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Remembering One's ID/E-ntity : E/ID Protein Regulation of T Cell Memory

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Abstract

Upon infection, $CD8^+$ T cells proliferate and differentiate into armed effector cells capable of eliminating the assaulting pathogen. Although the majority of the antigen-specific T cells will die as the immune response wanes, a few will survive indefinitely to establish the memory population and provide long-lived protection against reinfection. E protein transcription factors and their inhibitors, ID proteins, operate to balance expression of genes that control $CD8^+$ T cell differentiation through this process. Here, we discuss the role of ID2 and ID3 in promoting the generation and survival of effector and memory populations, particularly highlighting their reciprocal roles in shaping the $CD8^+$ T cell response unique to the inflammatory milieu. We further examine this coordinated control of gene expression in the context of additional transcription factors within the transcriptional network that programs $CD8^+$ effector and memory T cell differentiation.

Introduction

In response to infection, a naive pathogen-specific $CD8^+$ T cell undergoes a massive proliferative burst, during which one cell is capable of generating upwards of tens of thousands of progeny [1]. This expansion is concurrent with dramatic alterations in gene expression due to modifications in chromatin structure and expression of key transcription factors [2,3]. These changes also coincide with the acquisition of effector function including the capacity to secrete effector cytokines interferon (IFN)γ and tumor necrosis factor (TNF)α, and release cytolytic molecules such as perforin and granzymes to eliminate pathogen-infected cells [1,4,5]. This expanded population of $CD8^+$ T cells is a heterogeneous mixture of cells that include short-lived memory and effector T cells ((which can be identified by high levels of the surface receptor killer cell lectin-like receptor G1 (KLRG1) and low levels of Interleukin-7 receptor (IL-7Rα), CD127)), as well as memory-precursor cells (contained within the $KLRG1^{lo}CD127^{hi}$ population) [5]. As indicated by their monikers, most short-lived effector cells will survive as a population for only a matter of days during the height of the immune response, after which they undergo a rapid contraction phase. The majority of the $KLRG1^{lo}CD127^{hi}$ effector population, which contains memory-precursor cells, also succumbs to programmed cell death after infection. However, ~5% of the effector cells endure and persist in greater numbers than their naive

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precursors, and are transcriptionally programed to seed the long-lived memory pool providing protection against re-infection [1,5-7].

Of particular interest in the study of CD8+ T cell immunity are the transcriptional networks and targeted gene-expression changes that orchestrate the bifurcation of differentiation—mediating the short half-life of the effector cells versus the longevity of memory cells. While the integrated balance of expression and activity of T-BET, B lymphocyte-induced maturation protein-1 (BLIMP-1), signal transducer and activator of transcription (STAT) 4 and forkhead box O (FOXO) 3 have been shown to regulate effector cell differentiation, survival and contraction; eomesodermin (EOMES), B cell lymphoma-6 (BCL-6), T cell factor-1 (TCF-1), STAT3, and FOXO1 influence the generation and maintenance of memory cells [8-10] (Figure 1). Recently it was discovered that E and the inhibitor of DNA binding (ID) proteins also regulate the differentiation of both the shortlived effector and memory-precursor populations of CD8+ T cells [11-15]. This raises the possibility of an analogous role for these molecules in determining CD4+ T cell fate.

E proteins

E proteins are transcription factors in the basic helix-loop-helix (bHLH) family that control many aspects of lymphocyte biology [16]. Four different E proteins, E12 and E47 (splice variants of E2A), E2-2 and HEB, are present in mammals. E proteins can interact as homo- and hetero-dimers via their HLH domains and bind specifically to DNA at E-box-consensus sequences acting as transcriptional activators or repressors (Figure 2) [16,17]. The ability of E proteins to bind DNA and regulate gene expression is inhibited by the highly related ID proteins, which share the HLH domain and thus form heterodimers with E proteins, but lack a DNA-binding domain, preventing E protein function (Figure 2) [18,19].

