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2 # Title: The regulation of lymphocyte activation and
3 # proliferation
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Abstract

Activation induced proliferation and clonal expansion of antigen specific lymphocytes is a hallmark of the adaptive immune response to pathogens. Recent studies identify two distinct control phases. In the first T and B lymphocytes integrate antigen and additional costimuli to motivate a programmed proliferative burst that ceases with a return to cell quiescence and eventual death. This proliferative burst is autonomously timed, ensuring an appropriate response magnitude whilst preventing uncontrolled expansion. This initial response is subject to further modification and extension by a range of signals that modify, expand and direct the emergence of a rich array of new cell types. Thus, both robust clonal expansion of a small number of antigen specific T cells, and the concurrent emergence of extensive cellular diversity, confers immunity to a vast array of different pathogens. The *in vivo* response to a given pathogen is made up by the sum of all responding clones and is reproducible and pathogen specific. Thus, a precise description of the regulatory principles governing lymphocyte proliferation, differentiation and survival is essential to a unified understanding of the immune system.

Introduction

According to classic two-signal theory, lymphocytes face a binary decision when stimulated by antigen and must choose between tolerance (death) and activation (proliferation). A second signal is needed to tip the balance from one state to the other. Careful studies of the control of T and B cells are substantially modifying this view and replacing the binary decision with a quantitative signal integration model that tempers the overall strength and type of response to the nature of the threat. As a result, the magnitude and duration of the immune response must be seen as continuously variable. How this T cell behaviour is modulated, at molecular, single cell and population levels to achieve such a rich set of alternative outcomes remains under intensive investigation. **Figure 1** illustrates the control of lymphocyte proliferation as a two-stage process with each naive cell integrating activation signals, stochastic probabilities and ongoing signals to control the rich heterogenous population outcome.

Early programming cooperates with ongoing signal integration to control the response magnitude

In reviewing progress to date, it is helpful to distinguish two separate stages for lymphocyte activation. In the first, an autonomously programmed response, leading to multiple changes in

52 fate are motivated by the initial stimuli. T cells divide several times subsequent to removal of
53 stimuli. In CD8+ T cells this can be after a very brief initial exposure [1-4] whereas in CD4+
54 T cells a longer antigen exposure is required to commit cells to an autonomous proliferative
55 burst [5,6]. A similar initial autonomous clonal division is observed in B cells [7-9]. In the
56 second phase of control, the early cell programming is further modified by ongoing signals.
57 For example, T cells modify their own environment by producing the growth factor IL-2 that
58 can promote their continued division [10]. As signalling inputs can operate simultaneously on
59 both phases of the response it can be difficult to determine individual control mechanisms.
60 Costimulatory signals as well as cytokines and chemokines all play fundamental roles in
61 regulating one or more parameters that determine the final cell numbers. Additional features of
62 signal control, such as increased TCR affinity, enhanced dose, duration or mechanical
63 properties of TCR-pMHC contacts or prolonged antigen exposure also result in a greater
64 response magnitude, with increased rounds of cell division or greater recruitment of cells into
65 division [11,12]. In many cases, the early expansion rate of activated T cells is unchanged in
66 these systems. Instead, the duration of their expansion is increased [2,11,12] suggesting an
67 equal proliferation rate of activated cells with more cells dropping out of division or dying
68 sooner under weak stimulation conditions.
69
70 For T cells the extent of the initial proliferative burst is a major determinant of response
71 magnitude *in vitro* and *in vi*vo. The average number of divisions undergone can vary with
72 stimulation. T cells integrate all the signals they receive through the TCR, costimulatory
73 molecules and cytokines receptors to determine the size of the initial burst, referred to as their
74 initial ‘division destiny’ (DD) [13]**. Multiple contributions to DD, provided at the same time,
75 added arithmetically allowing predictions to be made for the final response. In this study the
76 authors demonstrate that many different combinations of costimulatory signals are capable of
77 adding to a significant response outcome. These experiments also highlight control of the
78 second phase of the T cell response: Cytokines such as IL-2 and IL-4 play both a major role in
79 initiating, as well as sustaining / extending cell division beyond their initial autonomous DD.
80 This maintenance was shown to be particularly important for T cells that have migrated to sites
81 of infection or inflamed tissue [14,15]. T cells modulate IL-2 production and integration of IL-
82 2 signals as a mechanism of paracrine communication in order to fine tune and optimise the
83 response magnitude [10,16,17]. Furthermore, although the proliferative effect of a particular
84 stimulus can act predominantly on initial programming, it may also have alternate modulatory
85 roles during the subsequent progression of the response. For instance, CD28 signalling

