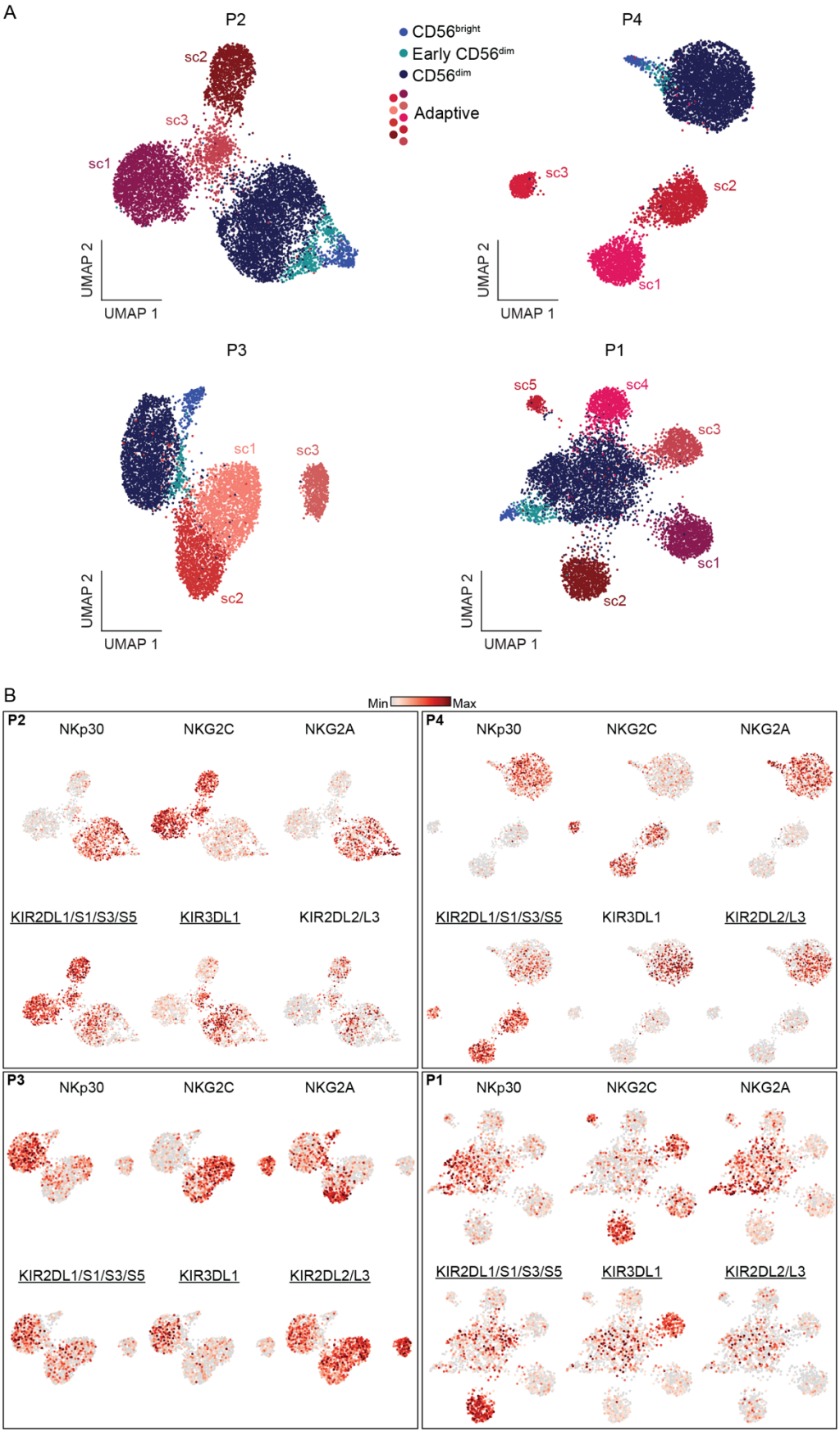

Figure 18 Donor-specific analysis identifies adaptive NK cell heterogeneity. (A) UMAP embedding of NK cells from four HCMV⁺ individuals analyzed by scATAC-seq. **(B)** Surface expression of the indicated proteins projected on the UMAP embeddings for each donor. Educating KIRs are underlined, see Table 5.

To assess the degree of convergence of the adaptive NK cell signature, we analyzed the overlap of differentially accessible regions between the total adaptive and the CD56^{dim} compartment across HCMV^{+} donors (P1, P2, P3, P4). This comparative analysis revealed that around half of all DARs were shared between at least three donors, similar to the signatures distinguishing CD56^{bright} from CD56^{dim} NK cells (Figure 19A-B). Consistent with the integration into a donor-overarching adaptive cluster (Figure 6A), this adaptive signature was shared by all adaptive subclusters, separating them from the corresponding conventional CD56^{dim} populations (Figure 19C). Many of the features identified in the integrated analysis were highly penetrant across donors and subclusters, such as the peak in the proximity of *AIM2* (Figure 19D), as well as strongly enhanced AP1 motif activity (Figure 19E), further supporting the idea that this convergent epigenetic signature reflects a coordinated program induced in response to signals received during HCMV infection.

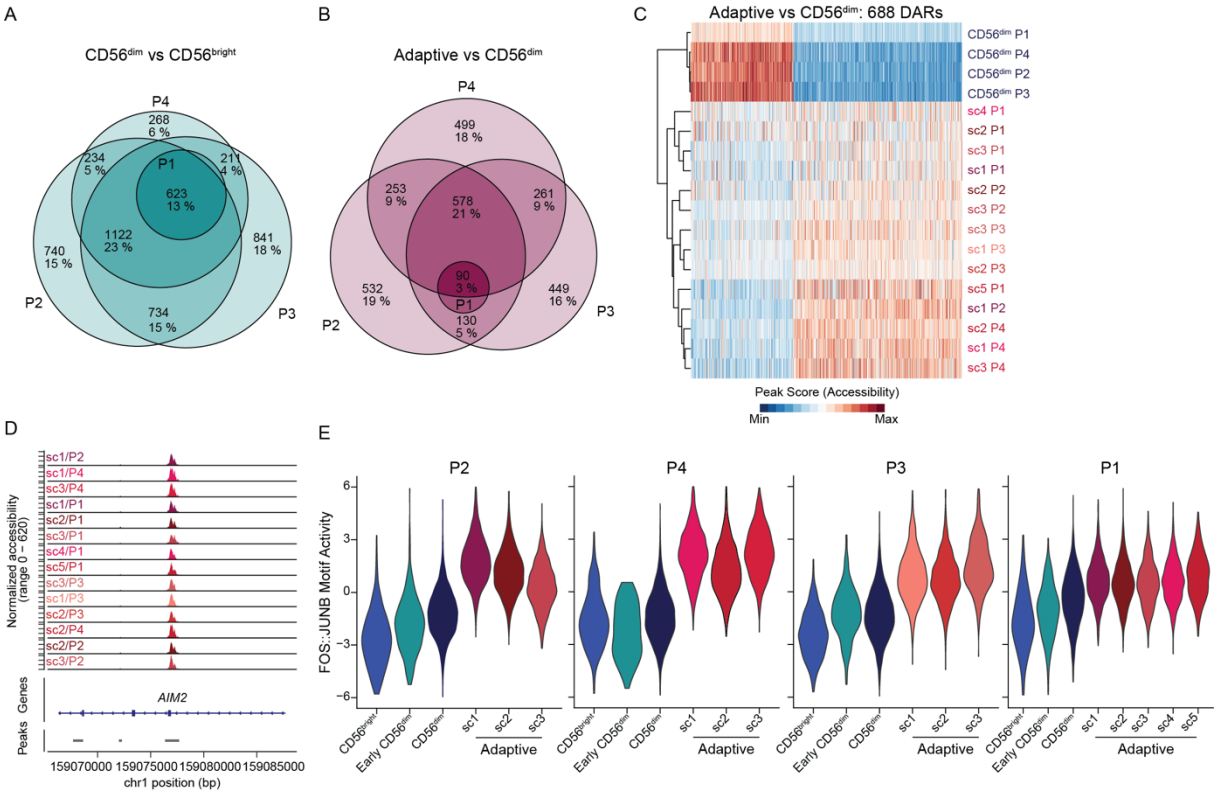

Figure 19 Convergent epigenetic features of adaptive NK cells. (A-B) Euler diagrams illustrating overlap of differentially accessible regions (DARs) across donors comparing CD56^{dim} and CD56^{bright} (A) or adaptive and CD56^{dim} NK cells (B). **(C)** Column-scaled accessibility of differentially accessible regions (DARs) shared between at least three donors comparing the total adaptive and CD56^{dim} compartments. **(D)** Accessibility of adaptive NK-cell defining *AIM2* region within each donor and adaptive subcluster. **(E)** FOS::JUNB motif activity within each donor.