E proteins are well-established regulators of thymocyte development and are required for proper control of progression, survival, proliferation and T cell receptor (TCR) rearrangements by T cell progenitors [16]. It is now clear that E proteins are also active in the early stages of mature T cell activation and induce expression of genes important for commitment to the memory lineage. E2A expression is upregulated by CD8+ T cells upon activation, and increased E protein DNA-binding activity is observed in antigen-specific CD8+ T cells early during infection (Figure 3) [20]. Deletion of E2A, E2-2, or HEB had minimal effects on the expansion and phenotype of CD8+ T cells responding to infection, indicating compensatory functions between E proteins in this context. However, deficiency in both E2A and HEB resulted in an increased frequency of KLRG1^{hi} terminally-differentiated effectors [20]. Activated CD8+ T cells lacking E proteins exhibited altered gene-expression profiles with upregulation of genes linked to early effector populations and activation (*CD28, Lymphocyte activation gene 3 (Lag3)*) and a downregulation of genes associated with memory formation (*Il7r*, *Eomes*) [20]. The genes identified to be differentially regulated upon loss of E proteins also possessed E2A-bound E-box sites in close proximity to their transcriptional start site (TSS), strongly suggesting direct regulation by E proteins [21]. Overall, these studies suggest that E proteins regulate transcription factors, cell-surface markers, and cytokine signaling early during CD8+ T cell activation to support memory-precursor formation [20].

Recent studies have provided a further link between E proteins and the regulation of other transcription factors central to cell-fate decisions of CD8+ T cells. E proteins are known to directly impact the expression of the FOXO transcription factors [21,22]. Interestingly, two family members, FOXO1 and FOXO3, have been recognized as important to memory formation [23-25]. While FOXO1 appears to be a key mediator of CD8+ T cells differentiating into long-lived memory cells [23], FOXO3 is suggested to function during

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the contraction phase of the T cell response [24-26]. E proteins have also been shown to regulate TCF-1 expression, a factor central in CD8+ T cell immunity [13,27]. TCF-1, also a likely E protein target, was shown to control *Eomes* expression and the differentiation, maintenance and function of CD8+ T cell memory [28]. From these studies, it is clear that E proteins play an important role in orchestrating the transcriptional network necessary for the generation of productive CD8+ T cell memory.

ID Proteins

There are four ID family members (ID1-ID4); with ID2 and ID3 emerging as the players relevant in shaping lymphocyte differentiation [16,18,19]. While ID protein-mediated regulation of E proteins is known to be crucial to lymphocyte development, the role of these factors in mature T cells is only beginning to be revealed. Importantly, a reciprocal relationship between ID2 and ID3 has been suggested in the differentiation of mature T cells during the response to infection [14]. Studies with knock-in reporter mice have shown that *Id2* is upregulated in effector CD8+ T cells while induction of *Id3* expression marked CD8+ T cells with memory potential [14].

ID2

Although downregulated early in infection, ID2 expression is upregulated at the peak and maintained in memory T cells, albeit at lower levels (Figure 3) [11,13,14,20]. ID2 plays important roles in the CD8+ T cell response to infection by mediating survival and differentiation of effector cells and repressing memory formation [11-13]. Naive CD8+ T cells lacking ID2 show normal activation, proliferation and differentiation into effector cells. However, they fail to accumulate through the course of infection with *Listeria monocytogenes*, Vesicular Stomatitis Virus and Lymphocytic Choriomeningitis Virus due to increased susceptibility to apoptosis [11]. The absence of cells to analyze following infection left it unclear how ID2-deficiency further affected CD8+ T cell responses. Two recent papers have begun to address this issue and show clear evidence that differentiation, as well as survival, are controlled by ID2 [12,13].