86 increases IL-2 production and sensitivity [18]. If IL-2 is blocked, the CD28 signal is still
87 effective at programming and promoting division destiny changes into naïve T cells but the
88 signal must be received prior to the first division to have an effect [13]. Later engagement of
89 CD28 alters downstream fate selection during the response without having any further
90 proliferative impact [19].
91
92 The regulatory precision of the initial burst of proliferation and return to quiescence by
93 stimulated T and B cells [9] suggested a common mechanism might be found. This proved
94 correct. The cell division-promoting proto-oncogene Myc [20]** is induced upon activation
95 and lost over time until a minimal threshold is crossed, and division ceases. Rather than diluting
96 by division, as was expected, Myc levels degraded over time at a predictable rate that was
97 faithfully passed on to daughter cells independently of division number. As expected for this
98 form of control, the level of Myc protein induced after activation was found proportional to the
99 strength and number of signals received by the cell, and its level highly correlated with
100 subsequent DD. Therefore, Myc translates the signals the cell receives into the time that each
101 founder cell is given to divide before returning to quiescence [20].
102 Furthermore, in addition of providing stimulatory signals on their own, inflammatory signals
103 such as IL-12 and IFN-α can increase sensitivity to IL-2 signalling [14,21,22]. Continuous
104 signalling via IL-2 or other cytokines slows the loss of Myc protein and therefore extending
105 the period of time for which the cells can divide [20] [23]* [24]. Understanding how these
106 factors function as part of a subcellular network, combining and cooperating to determine the
107 ultimate proliferative potential of an individual T cell remains a major objective in the field of
108 lymphocyte biology.
109
110 These studies may have a further counterpart in the germinal centre. During affinity maturation
111 germinal centre B cells travel from the light zone where they undergo positive selection to the
112 dark zone and undergo somatic hypermutation [25]. Interaction with Tfh cells during the
113 positive selection process provides proliferation and survival signals. In this context Myc
114 expression is induced through interaction with Tfh cells and is seen in a proportion of,
115 presumably, recently activated cells [26,27]. Affinity dependent stimulation is thought to
116 control proliferation and survival with low affinity B cells dying in the light zone while
117 dysfunctional BCR induced through mutation leads to cell death in the dark zone [28,29] [30]*.
118 In parallel with the control of T and B cell DD, it seems likely that higher affinity B cell clones
119 receive stronger stimuli and accumulate more Myc, extending their duration of proliferation in