In contrast to these largely shared aspects of adaptive NK cell chromatin accessibility, DARs between adaptive subclusters were much less conserved. Indeed, almost 80 % of these DARs were donor- and subcluster-specific (Figure 20A). Each adaptive subcluster showcased highly

unique open chromatin regions (Figure 20B-C), underlining their distinctive epigenetic make up.

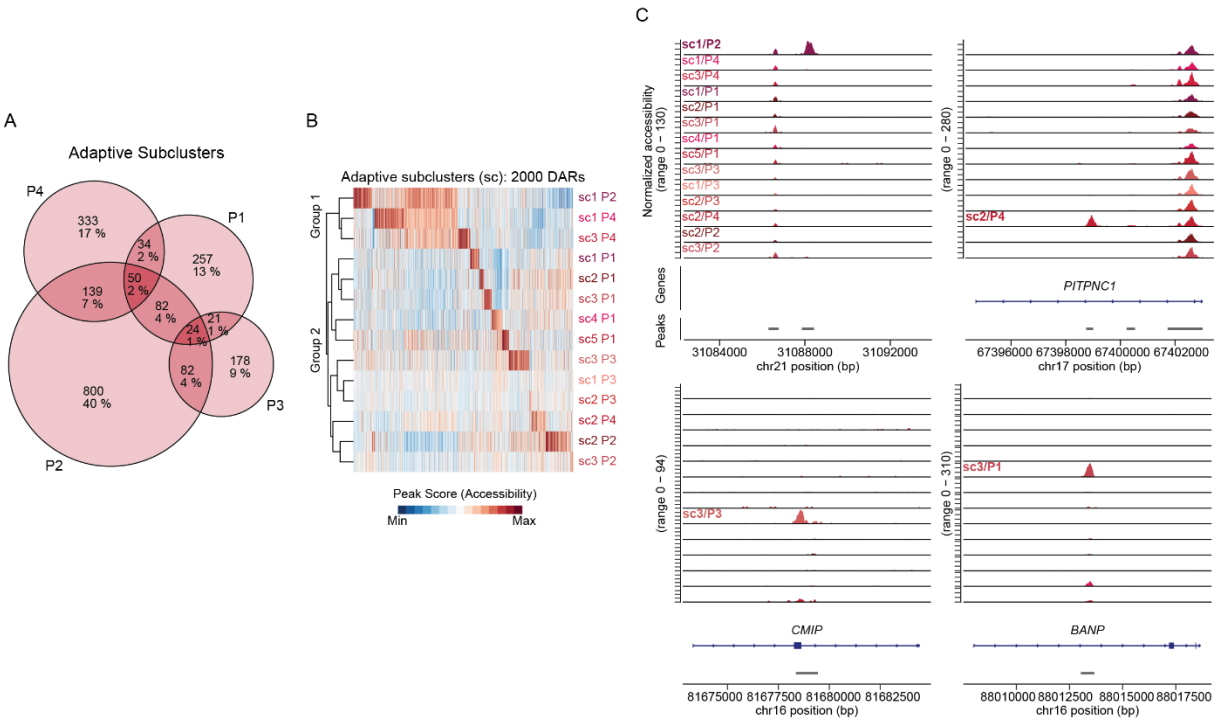

Figure 20 Divergent epigenetic features of adaptive NK cells. **(A)** Euler diagrams illustrating overlap of differentially accessible regions (DARs) across donors adaptive subclusters (sc). **(B)** Column-scaled accessibility of subcluster-defining DARs and hierarchical clustering of adaptive subclusters for all individuals. **(C)** Accessibility of representative subcluster-specific chromatin regions for each donor.

Nevertheless, hierarchical clustering divided the adaptive subclusters into two groups, mainly driven by overlapping signatures of three subclusters (Figure 20B). Importantly, when directly comparing the gene scores of the two groups, two clear signatures emerged, which had an intriguing overlap with those defining CD56^{bright}/early CD56^{dim} NK cells such as *RUNX2*, *BACH2, TCF7, IL7R* and *SELL* (encoding CD62L) on the one, and CD56^{dim} associated genes such as *GLI3* or *ZBTB16* on the other end (Figure 21A). Indeed, differential accessibility of the respective chromatin regions between the adaptive subcluster groups significantly correlated with the differences between early CD56^{dim} and CD56^{dim} NK cells (Figure 21B) and followed consistent patterns as exemplified for chromatin regions in the proximity of *TCF7* or *PCNT* (Figure 21C), suggesting conventional NK cell maturation signatures are preserved upon adaptive differentiation. This was also supported by the differential activity of CD56^{bright}/early CD56^{dim} associated motifs such as TCF7L2 on the one, and the CD56^{dim} associated CTCF motif on the other hand (Figure 21D). Strikingly, the group rather resembling CD56^{bright}/early CD56^{dim} NK cells was also marked by surface expression of CD62L (Figure 21E), consistent with its expression by CD56^{bright} and early CD56^{dim} NK cells within the conventional compartment (Juelke et al., 2010) (Figure 6A-D). Besides, the clusters which shared part of the CD56^{bright}/early CD56^{dim} signature had relatively even higher AP1 motif activity (Figure 21F), suggesting the two groups might also reflect a different

extent of adaptive chromatin remodeling. Overall, this donor-centric analysis revealed an unappreciated heterogeneity within the adaptive NK cell compartment. Heterogeneity was defined by a highly diverse set of unique, subcluster-specific peaks and signatures resembling gradients in conventional NK cell maturation, possibly reflecting the cell of origin.