Knell *et al.* determined that ID2 regulates CD8+ T cell responses at two levels by ensuring survival of effector cells as well as influencing effector to memory cell differentiation [12]. Consistent with the observation of defective survival, ID2-knockout effector CD8+ T cells displayed lower expression of the antiapoptotic molecules *B cell lymphoma-2 (Bcl-2)* and *serine protease inhibitor 6 (Spi6)*, and higher levels of the proapoptotic factors *Bcl2l11* (encoding Bcl-2-interacting mediator of cell death (BIM)) and *cytotoxic T-lymphocyte antigen 4 (Ctla4)* [11]. Closer examination identified >30 conserved E-box sites in the *Bcl2l11* promoter, and as such, TCR activation led to increased E protein-binding at these E-box sites in CD8+ T cells [12]. Interestingly, loss of BIM expression rescued the accumulation defect observed for ID2-deficient CD8+ T cells, further defining a role for ID2 in effector cell survival [12]. Strikingly, we found that ID2-deficient and “rescued” ID2-deficient CD8+ T cells almost completely lacked a terminally differentiated KLRG1^{hi} effector population during *Listeria monocytogenes* infection [12,14]. The effector cells that did form exhibited a cell-surface phenotype, cytokine and gene-expression profile consistent with memory-precursor cells [12]. In fact, microarray analysis revealed upregulation of several genes involved in memory cell formation or function including *Id3, Tcf7* (encodes TCF-1), and C*xcr3* [12].

In a second study, ID2-deficient CD8+ T cells were found to have a significant defect in formation of the terminally-differentiated KLRG1^{hi} population [13]. In this case, ID2-deficient CD8+ T cells accumulated normally during Influenza virus infection but not *Listeria monocytogenes* infection, suggesting that the specific inflammatory milieu or type

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of infection (systemic versus localized) may yield environments that differentially induce and/or require ID2 [13]. This study took advantage of the normal ID2-deficient $CD8^+$ effector population following Influenza infection to study how loss of ID2 impacts effector and memory cell differentiation, and found an inverse correlation between ID2 levels and memory recall potential [13]. Consistent with this observation, the transcriptional program of the responding $CD8^+$ T cells was influenced by ID2 expression; low or no ID2 resulted in high expression of characteristic memory cell-associated transcription factors, low expression of cytolytic molecules and altered integrin and chemokine receptors that promote localization in non-lymphoid tissues [13]. ID2 function appeared to be mediated through inhibition of E2A as the genes upregulated in the absence of ID2 largely contained E2A binding sites. Furthermore, shRNA knockdown of *Tcfe2a* expression partially compensated for ID2-deficiency, yielding increased expression of effector molecules and diminished expression of characteristic memory genes such as *Tcf7*, *Id3* and suppressor of cytokine signalling (*Socs)3* [13]. Conversely, *Tbet*, a key regulator of effector differentiation, was 2-fold reduced in ID2-deficient effector cells and rescue of expression by retroviral infection induced formation of $KLRG1^{hi}$ cells and somewhat restored granzyme and BLIMP-1 expression, suggesting ID2 is at least indirectly responsible for inducing *Tbet* which then supports the generation of short-lived effector $CD8^+$ T cells [13]. Collectively, these studies demonstrate that ID2 mediates both survival of $CD8^+$ T cells during the effector phase of infection and the differentiation of $KLRG1^{hi}$ cells, shaping the composition of the effector and memory populations.

