Thanks for the Memory!

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Thanks for the Memory!1
Susan L. Swain2

I am very lucky to have had the opportunity to work with excellent colleagues both at the University of California, San Diego (UCSD) in La Jolla, California, and more recently at the Trudeau Institute in Saranac Lake, New York. This talk is dedicated to all of my present and former colleagues.

Over the past decade, we have come to the understanding that naive CD4 T cells go through multiple stages of differentiation and expansion when they encounter Ag. The initial naive CD4 T cell has little direct function but acts as a precursor whose teleological role is to expand and differentiate into effector cells, which carry out multiple functions and also give rise to memory cells. The memory cells are retained and have both immediate effector cytokine production capacity as well as the ability to more rapidly respond and become secondary effectors. The progression of CD4 T cells through these stages of differentiation is tightly regulated. Responses of naive cells require efficient presentation of peptide Ag by specialized, activated APCs, which display costimulatory ligands that are necessary for the activation and initial proliferation of the naive T cells. Subsequent steps in division are driven by IL-2 (which is produced by the naive CD4 T cell early on) and by other growth and differentiation cytokines and signals. The primary effectors generated as a result of effective CD4 T cell priming in vitro are highly activated cells that begin cytokine and chemokine synthesis immediately upon re-exposure to Ag and secrete very high titers of these effector molecules (1). These same activities can be seen in vivo (2, 3). Many of the effectors, especially those that are Th1 cytokine polarized, die of activation-induced cell death soon after cytokine secretion. The stimulation of effectors is costimulation independent and requires much lower Ag expression on APC than do naive CD4 T cells. This presumably ensures that infected nonprofessional cells can elicit the helper response.

If effectors succeed in becoming resting cells without dying, they can become memory cells. Exposure to survival and growth cytokines that bind to the γc-IL-2R (IL-2, IL-4, and IL-7) and TGF-β block death and facilitate resting (4). IL-7 can play this role in vivo, but the role of other factors is unclear (5). What is clear is that Ag and class II recognition and further division are not required for the transition in the resting state and survival that leads to memory (6, 7).

Memory cells resemble effectors in that they respond very rapidly to restimulation by cytokine synthesis and initiation of division and require lower doses of Ag and less costimulation (8). However, they are resistant to activation-induced cell death like naive cells. Within 2–3 days of restimulation, the memory cells have expanded and become memory effectors that are so far indistinguishable from primary effectors.

Many of the conclusions above were reached from in vitro studies with model Ag. In 1996, we moved from warm and sunny UCSD on the beach of southern California to the Trudeau Institute in Saranac Lake with the dramatic climate of the northeast mountains. As well as a change in locations, we changed the approach we took to analyzing immune responses. Here we switched to in vivo studies and have now moved into models of infectious disease instead of model Ag. These have

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the advantage of relevance to biomedicine and they have other advantages as well. Because immune system evolution was driven by the selection pressure of infectious disease, we believe models of those diseases will reveal secrets about the immune system we will not uncover in model Ag systems. We want to understand the generation and regulation of CD4 T cells so that new and better vaccines can be developed.

Influenza virus is our pathogen of choice. Current vaccines will not protect us from a pandemic influenza (flu) outbreak because they rely on the generation of a strain-specific Ab, which will not cross-react with the new strains with different proteins of the exterior coat. It seems that most natural immunity to flu is also largely due to such Abs to hemagglutinin (HA)\(^3\) and neuraminidase (NA). However, T cells can recognize interior proteins, which change much less, and we imagine that heterotypic T cell immunity to common epitopes may be protective against newly emergent flu strains. There is some circumstantial evidence that natural immunity to shared flu epitopes moderates the virulence of flu.

The flu genome is segmented, with the nine individual proteins encoded by individual segments, resulting in reassortment if a cell is coinfected with two flu viruses. Also, flu is a RNA virus, so it lacks the proofreading mechanisms of DNA, leading to accumulation of mutations. Moreover, flu is endemic in both wild and domestic birds and pigs, as well as humans. Although transmission often has some species specificity, it is not strong. As a result, new strains do get generated from reassortment every year. Together these factors lead to multiple new strains every few years and sometimes these will have the dreaded combination of high transmissibility, high virulence, and unique surface HA and NA that result in a flu capable of causing a pandemic because humans have little effective immunity. We will face pandemic flu at some time and the current avian flu (‘bird flu’) could be the donor strain for the new HA proteins (H5 or H7) and virulence factors. The 1918 “Spanish” flu resulted in one-third of the U.S. population infected, >20 million deaths, and a 10-year plunge in expected lifespan from 55, before the pandemic, to 37 years of age.

With modern transportation, disease and deaths from a new pandemic would be worldwide and could be catastrophic. Therefore, we set out to test whether CD4 T cell immunity can provide a level of protection from flu that would justify adding to current vaccines, proteins and adjuvant that will generate CD4 T cell memory.