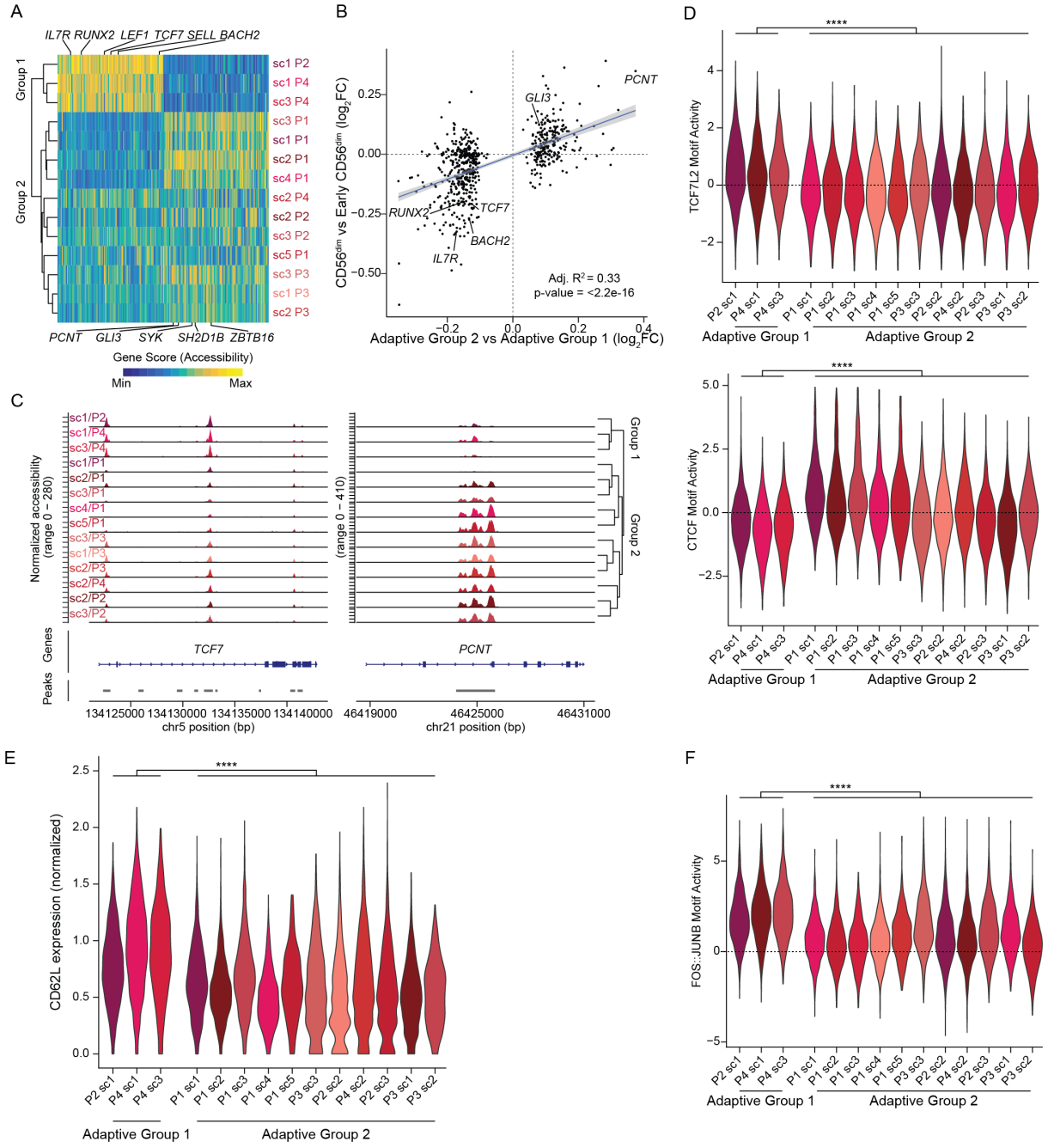

Figure 21 Adaptive subclusters can be dissected into two groups reflecting NK cell maturation. **(A)** Top 100 differential column-scaled gene scores for each adaptive subcluster group by Wilcoxon rank sum test. **(B)** Linear correlation between DARs comparing the two adaptive subcluster groups and CD56^{dim} to early CD56^{dim} NK cells; p-value from F-test. **(C)** Accessibility of adaptive group-defining regions within *TCF7* and *PCNT* for each donor and adaptive subcluster. **(D)** Normalized CD62L surface expression per cluster; Wilcoxon rank sum test. **(E)** TCF7L2 and CTCF motif activity for each donor and adaptive subcluster; Wilcoxon test. **(F)** FOS::JUNB motif activity for each donor and adaptive subcluster; Wilcoxon test. **** $p < 0.0001$.

4.6 Clonal expansion underlies divergent signatures of adaptive NK cell subclusters

In contrast to the convergent remodeling at key adaptive genes and a pronounced enrichment of API motifs, the large number of unique peaks detected only in individual adaptive subclusters represents a diversification that is difficult to reconcile with a coordinated differentiation program. Based on these observations, we hypothesized that this apparent diversification might result from the expansion of individual naive NKG2C^{+} cells with unique accessible chromatin regions. Expansion of rare founder cells would amplify these regions beyond the detection threshold, while at the same time reducing the epigenetic heterogeneity of their progeny. Hence, as a measure for cluster heterogeneity, we analyzed the average distance between the k nearest-neighbors within the latent semantic indexing (LSI) space representing their chromatin accessibility landscape (see section 3.4.2.6). Notably, all adaptive subclusters were less heterogeneous compared to their corresponding conventional CD56^{dim} compartment, as demonstrated by a uniformly lower average distance (Figure 22A). This heterogeneity measure was robust over a wide range of values for k (Figure 22B), indicating a focused epigenetic profile of individual adaptive NK cell clusters, potentially resulting from a cell selection bottleneck during recruitment, followed by clonal expansion.

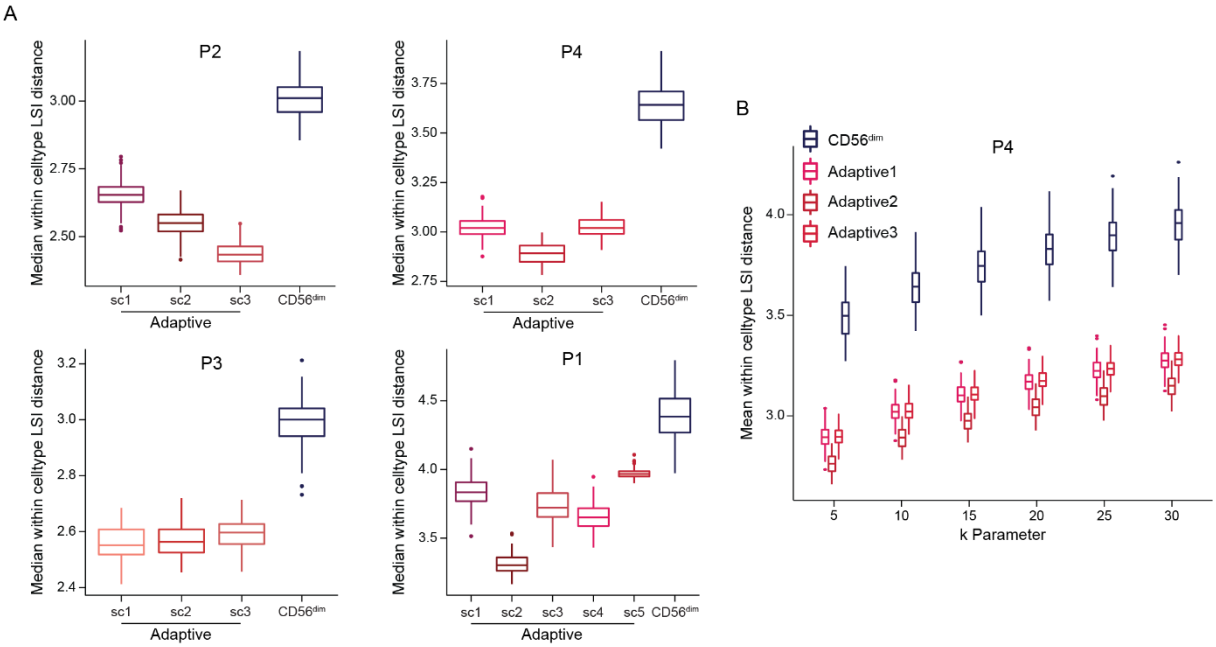

Figure 22 Epigenetic focusing of adaptive NK cells. (A) Cluster heterogeneity as assessed by measuring the median distance of 200 randomly sampled cells to their 10 nearest neighbors and repeating this process 100 times. **(B)** Cluster heterogeneity of donor P4 NK cells as assessed in (A), the k parameter was varied from 5-30 to assess robustness of this heterogeneity metric.

To test the hypothesis that divergent epigenetic profiles characteristic of adaptive subclusters are associated to clonal expansions, we applied a recently published method that enables detection of somatic mtDNA mutations as part of a modified scATAC-seq protocol (see section 3.3.5),

exploiting these mutations as endogenous barcodes to reconstruct clonal relationships and concomitantly linking them to epigenetic and cell surface phenotypes (Lareau et al., 2021; Ludwig et al., 2019; Mimitou et al., 2021). The usage of long, overlapping paired-end sequencing reads eliminates sequencing errors, as it enables to filter for mutations called independently on both strands (see section 3.4.2.4). Applying this method to four HCMV$^+$ and three HCMV$^-$ donors, we identified informative mtDNA mutations by high strand-concordance and variance-to-mean ratio (Figure 23).

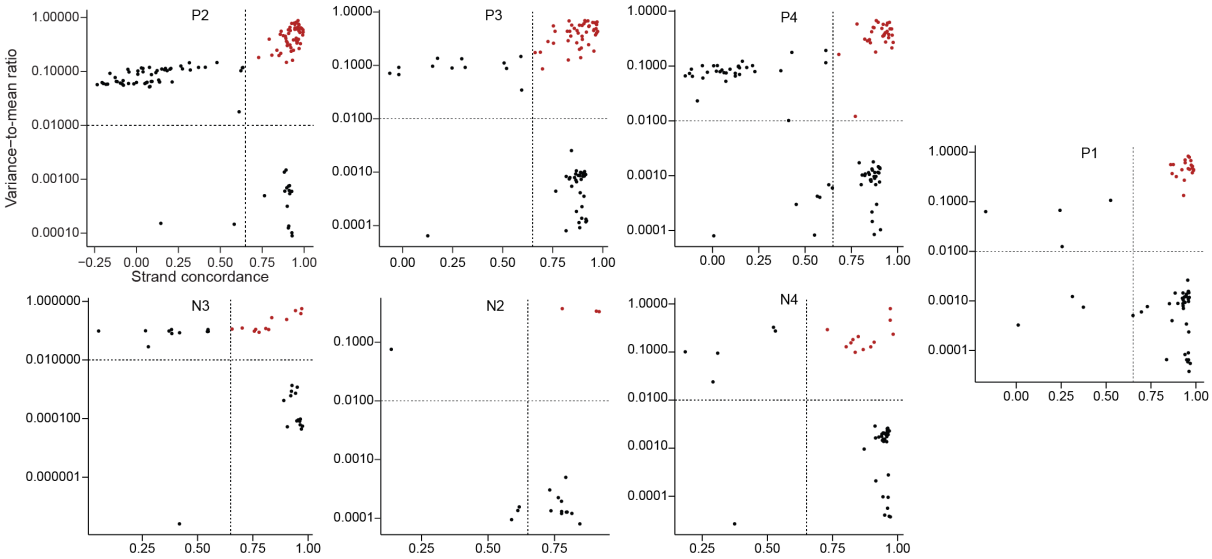

Figure 23 Identification of informative mutations. Variance-to-mean ratio and strand-concordance for mutations called by mgatk in each donor.