ID3

ID3 is expressed at its highest level in naive $CD8^+$ T cells and is rapidly downregulated upon activation. Expression of ID3 later increases during contraction of the effector response and coincides with the appearance of memory $CD8^+$ T cells, effectively acting as a marker of memory-precursor cells (Figure 3) [14]. Defining a role for ID3 in T cell differentiation has been hindered by the complexity of the ID3-knockout mouse model. While viable, ID3 germline-knockout mice have a multifaceted phenotype that includes an outgrowth of $V\gamma1.1^+V\delta6.3^+$ T cells in the thymus and spleen, excessive IFNγ and interleukin (IL)-4 production by these innate-like cells [26,29-32], as well as T cell-dependent Sjögren's autoimmune syndrome [33,34]. In light of this confounding phenotype, studies have utilized ID3 heterozygous GFP-reporter mice and mixed-bone marrow chimeras to circumvent extrinsic effects of ID3-deficiency. Importantly, at the height of the $CD8^+$ T cell response, cells with little to no expression of ID3 were chiefly $KLRG1^{hi}CD127^{lo}$ short-lived effector cells, while those expressing the highest level of ID3 were largely $KLRG1^{lo}CD127^{hi}$ memory-precursor T cells producing more IL-2, IFNγ and TNF [14]. This functional divergence based on ID3 expression was further supported by transfer of $ID3^{hi}$ and $ID3^{lo}$ populations sorted from the peak of infection, which revealed that the $ID3^{hi}$ cells preferentially differentiated into memory $CD8^+$ T cells and that ID3 expression preceded other traditional phenotypic indicators of memory potential [14]. To investigate whether ID3 is a marker of memory-precursor $CD8^+$ T cells at the transcriptional level, global gene-expression analyses was performed at effector and memory time points. Hallmark $CD8^+$ T cell memory transcripts were expressed by $ID3^{hi}$ effector cells as early as day 5 of infection (*IL7r, Sell, IL3, Bcl2 and Ccr7*,) whereas transcripts indicative of terminal differentiation were downregulated (*Gzmb, Klrg1, and Prdm1*) [14]. Indeed, analysis of either ID3-wildtype:ID3-knockout mixed-bone marrow chimeras or adoptively transferred ID3-deficient melanoma-specific TCR-transgenic (pmel-1) $CD8^+$ T cells, both revealed that loss of ID3 led to defects in the formation and survival of a long-lived memory, despite having little effect on population expansion [14,15]. Conversely, ectopic expression of ID3 in pmel-1 $CD8^+$ T cells increased their accumulation following secondary viral challenge and enhanced survival after challenge with B16 melanoma compared to controls [15]. Taken

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together, this study concluded that the formation of a functional memory compartment is dependent on ID3 expression and forced expression of ID3 improved the secondary response through an increase in the number of effector-memory T cells.

Coordination of ID2 and ID3

ID2 and ID3 appear to have distinct roles in $CD8^+$ T cell differentiation; however, it is the timing of their expression that is required to regulate E protein activity and orchestrate the transition from naive to effector or memory cell (Figure 3). Interestingly, in the absence of ID2, ID3 is significantly upregulated in effector cells; however, the reverse does not occur and ID2 remains unchanged in ID3-deficient $CD8^+$ T cells, indicating that ID2 regulates expression of ID3 [14]. Consistent with this, E-box binding sites have been identified in the *Id3* locus [13,21,35]. Thus, factors that control *Id2* expression will likely also inversely impact *Id3* expression via ID2-mediated inhibition of E protein activity.

Though the regulation of ID3 by ID2 needs to be explored further, the direct repression of ID3 by BLIMP-1 has been demonstrated [15]. BLIMP-1 expression is inversely correlated with that of ID3 as well, with chromatin immunoprecipitation (ChIP) analysis showing BLIMP-1 bound to the *Id3* promoter region [15]. Since ID3 is required for the persistence of long-term memory cells, BLIMP-1 presumably represses *Id3* transcription and prevents the over-accumulation of memory T cells. In support of this idea, ID3 retroviral overexpression, where BLIMP-1 cannot efficiently repress *Id3*, resulted in a blunted contraction phase and increased persistence of effector cells into the memory phase [15].

Extrinsic factors also influence the balance of ID2 and ID3 levels within a differentiating $CD8^+$ T cell, dictating its fate. In support of this, cytokine signaling through STAT4 and 5 was demonstrated to be important for the modulation of ID2 and ID3 expression by $CD8^+$ T cells. Using the ID2- and ID3-reporter mice, we found that activation of $CD8^+$ T cells in the presence of IL-2, IL-12 and IL-21 upregulated *Id2* expression but lead to downregulation of *Id3*. Furthermore, ChIP analysis identified STAT-binding sites in the *Id2* promoter [14]. Such environmental control of ID protein expression may provide an explanation for infection-specific differences observed in ID2-mediated $CD8^+$ T cell survival [11,13]. During Influenza infection, loss of ID2 does not impair the accumulation of antigen-specific cells as compared to other systemic, highly inflammatory infections [11,12]. The cytokine profile of Influenza infection may not induce significant upregulation of ID2; however, at the same time, ID3 expression would not be substantially decreased, so it may function in a compensatory manner to control the transcriptional program. This would include providing protection from elevated BIM levels and apoptosis. Thus, the information provided by the infection itself may instruct the differentiating T cells to generate effector and memory populations optimal for the response.