Fortunately we had available a TCR transgenic mouse, which recognizes the HA of a common strain of flu —PR8, originally made by Lo and colleagues (9). This gave us the ability to use an adoptive transfer model in which we could visualize the response of the TCR transgenic donor naive CD4 cells in an intact host. We knew, from many early studies, that CD4 and CD8 T cells were needed for optimum flu clearance in a primary response, that B cells could also be important, and that the most efficient secondary protection is seen when there is circulating neutralizing Ab present. Flu virus in nature enters the respiratory tract because an aerosol is inhaled, and the virus infects lung epithelium in the airways or it infects via the mouth when it is touched after contact with virus on some other sur-

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\(^3\) Abbreviations used in this paper: HA, hemagglutinin; NA, neuraminidase; DLN, draining lymph node; BAL, bronchoalveolar lavage; LN, lymph node.
Many of the donor-derived effectors can produce IFN-γ as detected by intracellular cytokine staining, but only some effectors in the lung produce the cytokine ex vivo without the need for restimulation (1). The fact that there is a large cohort of highly differentiated, cytokine-secreting CD4 effector cells in the lung suggests these CD4 effectors may be important mediators of viral clearance, a role previously ascribed primarily to the CD8 effector population. Because CD4 T cells can be specific for heterotypic determinants shared among flu strains, we have analyzed in detail what mechanisms CD4 effectors can use to provide protection.

To evaluate potential CD4 effector mechanisms, we analyzed the effects of 4-day in vitro generated flu-specific Th1 CD4 effectors, as well as those generated in vivo. The in vitro generated effectors are free of other cells, migrate to the lung upon transfer, and share the phenotypic and cytokine-producing phenotype of their in vivo counterparts. The effectors were introduced into BALB/c hosts just before infection with a lethal dose of PR8 influenza (5 LD₅₀). Survival, weight loss, and viral titer were evaluated for 2–3 wk. Because IFN-γ is necessary for flu clearance, we routinely compared effectors generated from control HNT TCR transgenic mice to ones from IFN-γ knockout HNT donors. Hosts in the group without added donor effectors lost weight quickly and were moribund by day 10. In contrast, in hosts given either normal or knockout CD4 Th1, the weight loss started to reverse at day 8, and hosts all survived and regained weight, indicating a dramatic protective effect of the Th1 effectors. A similar pattern was seen in T-deficient, nu/nu hosts, indicating that neither host CD4 or CD8 T cells was necessary for protection. The introduction of CD4 effectors caused a control of viral growth that was apparent as early as 2–3 days (D. Brown, unpublished observations).

The Th1 effectors were specifically cytotoxic to flu-infected targets, and this cytotoxic activity was IFN-γ independent but perforin dependent. Thus, we suspect that granular exocytosis may explain some of the anti-flu effects of the CD4 T cells. When we examined the different components making up the in situ response to flu, we found that Ab was barely detectable until day 8, with titers rising thereafter. However, the CD4 effectors could be inducing an earlier B cell response. To evaluate the need for host B cells, we introduced CD4 effectors into B cell-deficient JHD mice. The early response to flu, up to day 8, was similar to that in wild-type hosts, with a cessation of weight loss by day 5. However, B cell-deficient hosts began to lose weight again after day 8, and they succumbed by day 13–15. Thus, we suggest that the effectors were working to give a one-two punch. First, we postulate they killed off virally infected cells, gaining time for an adequate B cell response to develop so that B cell-produced Ab could clear virus. Subsequent studies support this mechanism and indicate that in vivo generated effectors are also able to protect using these mechanisms and at least one IFN-γ-dependent mechanism (D. Brown, unpublished observations).

This strengthens our conviction that vaccines which induce CD4 and CD8 T cell priming should provide some measure of protection even when Ab to the particular strain of HA are not initially present.

Earlier studies in the laboratory had indicated that CD4 effectors can transition to memory cells without the need for further Ag or MHC recognition or for division (6, 7). That finding suggested to us that the progressive differentiation and epigenetic modification that results in programming the effectors, which occurs as naive cells become effectors, ceases when Ag disappears. In the model we have been examining, the effectors we see are quite heterogeneous in the periphery. Therefore, we wondered if effectors at multiple stages in their progressive differentiation had the capacity to give rise to memory cells and if different subsets of memory cells were in fact a consequence of heterogeneity at the effector level being retained through the transition to memory. To evaluate this possibility, we first followed effectors as they became memory cells.