In the HCMV$^+$ donors, a number of these mutations were specifically enriched in the adaptive NK cell compartment (Figure 24). Importantly, clonotypes defined by individual mutations (Figure 25A, see section 3.4.2.4) were significantly associated not only to adaptive NK cells as a whole but to specific subclusters, demonstrated by the χ^2-statistic of the observed compared to a randomly permuted clonotype-cluster relationship (Figure 25B-D). This striking concordance between mitochondrial mutations and epigenetic identities reveals the inheritance of epigenetic states as a defining clonal mark. Conversely, such an association was absent in the conventional NK cell compartment (Figure 25D-F), suggesting these mitochondrial mutations do not flag clonal NK cell expansions, but might instead be present already on the progenitor level. Similarly, there was no significant association between clonotypes and epigenetic identities in HCMV$^-$ donors (Figure 25E), underlining the specific clonal expansion of NKG2C$^+$ NK cells in response to HCMV infection.

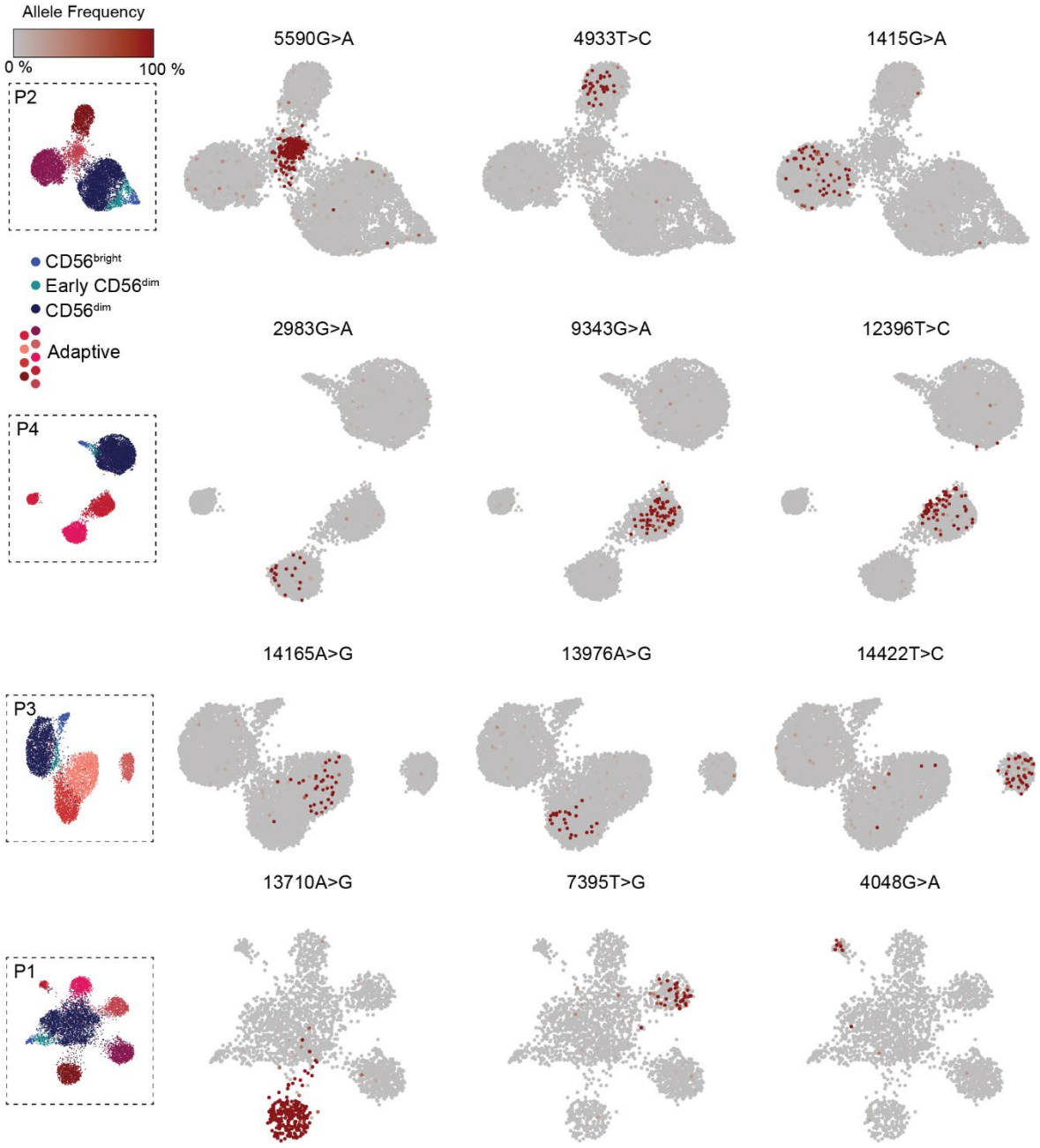

Figure 24 Clonotype-defining mutations are enriched in the adaptive NK cell compartment. Allele frequency of representative somatic mtDNA mutations projected onto UMAP embedding for each HCMV$^+$ donor.

Donor-specific patterns further highlighted the degree of clonal expansion of the adaptive subclusters. For example, an exceedingly large fraction of cells in subcluster 3 of donor P2 carried the 5590G>A mutation at near homoplasmic frequencies (Figure 24), coinciding with expression of a second self-MHC-specific KIR3DL1 which was absent from the other adaptive subclusters in this individual (Figure 18B). Similarly, the clonotype marked by the 13710A>G mutation in donor P1 constituted virtually all cells of the KIR2DL1/S1/S3/S5 expressing subcluster 2, whereas subcluster 3 was enriched for the 7395T>G mutation and specifically expressed KIR3DL1 (Figure 24, Figure 18B). These skewed receptor profiles thereby represent a specific clonal feature related to

adaptive subpopulations, as was postulated based on phenotypic patterns alone (Béziat et al., 2013; Schlums et al., 2015).