The regulation of ID2 and ID3 expression and their relative levels over time within a responding T cell is likely a major determinant of its fate. Early during infection, E protein expression is upregulated, coinciding with downregulation of ID2 and ID3. This allows E proteins to target gene expression, inducing formation of memory precursors [11,14,15,20]. As the T cell response peaks, ID2 protein levels increase, possibly induced through cytokine signaling. E protein activity is then inhibited, permitting the survival and differentiation of late $CD8^+$ effector cells [13,20]. Reciprocally, cells expressing ID3 and low levels of ID2 are memory precursors (Figure 3). Although both ID2 and ID3 are thought to similarly function by repressing E protein activity, there is a clear distinction in the role they play in $CD8^+$ T cell differentiation. This could be a result of variation in E protein binding, such as a preference for specific homo- or heterodimers or the affinity with which the interaction

takes place. Further studies clarifying the downstream binding partners of ID2 and ID3 will be necessary to interpret their individual contributions to $CD8^+$ T cell responses.

E and ID proteins in $CD4^+$ T cells

In contrast to $CD8^+$ T cell differentiation where memory subsets have been defined in substantial detail, the gene-expression and phenotypic changes that $CD4^+$ effector T cells undergo during memory formation is less clear. Since effector $CD4^+$ T cells can differentiate into multiple helper (Th) populations [36], additional complexity exists and analysis of memory formation from each effector subset needs to be established. As in the case of $CD8^+$ T cell responses, relative ID2 and ID3 levels may act as novel markers of early $CD4^+$ T cell memory-precursors, in addition to regulating gene-expression programs that govern effector versus memory cell formation. Recently, it was demonstrated that *Id2* was highly expressed in the Th1 population, whereas *Id3* transcript was almost exclusively expressed in the T follicular helper (Tfh) population after infection [37]. Another study demonstrated that ID3-deficiency resulted in aberrant formation of effector-memory-like $CD4^+$ T cells suggesting that ID3 is important for the maintenance of the naïve state. Furthermore, ID3-deficiency led to the upregulation of Tfh markers at the RNA (*Bcl6*) and protein level (CXCR5, ICOS and PD-1), as well as elevated IFNγ and IL-4 production following stimulation [35]. Further, ID2 and ID3 have been examined in models of $CD4^+$ T cell-mediated autoimmunity. ID2 was shown to be an important factor in the development of experimental autoimmune encephalomyelitis with high ID2 expression in the most encephalitogenic $CD4^+$ T cells [38]. ID2-deficiency led to decreased numbers of effector $IL17A^+IFN\gamma^+CD4^+$ T cells due to reduced proliferation, and similar to $CD8^+$ T cells, increased apoptosis. ID2 also appeared to be important in mediating cytokine production in this system by regulating expression of the repressor SOCS3 [38]. ID3 has also been implicated in the TGFβ1-dependent reciprocal regulation of T regulatory (Treg) and Th17 development [39-41]. In the absence of ID3, Tregs do not properly develop as a result of an inability to inhibit GATA-3 binding to the *Foxp3* promoter; instead, Th17 differentiation is increased both *in vitro* and *in vivo* in a model of asthma [39]. These studies suggest that the balance between ID2 and ID3 will also be important in the fate decisions of mature $CD4^+$ T cells as they respond to infection and differentiate into distinct effector and memory populations.

Conclusion

The idea that pairs of transcription factors function in a coordinated and, at times, opposing fashion has been proposed for T-BET/EOMES, BLIMP-1/BCL-6, ZEB1/ZEB2 and STAT3/STAT4 [8]. Similarly, it is becoming clear that E and ID proteins work to balance the generation of short-term effector-memory versus long-lived memory populations. Over the course of the $CD8^+$ T cell response, ID2 and ID3 also demonstrate reciprocal expression and function, suggesting a complex interplay in their regulation of E protein activity that is not yet fully understood. Ultimately, it is how these pairs function together to direct the transcriptional programs required for mature T cell differentiation that is of greatest interest. Future studies identifying the direct gene targets for each of these transcriptional regulators in the context of global gene-expression changes will be critical for assembling the comprehensive transcriptional network governing $CD8^+$ T cell immunity.