120 the dark zone and therefore licensing these clones for more extensive somatic hypermutation
121 [29].
122
123 **Clonal concordance, probabilistic events and fate inheritance shape the response**
124 The proliferative and phenotypic profile of a responding T cell population is highly
125 heterogeneous, even under highly controlled *in vitro* conditions. Despite this extensive
126 diversity, there is a remarkable concordance of the proliferative fate within a clonal family
127 while a considerable disparity is observed between different clones even in response to
128 identical stimulation conditions [31] [32]* [33]*. *In vivo* studies have also demonstrated
129 extensive heterogeneity in clone size and a distinct correlation between clonal proliferative
130 potential and cellular phenotype [34-39]. The phenotypic correlation with clonal burst size
131 points to a role for both heritability and division as determinants of the emergence of cellular
132 heterogeneity [35,36,40]. This is complemented by studies that have elucidated a role for
133 division progression in regulating specific components of T cell differentiation, such as
134 cytokine production, cytotoxicity and surface marker expression [40-45].
135
136 Although there is a clear influence of clonal membership on division progression and
137 phenotype, *in vivo* studies demonstrate substantially greater intraclonal diversity than the
138 striking concordance observed *in vitro* [32,37,38]. TCR signal quality strongly determines the
139 response outcome on a population basis, and although weakly stimulated cells expand less,
140 many are still able to acquire effector functions and differentiate into memory cells [11,46,47].
141 Therefore the fate of T cell clones is not controlled by the TCR ligation quality alone, but TCR
142 ligation works in concert with quantitative integration of additional signalling and stochastic
143 events to determine the fate outcome of the clonal progeny [13,33,35] [46]** [48] [49]*
144 [50,51].
145
146 Many of the above studies highlight early stochasticity and familial heritability as key drivers
147 of emergent heterogeneity. Several different models have been proposed to explain the
148 diversity observed in T cell fates. The concept of asymmetric division of the founder cell
149 resulting in two distinct fate outcomes of the daughter cells and their progeny has been
150 proposed as a determinant of T cell fate and population heterogeneity [5,52-54]. Recent studies
151 have proposed a role for the polarised segregation of Myc and subsequent asymmetric
152 inheritance of metabolic programming as a determinant of CD8+ T cell fate selection [55,56].
153 However, the uneven inheritance of Myc between first-division T cells is at odds with its role

154 as a regulator of the highly symmetrical clonal phenomenon of DD [20]. In an alternative
155 model the strong familial concordance in concert with early stochasticity are sufficient to
156 describe the emergence of the clonal diversity [46,57]. Computational descriptions of clonal
157 heterogeneity have also highlighted the capacity to resolve patterns of T and B cell
158 diversification without the requirement of asymmetric fate segregation [50,58]. Furthermore,
159 impairment of the capacity of cells to polarise their contents does not hinder the generation of
160 lymphocyte diversity [59].
161

162 **Recruitment into division as a first step directing the response magnitude**

163 The number of cells recruited into an immune response is another key determinant of response
164 magnitude. Two main factors determine the number of cells recruited into division: firstly, the
165 induction of a new survival program driven by the strength of stimulation selecting for strongly
166 activated cells to survive [60]. Secondly, whether the surviving cells reach the activation
167 threshold to enter proliferation. On a single cell level this threshold is controlled by the sum of
168 TCR affinity and dose [12] [61]* [62,63] and other signals received by the cells. Consistent
169 with the mechanism of signal addition, IL-2 or additional costimulation increases this precursor
170 frequency and promotes the entry of weakly stimulated cells into division [61,64-66]. Similarly
171 stimulation through the costimulatory receptor CD27 lowers the affinity threshold required for
172 activation, recruiting more low affinity clones into the response, potentially as a measure to
173 broaden the subsequent memory pool repertoire [67].
174 On a population level, whether a response is observed is determined by the sum of all individual
175 outcomes and the complex interaction between these cells and the molecules they produce.
176 This can be described as a collective decision made by the T cell population [68]. IL-2 produced
177 by strongly activated cells plays a critical role in this activation phase, as some low affinity T
178 cells can reach the threshold to enter division through integration of IL-2 produced by strongly
179 activated cells. The response rate of a mixed population of high and low affinity cells can be
180 modelled and predicted accurately using a dynamic system incorporating IL-2, IL2R and PIK3
181 levels controlling the accumulation of Cyclin D to reach the threshold of cell cycle entry [69]**
182 demonstrating how the signal integration by individual cells controlling their fates fine tunes
183 the overall response on the population basis.
184

185 **Survival as an additional and independent mechanism shaping the immune repertoire**

186 The survival of activated T cells critically underpins the ability to form an immune response.
187 This process is carefully regulated by a quantitative balance between pro- and anti-apoptotic