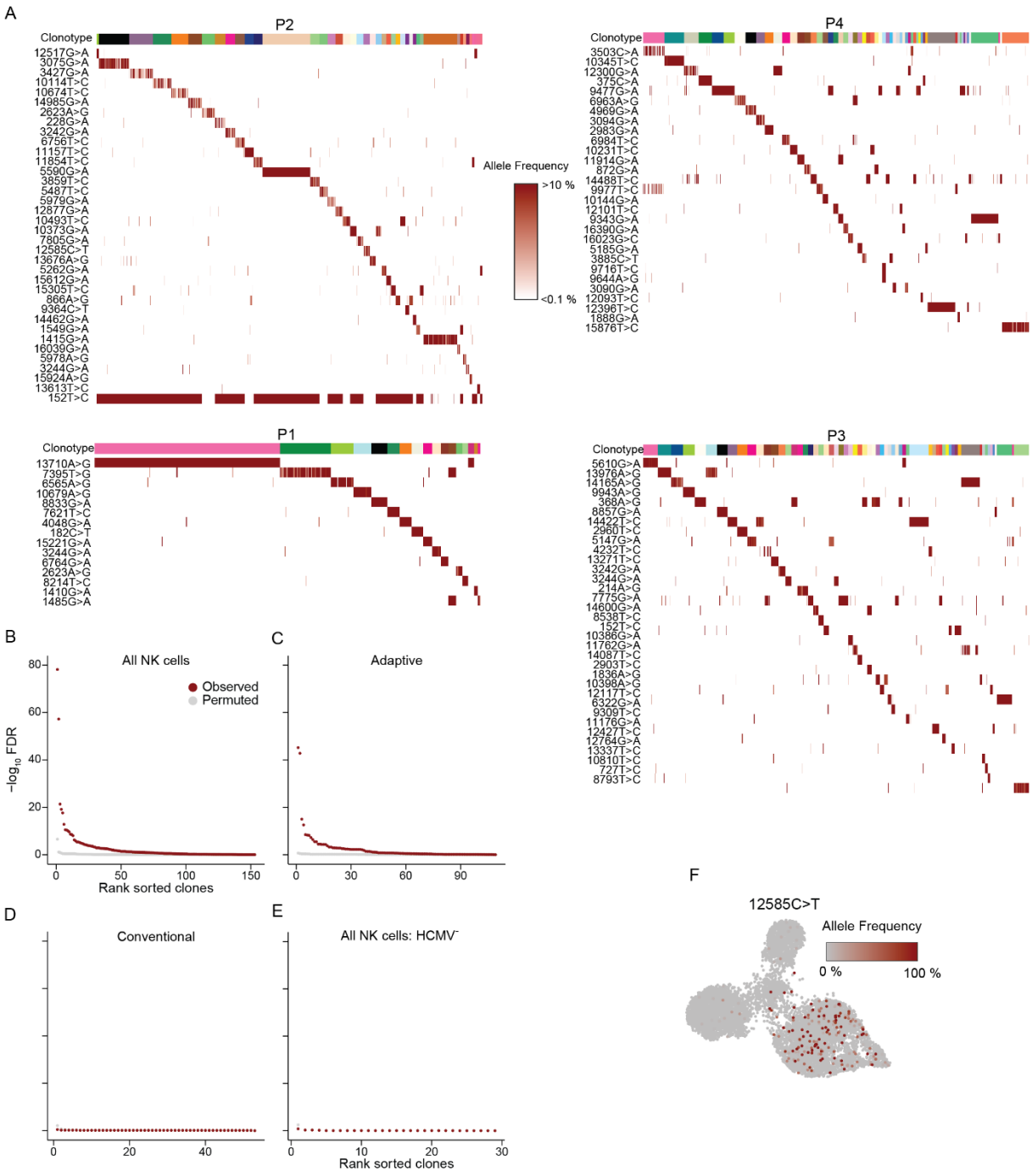

Figure 25 Clonotypes are significantly associated to adaptive subclusters. (A) Clonotypes defined by clustering on per-cell allele frequency for all high-confidence variants. **(B-E)** Association of clonotypes to clusters defined by chromatin accessibility in HCMV⁺ donors for all NK cells (B), adaptive NK cells (C) and conventional NK cells (D) and for HCMV⁻ donors (E); false discovery rate (FDR) from χ^2-test for the observed and randomly permuted clonotype-cluster relationships for all donors. **(F)** Allele frequency of representative somatic mtDNA mutation within the conventional compartment of donor P2.

Finally, association of clonotypes to individual subclusters defined by chromatin accessibility highlighted the epigenetic similarity of cells belonging to one clonotype. To assess this more

closely, we analyzed the chromatin profiles of adaptive clonotypes and found a significant association between individual open chromatin regions and clonotypes (Figure 26A). Importantly, we found regions that were uniquely accessible in individual donors and clonotypes (Figure 26B), while a part of this diversity was again driven by the opposing accessibility in the proximity of genes associated with conventional NK cell maturation such as *IL7R* and *GLI3* (Figure 26C), suggesting that these signatures are indeed clonally inherited. Together, we demonstrate that subclusters of adaptive NK cells contain individual clonotypes with private chromatin accessibility profiles, strongly supporting clonal expansion as a driving force of adaptive NK cell generation and epigenetic diversification.

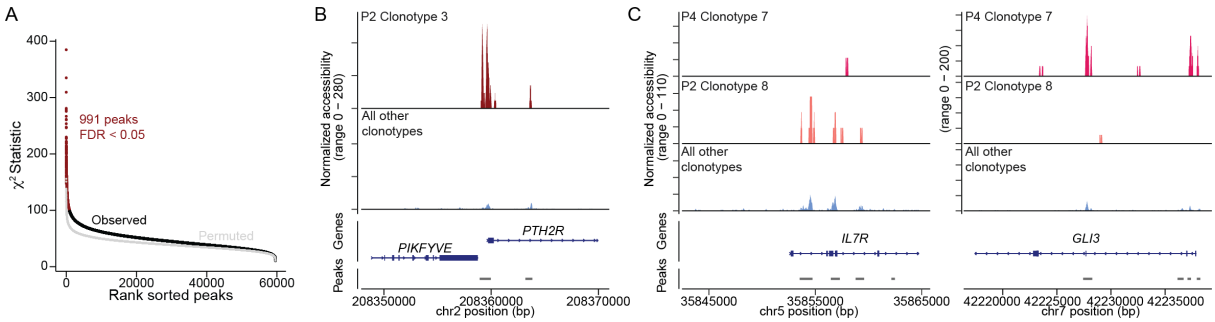

Figure 26 Clonotypes exhibit unique epigenetic profiles. (A) Association of clonotypes to open chromatin regions as assessed by χ^2-test for the observed and randomly permuted clonotype-peak relationships. **(B-C)** Representative open chromatin regions specifically associated to individual clonotypes.

4.7 Adaptive NK cell clonotypes are stably maintained over time

The specific association of non-overlapping clonotypes to adaptive subclusters suggests a degree of clonal and epigenetic stability. To test this hypothesis, we performed a longitudinal follow-up analysis of three HCMV$^+$ donors after 11, 7, and 19 months, respectively. Clusters defined by chromatin accessibility were unchanged between the two time points, supporting stable maintenance of the frequency and composition of the adaptive NK cell pool down to the subcluster level (Figure 27A-C). Further, the subcluster-specific open chromatin regions remained stable in this timeframe (Figure 27D-I).