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Highlights

- E and ID proteins control many aspects of $CD8^+$ T cell differentiation.
- ID2 mediates effector cell survival and differentiation to memory.
- ID3 marks cells as memory precursors and maintains the longevity of these cells.
- ID2/ID3 coordination, controlled by the infection milieu, shapes the $CD8^+$ T cell response.

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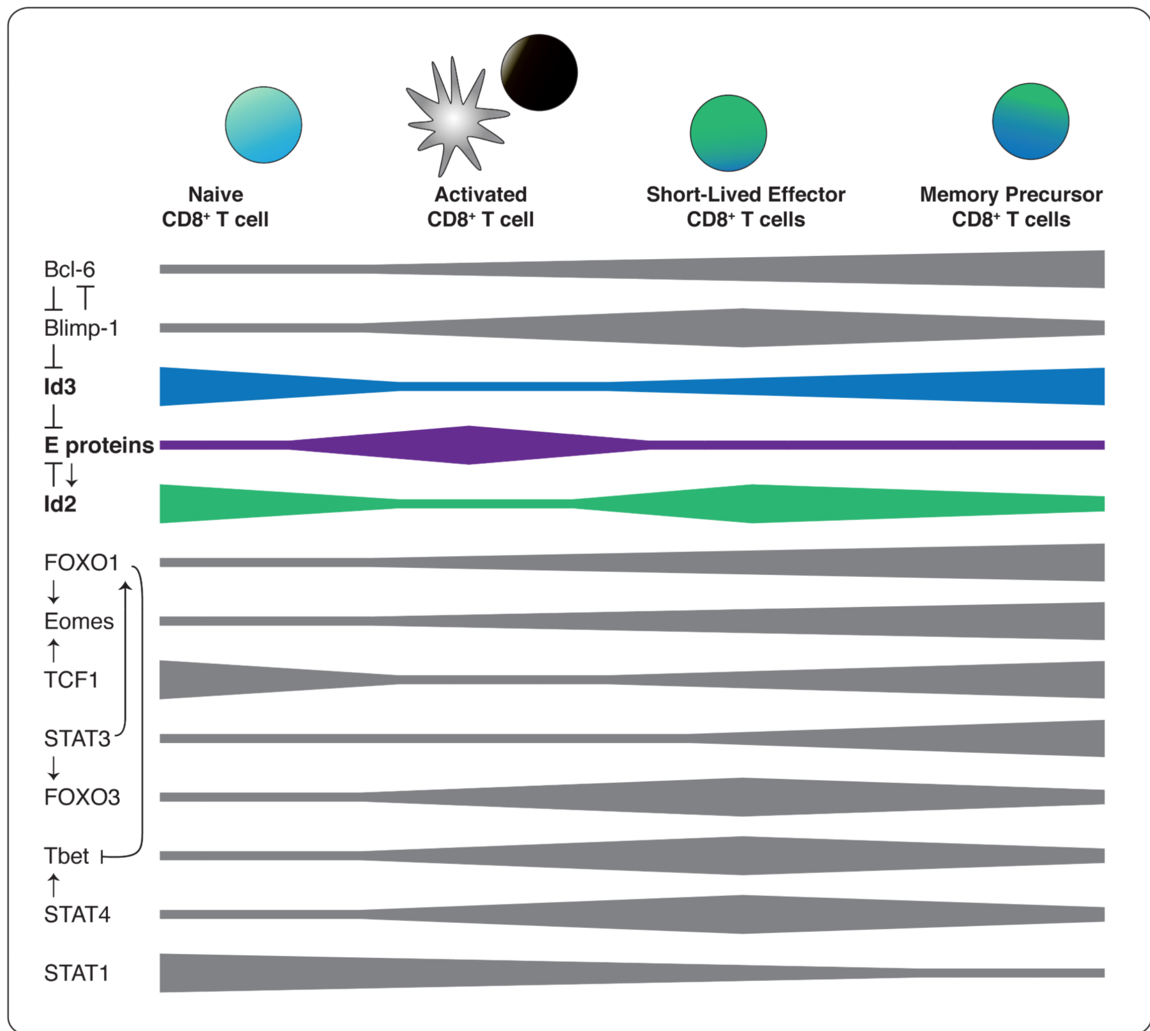

Figure 1. Interplay of transcription factor networks during CD8$^+$ T cell activation and differentiation

Width of bar indicates transcription factor activity and/or expression.