In a number of kinetic analyses, we had found that effectors become resting cells within 2–3 days (5) and that they become virtually indistinguishable from later memory cells within 4 wk of initial infection. When we compared the patterns of heterogeneity at the peak of effector response (day 7) with those at 5 wk, we saw substantial retention of the patterns for a number of markers, including division (CFSE intensity), CD62L, and CD49d expression, as well as cytokine profile (data not shown). Most strikingly, the heterogeneous pattern in secondary lymphoid tissues remained in contrast to a much more homogeneous pattern in the lung and BAL (E. Roman, unpublished observations). These results reinforced our hypothesis, but because we were not following a defined population, the results could be subject to other interpretations.

To explore further the concept that distinct effector populations are responsible for distinct memory subsets, we primed naive TCR transgenic CD4 cells in vivo in adoptive hosts and then recovered sorted CD4 effector cells from the DLN vs lung. These cells were then transferred to a second host. The initial populations and the donor cells in the second host were subsequently analyzed. The initial populations closely followed those seen in situ. After transfer of DLN cells, we could detect donor cells in the spleen and LNs of secondary hosts, but no cells were detectable in lung. The “memory” donor cells in spleen and LNs were still heterogeneous for CFSE and CD62L (data shown) and for CD49d and cytokine production (data not shown). In contrast, the effector cells initially recovered from the lung were detected in the lung as well as the spleen and LNs and were more homogeneous in all sites. There was some up-regulation of CD62L on a small fraction of lung-derived effectors detected in spleens and LNs of adoptive hosts (E. Roman, unpublished observations). Although this is not definitive proof that all memory heterogeneity stems from different programs already expressed at the effector stage, it does strongly suggest that effectors at various stages in a scheme of progressive differentiation can contribute to the memory pool and is consistent with the suggestion that different effector subsets beget corresponding, related memory subsets (Fig. 1). This is important because it suggests that if we sample effectors produced in response to vaccination when they are readily detectable, what we see will be related to what we will retain as memory.

What factors influence the effector and memory populations we generate? Recent published studies from our lab indicated that repeated stimulation with Ag leads to less functional effectors both in vitro and in vivo (12). Obviously an infection leads to a large amount of pathogen, present for some time. What is the consequence for the naive T cell response of being exposed to this large amount of Ag for a fairly long time? How long in fact are flu Ag present in such a form as to stimulate naive CD4 T cells? To address these questions we introduced naive CD4 TCR transgenic cells (indicator cells) at different intervals after...
flu infection and monitored division, effector generation, and subsequent memory. To our surprise, sufficient presentation of viral Ag to drive naive CD4 cell division persisted for >3 wk. Non-cross-reacting flu did not stimulate the flu-specific naive cells and non-flu-specific naive cells did not respond. However, generation of flu-specific, IFN-γ-producing effectors was greatest when naive cells were introduced by 6 days, with very few fully differentiated (CD62L⁻), fully divided effectors recovered when naive cells were introduced by 6 days, with very few fully differentiated (CD62L⁻), fully divided effectors recovered from the lung after transfer beyond 8–10 days (13).

This suggested that residual Ag presentation beyond the time of flu clearance (day 8 or so) was limiting and could promote only partial differentiation of responding T cells. We wondered if these less differentiated responding cells could nonetheless become memory cells, as suggested by our theoretical model. To test this we transferred indicator donor naive cells at 0, 1, 2, and 3 wk following flu infection and compared their recovery as effectors at day 7 and as memory at day 28. As expected, we found the largest numbers of effectors were recovered following transfer at day 0 and at 1 wk, with many fold fewer after transfer at 2 or 3 wk. However, memory recovery exhibited a different pattern. Surprisingly, more donor memory cells were recovered after donor cell transfer at day 7 than at day 0, and despite the much lower effector numbers, memory cells were still detectable when donor cells were transferred 2 and 3 wk after viral infection (13). This suggested that the less differentiated CD4 T cells actually were more efficient at becoming memory cells than the most differentiated effectors. Moreover, these results raised the possibility that newly exported, or perhaps even previously unstimulated, naive CD4 T cells could be recruited into a responding cell pool after viral clearance and that such cells might play an important role, as they had potential for preferential survival and transition to memory cells.

We believe this set of results suggests it is time to consider some different strategies in making vaccines for influenza and perhaps other viruses. First, given the fact that CD4 as well as CD8 cells can contribute to protection, vaccines that are designed to stimulate T cell immunity to shared, invariant flu determinants, as well as those that stimulate Ab-mediated response to shifting HA and NA, could provide broader protection. Second, in evaluating vaccine efficacy, the induction of T cell effectors as well as B cell Ab should be assessed.

Third, while memory may reflect the effector response, some component of memory may actually develop later, suggesting that it might be wise to evaluate CD4 T cell response several weeks after peak effector response. Finally, our results suggest that responses to replicating pathogen that are present in high concentration and/or for prolonged times may result in the generation of exhausted effectors that do not contribute to memory.

We believe that further insights into how memory is generated and how protection is achieved can lead to new and better vaccine strategies to protect us against pandemic flu and other dangerous pathogens.

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