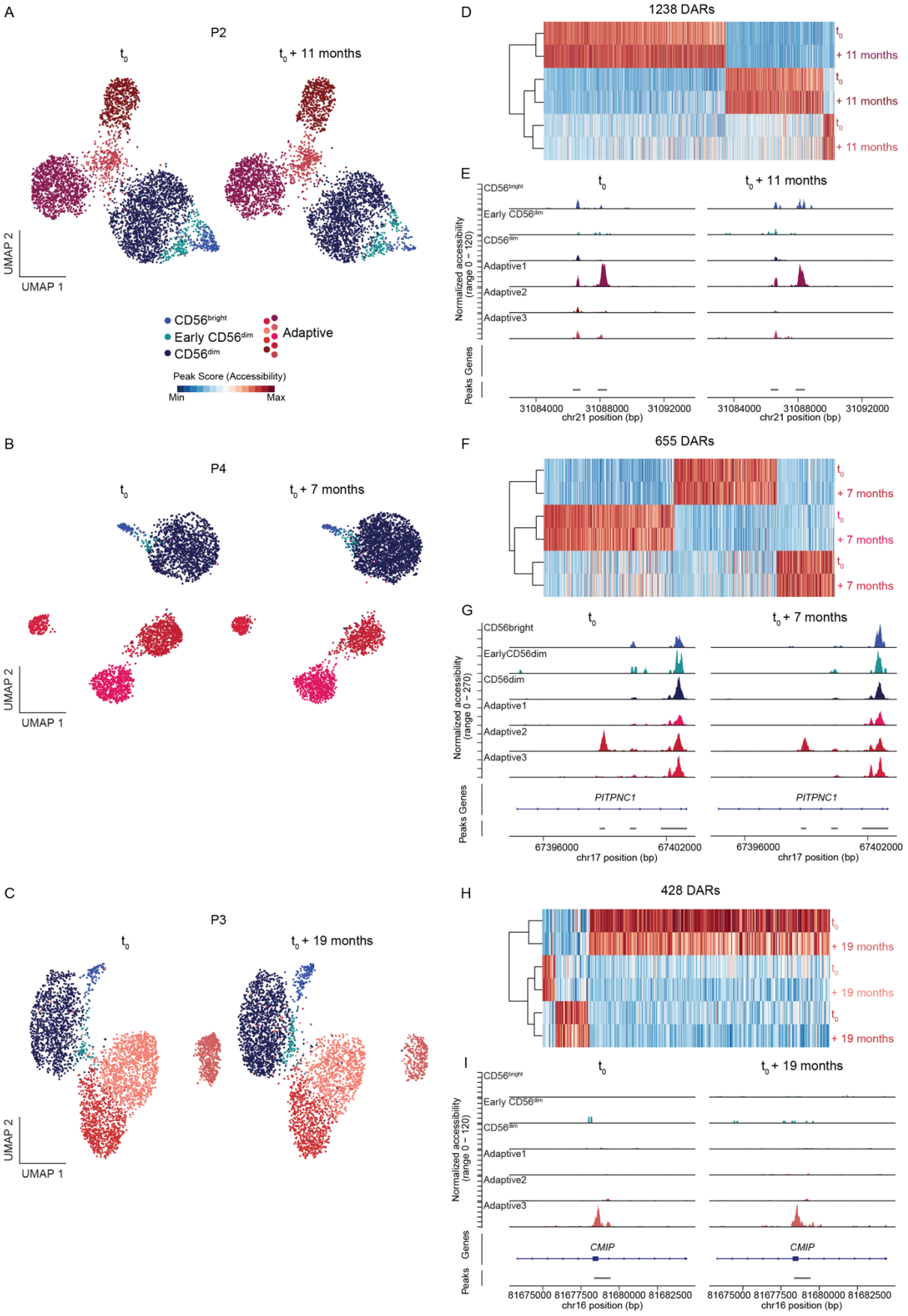

Figure 27 Epigenetic stability of adaptive NK cell expansions. (A-C) UMAP embedding of NK cells from HCMV⁺ donors P2, P4 and P3 analyzed by scATAC-seq at two different time points. **(B-I)** Stability of overall (D,F,H) and representative (E,G,I) subcluster-defining differentially accessible regions (DARs) over time; column-scaled.

Importantly, the same clonotype-defining mutations were detected at both time points and remained associated to their original subclusters (Figure 28A-C), highlighting their persistence and demonstrating that adaptive subclusters represent independent and stably maintained clonal expansions. Along these lines, even frequencies of clonotypes significantly associated to the adaptive compartment were stably maintained over time (Figure 28D-F), which was further supported by an unskewed total clonotype distribution across time points (Figure 28G-I). Overall, the longitudinal follow-up demonstrates the long-term clonal maintenance of adaptive NK cells with stable chromatin accessibility profiles in progeny arising from individual clonal founders, which are key features of "epigenetic memory" as previously ascribed to the adaptive immune system.

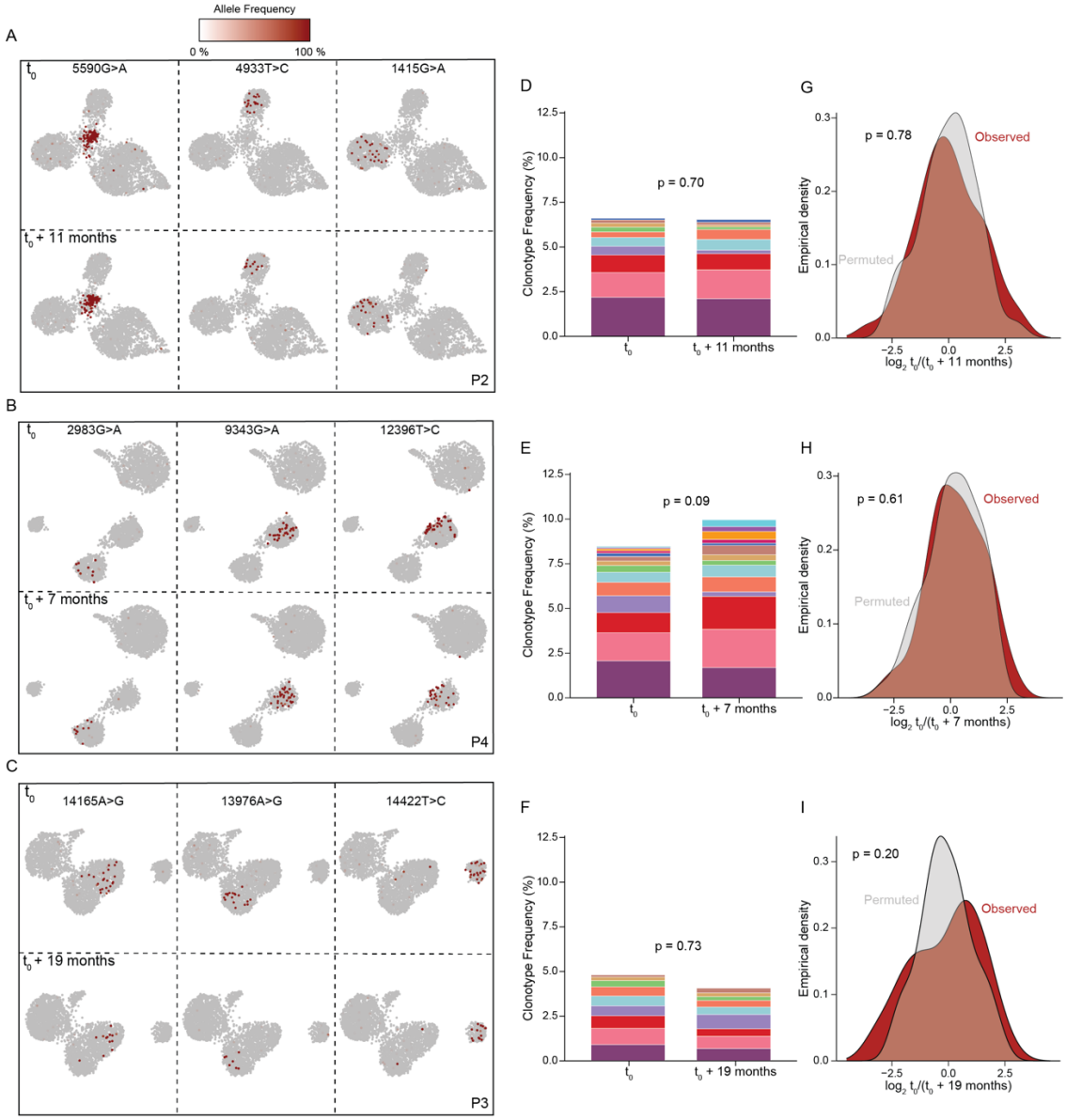

Figure 28 Clonal stability of adaptive NK cell expansions. (A-C) Representative clonotype-defining mutations projected onto UMAP embeddings at the two time points. **(D-F)** Clonotype frequency of adaptive NK cell clonotypes within total adaptive NK cell compartment over time; Fisher's exact test. **(G-I)** Observed and permuted distribution of clonotype $\log_2$ fold-changes between time points; Kolmogorov-Smirnov test.

5 Discussion

Since the initial discovery of adaptive NK cells in humans (Gumá et al., 2004), systematic interrogation of their phenotypic, transcriptional and epigenetic traits has revealed conserved signatures driven by coordinated changes in their gene regulatory networks, such as downregulation of the TF PLZF (Schlums et al., 2015), or increased activity of the T cell lineage TF BCL11B (Holmes et al., 2021). Studies on the heterogeneity of adaptive NK cells have remained limited to the diversification of signaling adaptor expression between and within individuals (Schlums et al., 2015). Here, we provide the first integrated, multi-omic single-cell analysis of human NK cells, enabling us to study both, convergent and divergent aspects of adaptive NK cell biology. Altogether, this study is to my knowledge the first demonstration of clonal expansion and long-term persistence of an innate immune population in a memory state to a naturally occurring infection, introducing clonal selection and epigenetic inheritance as mechanisms that shape human innate immune composition for optimized secondary responses.