188 members of the Bcl-2 family proteins of the intrinsic apoptotic pathway (reviewed in [70]). A
189 survival program is induced after T activation, that is distinct from their naïve survival program
190 and operates simultaneously but independently to the proliferation program [71,72]. Both pro-
191 survival and pro-apoptotic proteins are induced by T cell activation signals through the TCR,
192 costimulatory molecules (i.e. CD28) or cytokines such as IL-2 [60,73-75]. A similar
193 quantitative switch in survival programs occurs in B lymphocytes [70,76]. In cells receiving
194 strong signals this balance favours a pro-survival state, however low affinity populations are
195 more sensitive to shaping through differential survival as stimulation with a lower affinity TCR
196 or a reduction in costimulatory signals and cytokines, favours death and the elimination of
197 weaker responders [75,77-79]. This points to a key function in the regulation of the response
198 quality.
199 As the lymphocyte survival program is initiated by many of the same signals as proliferation it
200 is often difficult to distinguish the relative importance of contributions of these processes in
201 shaping the immune response; however several lines of evidence suggest their regulation is
202 independent, and can be uncoupled. For instance, CD28 signalling and other growth factors
203 promote survival in the absence of proliferation [80]. CD8+ T cells deficient in the kinase Erk2
204 [81] or the transcription factor Bach2 [82] have been shown to have no defect in proliferative
205 potential, but have a reduced response magnitude due to impaired survival. Furthermore the
206 rate and extent of clonal expansion in strongly stimulated cells is not greatly impacted by cell
207 death. When pro-apoptotic molecule Bim is deleted or pro-survival molecule Bcl-2 is
208 overexpressed in T or B lymphocytes they undergo the same number of divisions in response
209 to a given stimulus irrespective of the enhanced cell survival [13,20,83,84]. A similar effect
210 can be observed in vivo, with Bim-deficient CD8+ T cells expanding to the same extent but
211 taking longer to contract [85], further demonstrating the independence of cell division and
212 survival. The significance of this is highlighted by the consequences of changes to either
213 parameter. The combined effect of small changes in survival or DD time synergise to a greatly
214 enhanced response when applied in combination and can be predicted by combining the
215 probability distributions for each timer [20].
216
217
218 **Conclusions:**
219 When the number of alternative cell fates, and the large number of known modifiers are
220 enumerated, the regulation of T and B cell responses appears impossibly complex. When
221 combined with the fine detail of cellular niches in different tissues, and the potential for

222 transient and persistent signal exposure, it is easy to imagine a daunting combinatorial problem
223 for control. This pessimistic view is at odds with the general observation that immune responses
224 are typically robust and reproducible, and many features of cell responses can be recreated in
225 much simpler *in vitro* environments.
226 In resolving this paradox, we suggest that a timed cellular program, that includes an automated
227 return to quiescence and even eventual death, can serve as a powerful new paradigm for
228 interpreting the complex control of T, B and GC cell responses. Manipulation of this core
229 cellular program by multiple modifying inputs provides the foundation for building a
230 reproducible, but highly regulated system. Such a model can also help explain how the present
231 complex system may have evolved from more primitive developmental states that utilised a
232 higher level of cellular autonomy to affect an adaptive immune outcome [7]. We envisage that
233 continued work on this activation paradigm with increasingly quantitative tools will deliver
234 scalable models with the power of prediction and significant potential for immune system
235 control.
236
237
238
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Figure legends

Figure 1. An autonomous self-limiting response underlies T and B cell activation. All lymphocytes receive and process a large number of signals from their environment, antigen and accessory cells. These signals serve to set timers for the burst of division and the eventual death of these cells, as well as either division-linked or timed differentiation changes. Similar cells do not perceive signals in identical manner, leading to clonal family dependent variation that may have a stochastic basis (1). All cell functions governing this initial internal immune program can be affected by external signals, and further fate changes regulated by division or time ensures sensitive and broad ranging fate control (2, 3).