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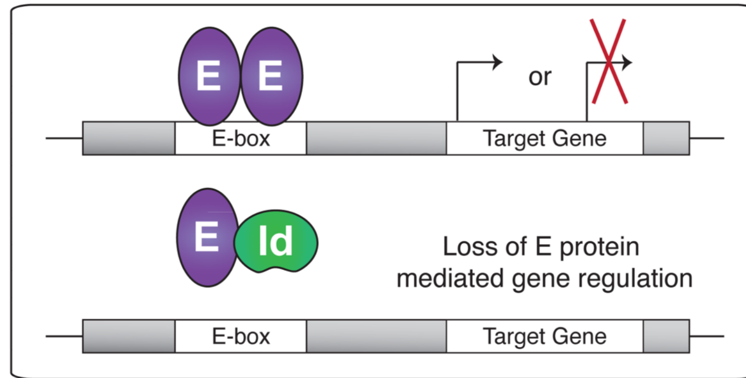

Figure 2. E protein activity is regulated by Id proteins

E protein dimers bound to DNA can activate (top) or repress gene transcription. When E proteins heterodimerize with Id proteins, DNA binding is inhibited, also blocking target gene transcription (bottom).

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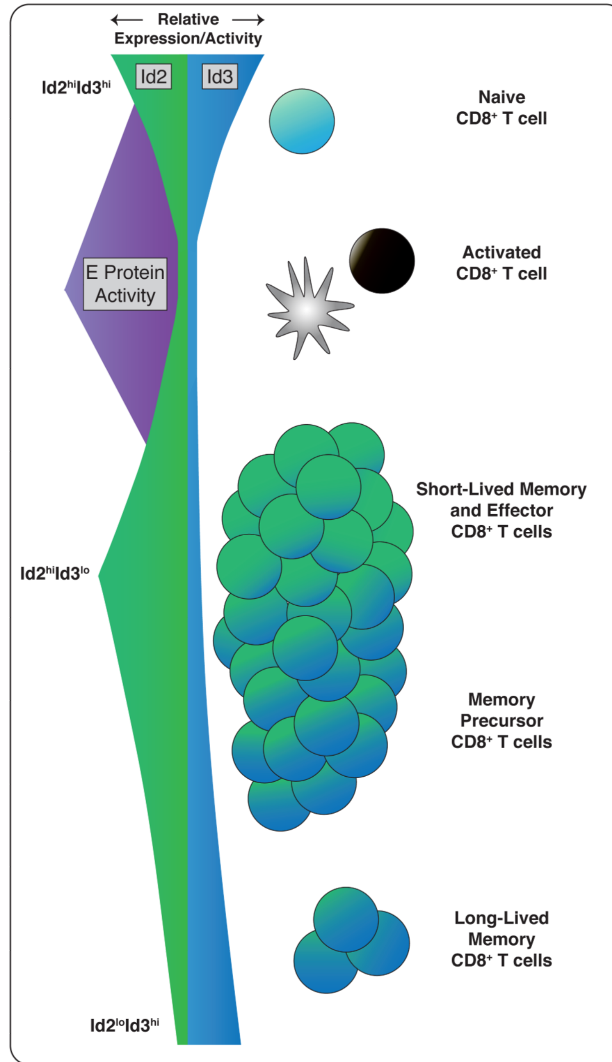

Figure 3. E and Id proteins show coordinated expression during CD8$^+$ T cell activation and differentiation

Id2 and Id3 levels are downregulated upon CD8$^+$ T cell stimulation, coincident with an increase in E protein DNA-binding activity. Id2 expression increases at the peak of infection, promoting survival and terminal differentiation. Conversely, Id3 expression is downregulated at the peak of infection, but increases to mark memory precursors and maintain the long-lived memory pool.