5.1 AP1 TFs induce the inflammatory memory signature

Our finding of a strong enrichment of AP1 motifs in adaptive NK cell chromatin extends observations from memory T cells (Moskowitz et al., 2017), B cells (Scharer et al., 2018), trained macrophages (Ostuni et al., 2013), murine Ly49H$^+$ memory NK cells (Lau et al., 2018), trained hematopoietic (de Laval et al., 2020) and epithelial stem cells (Larsen et al., 2021), further supporting the concept of a conserved inflammatory memory signature with active participation of AP1 TFs in its induction and maintenance (Larsen et al., 2021; Yukawa et al., 2020). Here, we demonstrate that HCMV infection can imprint this characteristic global chromatin remodeling indicative of inflammatory memory in human NK cells, revealing a surprising conservation of this signature across stimuli and species. Previous reports in mice (Nabekura and Lanier, 2016; Sun et al., 2012) and our own studies in humans have implicated a central role for pro-inflammatory cytokines IL-12 and IL-18 in adaptive NK cell differentiation, together with activation by HCMV-derived peptides via CD94/NKG2C and co-stimulation via CD2, especially for low-affinity peptides (Hammer et al., 2018b; Liu et al., 2016). Consistently, we demonstrate that synergistic activation of naïve NGK2C$^+$ NK cells via CD94/NKG2C and pro-inflammatory cytokines most closely recapitulated the adaptive NK cell signature observed *ex vivo* and induced the most pronounced activity of AP1 TFs. What remains completely open is which of the many bZIP family members are involved in the epigenetic remodeling that differentiates conventional into adaptive NK cells. Intriguingly, both, our *de novo* motif analysis of adaptive NK cell chromatin *ex vivo* and the motif enrichment after *in vitro* activation pointed to not only AP1 but also IRF family

TFs, suggesting similar mechanisms are at play as in T cells (Glasmacher et al., 2012; Li et al., 2012; Pham et al., 2019). In this context, it would be especially interesting to assess whether differential signal strength as imposed by different HCMV-derived peptides might not only result in different frequencies of cells recruited into the response but also in qualitative differences in gene expression and chromatin remodeling. Such a direct link has been demonstrated between TCR signal strength and binding of BATF-IRF4 to two classes of enhancers differing in their affinity for the TF complex (Iwata et al., 2017). Therefore, it would be exciting to assess whether such a mechanism also applies to signaling induced by NKG2C and especially which genes might be subject to signal-strength-dependent regulation in NK cells.

5.2 Adaptive NK cell heterogeneity reflects their clonal expansion

Besides these signal-induced, programmed differences between conventional and adaptive NK cells, our donor-specific analysis revealed two different layers of adaptive NK cell heterogeneity. First, we identified two dominant underlying signatures conserved across adaptive NK cells from HCMV$^+$ donors, which intriguingly resembled maturation differences within the conventional NK cell compartment. Second, we unveiled unique, donor-specific, i.e. private open chromatin regions characterizing individual subclusters. Based on our finding of the high degree of clonality of adaptive NK cell expansions, we propose that both of these levels of heterogeneity can be explained by clonal inheritance of epigenetic traits, enabling an epigenetic founder effect. In population genetics, founder effects are an important evolutionary mechanism driven by chance events in which a small group of individuals breaks off from a larger population to establish a new colony, resulting in the stochastic skewing of allele frequencies commonly referred to as genetic drift. Analogously, epigenetic drift affects dynamics of epigenetic marks, such as DNA methylation or histone marks during aging and cancer (Ermolaeva et al., 2018). Along these lines, recruitment of individual NK cells into the adaptive response and their massive expansion might represent a clonal bottleneck, skewing the distribution of open chromatin regions. Hence, the two layers of adaptive NK cell heterogeneity likely reflect the degree of diversity and maturation stage within the original naïve NKG2C$^+$ pool, before activation and acquisition of the superimposed adaptive differentiation program. Our observation that naïve NKG2C$^+$ NK cells span the whole spectrum of CD56^{bright}, early CD56^{dim} and CD56^{dim} NK cells suggests that they might be recruited into the adaptive response at different stages of differentiation, preserving a part of this initial epigenetic identity. This is consistent with findings in mice, where pre-existing NK cell subtypes, characterized by expression of CD62L, CD27 and CD160, were preserved after MCMV-induced expansion (Flommersfeld et al., 2021). Concerning the unique open chromatin

regions associated to adaptive NK cell subclusters, we envisage that they become evident due to the drastic expansion of individual NK cell clones. The highly specific association of such peaks to adaptive subclusters and clonotypes in the context of an overall reduced heterogeneity of adaptive populations support our concept of a founder effect contributing to the specific features of adaptive NK cells. Consequently, we propose that what appears like an epigenetic diversification macroscopically, manifesting for example in variable expression of signaling adaptors (Schlums et al., 2015), is in fact the result of epigenetic focusing driven by clonal expansion, while exposing the diversity of the conventional NK cell pool. The co-existence of several oligoclonal expansions with unique features creates the picture of a diversified adaptive NK cell population, while heterogeneity within these expansions is reduced due to their clonal origin.

Furthermore, it would be exciting to understand whether the epigenomic heterogeneity of adaptive NK cell clones also translates into functional differences. While a larger number of DARs between adaptive subclusters and clones lies in the proximity of genes with unclear role in lymphocyte biology, there are some exceptions. Especially components of the maturation gradient such as the self-renewal associated *TCF7, LEF1* or cytokine receptors such as *IL7R* might point towards differences in proliferative ability or responsiveness to external signals between clones, suggesting a similar division of labor might apply as for memory T cells (see section 1.2.5).

Moreover, the concordance between epigenetic identities and clonal origin might provide an opportunity to develop new analytic tools to estimate clonality more accurately. While the mitochondrial genotyping approach has been instrumental to come to the conclusions presented in this work, it relies on the presumably stochastic process of acquisition of mitochondrial mutations at high enough frequencies that enable clonal distinction. Hence, we likely underestimate the clonality of the adaptive NK cell pool and are not able to make clear statements on the size of the founder population or the clonal composition of the compartment as a whole. However, the combined measurement of mtDNA mutations and chromatin accessibility might inform new analytic methods which segregate clonotypes based on their epigenetic profiles, using the clones that can clearly be identified by their mutational barcodes as quality control, to gain a more holistic understanding of clonal composition.

5.3 Drivers of clonal success

Importantly, our observation of the clonal expansion of adaptive NK cells enables us to generalize some of the mechanisms driving clonal expansion and their consequences within the immune system as a whole. One such unifying pre-requisite for clonal expansion is the competitive recruitment of a limited number of cells to emerge as clones from a diverse population. Whereas

the expression of uniquely rearranged antigen receptors represents an obvious clonal bottleneck for classical adaptive immune cells, selection mechanisms for NKG2C$^+$ NK cells appear less obvious, as their main "antigen receptor" is invariant. However, other cell-intrinsic features of NK cells are strongly diversified, foremost their combined cell surface receptor expression profiles, with more than 30,000 appreciable phenotypes (Horowitz et al., 2013). This variegated receptor expression and their cooperative as well as counteracting signaling pre-dispose individual NK cells to respond to a given combination of stimuli, leading to selection of optimal receptor combinations. Along these lines, the low frequency of cells expressing NKG2C before HCMV-infection is a first limitation of the original pool able to respond to HCMV peptides. Further, only NKG2A$^-$ NK cells underwent remodeling in response to peptide and cytokine stimulation that resembled adaptive NK cells the closest, suggesting that NKG2A expression further limits the pool of naïve NKG2C$^+$ cells optimally equipped to respond to HCMV. Expression levels of NKG2C might also contribute to selection, as adaptive NKG2C$^+$ NK cells generally express higher levels of NKG2C than their naïve counterparts (Lopez-Vergès et al., 2011) and a similar mechanism occurs for Ly49H$^+$ NK cells in mice (Adams et al., 2019; Grassmann et al., 2019). Expression of other receptors that are engaged during HCMV infection or homeostasis such as CD2 or self-MHC-specific KIRs likely also contribute to this selection, as these are almost always expressed on adaptive NK cells (Béziat et al., 2013; Liu et al., 2016). Especially the mechanism for selection of self-MHC-specific KIR$^+$ remains completely unknown. Given the data in mice, where unlicensed NK cells expand more vigorously under the influence of inflammatory cytokines induced by MCMV (Orr et al., 2010), it would be interesting to understand whether KIR expression biases the recruitment or is a feature that is established later in the course of infection. Besides variable expression of cell surface receptors which influence naïve cell recruitment, clonal selection can be influenced by further cell intrinsic factors, including complex biological traits that are heterogeneously distributed among cells before or after activation, such as proliferation and differentiation kinetics (Buchholz et al., 2013; Grassmann et al., 2020), metabolic capacity (Hartmann et al., 2021) or genomic fitness (Karo et al., 2014).

Apart from cell-intrinsic mechanisms, T cell memory studies have highlighted the contribution of extrinsic factors, such as signal strength, duration and quality, in shaping the size and diversity of the naive population recruited into the response (Gett et al., 2003). Our own work has highlighted the role of peptide-affinity and CD2 co-stimulation for determining expansion of NKG2C$^+$ NK cells (Hammer et al., 2018b). Future studies are required to address how activation strength and co-stimulation affects clonal composition and phenotype of adaptive NK cell expansions.

The maybe most important implication of the present work is that clonal selection might act on inheritable epigenetic states, which in turn could contribute to clonal success. That such non-genetic mechanisms of inheritance can have a fitness effect was recently demonstrated in the context of tumor cell resistance against anti-cancer therapeutics (Shaffer et al., 2020). Future studies extending the number of donors and analyzed clonotypes will be instrumental to identify reoccurring patterns indicative of a selective advantage. The challenge will be to distinguish the adaptive NK cell features modulated by external signals from those conferring a selection advantage within the naïve repertoire, and the ones that are stochastic passengers of the clonal expansion process. In addition to increasing sample size, studying adaptive NK cell expansion in a more dynamic fashion to follow effector cells during acute infection into the memory phase might enable to identify individual genes or higher-order programs that are predictive of long-term clonal success, similar to what has recently been performed for MCMV-specific NK and T cells in mice (Grassmann et al., 2020; Riggan et al., 2022). These analyses might provide new concepts and general insights into drivers of clonal success in different cell types, independent of receptor-specificity.

5.4 Adaptive NK cell maintenance

A further pre-requisite to observe the pronounced clonality and characteristic epigenetic features of adaptive NK cells at steady state is their long-term maintenance and epigenetic stability. Prolonged survival of adaptive NK cells has already been postulated based on the observation of stable expression of KIR and signaling adaptor patterns (Schlums et al., 2015). Recently, evidence from patients with mutations in *GATA2* or *PIGA* further supported this concept (Corat et al., 2017; Schlums et al., 2017). Here, we were able to demonstrate the long-term persistence on the clonal level for at least 19 months. The remarkable clonal stability within this time frame might potentially suggest a prolonged half-life compared to the approximately two weeks that have been reported for conventional NK cells (Zhang et al., 2007), although the exact cellular mechanisms enabling long-term clonal maintenance remain to be defined. Future studies with repeated and prolonged sampling will address the stability and extinction kinetics more quantitatively and assess the potential contribution of homeostatic proliferation and cell longevity in maintaining the adaptive NK cell pool, similar to what has been reported for memory T cells (Akondy et al., 2017). Deuterium-labeling would be a promising approach to perform such studies, although the low rate of proliferation would complicate these studies and require long labeling periods for sufficient label uptake. It also remains open whether there are specialized

subsets within the adaptive NK cell compartment that maintain the pool and if so, where they are located.

5.5 Evolutionary considerations

There is evidence that NK cells have already evolved within invertebrates, indicated by cell populations with natural cytotoxic activity in different *Echinodermata* or *Tunicata* species (Lin et al., 2001; Parrinello et al., 1993). That these might indeed have a common evolutionary origin is suggested by the homology of a C-type lectin receptor in the urochordate *Botryllus schlosseri* to CD94 and NKR-P1 (Khalturin et al., 2003). Intriguingly, this receptor is expressed by a population of granulated circulating cells and is upregulated during an allogeneic rejection response. As the present work demonstrates the ability for clonal expansion and persistence by innate lymphocytes, it would be interesting to study how far back in evolutionary history these adaptive mechanisms might have evolved. If already ancestral NK cells had variegated expression patterns of NK cell receptors, one could go as far as speculating on a driving role for the evolution of clonal expansion as immune mechanism. It seems logical to me that the mechanisms for clonal expansion and inheritance of effector programs would have to precede the evolution of genetically diversified receptor systems, as the handful of adaptive lymphocytes specific to a given antigen would likely not be efficient enough to control infections without being able to give rise to substantial progeny. The concept of a common ancestor capable of clonal expansion would also fit with the independent evolution of the functionally highly similar but evolutionary completely divergent adaptive immune systems of *Agnathans* (Cooper and Alder, 2006). Receptor diversification systems in various invertebrate organisms might also support this idea (Cannon et al., 2002; Watson et al., 2005; Zhang et al., 2004). Now that the tools are available to study clonality across a range of cell types, they could be utilized to fill these gaps of our understanding of evolutionary history.

5.6 Limitations of the study

Apart from the already discussed limited ability to perform absolute quantification due to the stochastic nature of mtDNA mutation acquisition, we can also not exclude a functional impact of the mutations, especially at high allelic frequencies. However, some of the mutations associated with the biggest clonotypes such as 13710A>G or 4048G>A are common polymorphisms, synonymous mutations in coding genes such as 14422T>C or 12396T>C, or rated as likely benign

mutations of tRNA genes by available tools, as is the case for 5590G>A (Lott et al., 2013), suggesting at least these examples have, if at all, only a limited functional impact. All of the samples assessed in this study were acquired from healthy blood donors at steady state, so we were not able to perform any dynamic assessments of the expansion or contraction of adaptive NK cells in the context of primary infection or reactivation. While this study was focused on cells from peripheral blood, different parts of the adaptive NK cell life cycle might take place in tissues, as cells with a partially overlapping phenotype and transcriptome have been described in lung, bone marrow or liver (Brownlie et al., 2021; Marquardt et al., 2015; Yang et al., 2019). Future studies will be required to address these points.

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7 Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
ADT	antibody-derived tag
AICE	API-IRF composite element
ASAP-seq	scATAC with Select Antigen Profiling by sequencing
β2m	β_2-microglobulin
BCR	B cell receptor
bZIP	basic leucine zipper domain
CIML	cytokine-induced memory-like
CITE-seq	Cellular Indexing of Transcriptomes and Epitopes by sequencing
CLP	common lymphoid progenitor
CLR	centered log ratio
CMP	central memory precursor
CMV	Cytomegalovirus
CNS1	Conserved non-coding sequence 1
DAG	diacylglycerol
DAR	differentially accessible region
EBV	Epstein-Barr virus
FDR	false discovery rate
GEM	gel bead in emulsion
GPI	glycosylphophatidylinositol
HCMV	human Cytomegalovirus
$HCMV^+/HCMV^-$	HCMV-seropositive/negative
HIV	human immunodeficiciency virus
HLA	human leukocyte antigen
HSPCs	hematopoietic stem and progenitor cells
HTO	hashtag oligo
IFN	interferon
IL	interleukin
ILC	innate lymphoid cell
IP_3	inositol 1,4,5-trisphosphate
ITAM	immunoreceptor tyrosine-based activating motif
ITIM	immunoreceptor tyrosine-based inhibitory motif
KIR	killer immunoglobulin receptor
LDTF	lineage-determining transcription factor
LFL	VMAPRTLFL peptide
LSI	latent semantic indexing

MCMV	murine Cytomegalovirus
MHC	major histocompatibility complex
MPEC	memory precursor cells
mtDNA	mitochondrial DNA
mtscATAC	mitochondrial scATAC
NK	Natural Killer
PAMP	pathogen-associated molecular pattern
PBMCs	peripheral blood mononuclear cells
PCA	principal component analysis
PRR	pattern recognition receptor
RhCMV	rhesus Cytomegalovirus
scATAC-seq	single-cell assay of transposase-accessible chromatin sequencing
scRNA-seq	single-cell RNA sequencing
SLEC	short-lived effector cells
SRTF	signal-regulated transcription factor
TCM	central memory T cell
TCR	T cell receptor
TEM	effector memory T cell
TF	transcription factor
TF-IDF	term frequency-inverse document frequency
TH	T helper cell
TLR	Toll-like receptor
TRM	resident memory T cell
TSCM	stem cell-like memory T cell
TSS	transcription start site
UMAP	uniform manifold approximation and projection
WT	wildtype
YFV	yellow fever virus

8 List of figures and tables

9 Eigenständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst habe und sämtliche Quellen, einschließlich Internetquellen, die unverändert oder abgewandelt wiedergegeben werden, insbesondere Quellen für Texte, Grafiken, Tabellen und Bilder, als solche kenntlich gemacht habe. Ich versichere, dass ich die vorliegende Abschlussarbeit noch nicht für andere Prüfungen eingereicht habe. Mir ist bekannt, dass bei Verstößen gegen diese Grundsätze ein Verfahren wegen Täuschungsversuchs bzw. Täuschung gemäß der fachspezifischen Prüfungsordnung und/oder der Fächerübergreifenden Satzung zur Regelung von Zulassung, Studium und Prüfung der Humboldt-Universität zu Berlin (ZSP-HU) eingeleitet wird.

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