NK cell memory: discovery of a mystery

Ulrich von Andrian recounts how an unexpected experimental result called into question a well-established concept in immunology: the mechanism of immune memory. Follow-up experiments revealed that NK cells can mediate antigen-specific adaptive immune responses.

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"There must have been a mistake!" This was my first reaction when Mahmood Goodarzi showed me a most unexpected set of experimental results. It was the summer of 2003, and we were investigating the traffic signals that mediate the recruitment of effector T cells to peripheral sites of antigen (Ag) challenge. Mahmood, a postdoctoral fellow, had set out to explore this phenomenon in the murine bladder and was finalizing experiments for our first manuscript on this topic. Our initial rationale for choosing the bladder for our studies was that the default clinical therapy for non-muscle invasive bladder cancer had been the intravesical instillation of Bacillus Calmette–Guerin (BCG), which results in tumor remission in a majority of patients[1]. Although the exact mechanism of action of BCG therapy was unknown, the treatment had been shown to elicit a cytotoxic lymphocyte response in both patients and mouse models[2–4]. Thus, we asked whether and by what mechanisms local challenge of the bladder with an Ag could induce T cell recruitment.

We understood that naive T cells must first be stimulated by a cognate Ag in a lymphoid organ to acquire the prerequisite traffic molecules to subsequently migrate to peripheral tissues[5]. The specific recruitment signals for T cells vary between tissues and were unknown for the bladder. Therefore, our experimental plan called for a sensitization step whereby an Ag was applied to the skin to elicit a population of effector T cells in the draining lymph node (LN). Once these effector cells had left the LN and entered the blood stream, we hoped that a second exposure to the same Ag injected through a catheter into the bladder lumen would cause the circulating cells to accumulate in the bladder wall. We reasoned that, by using blocking antibodies and mutant mouse strains to disable candidate traffic molecules, we could dissect the molecular mechanisms of T cell recruitment to the bladder.

Our choice of Ag was dinitrofluorobenzene (DNFB), a chemical hapten that forms covalent adducts with self-proteins when applied to the epidermis[6]. The first encounter with haptens usually elicits only a mild and transient inflammatory irritant response in the exposed skin. However, according to theory, some of the chemically altered proteins are transported to cutaneous LNs, where dendritic cells (DCs) process and present hapten-modified peptides as neoantigens to lymphocytes to establish lasting immunological memory[7]. Subsequent rechallenge of the skin with a much smaller dose of the same hapten then elicits rapid recruitment of Ag-experienced T cells, resulting in a vigorous inflammatory response known as contact hypersensitivity (CHS). Many people are painfully aware of the symptoms of CHS, which manifests clinically as allergic or contact dermatitis that can be elicited by substances in certain plants, but also by many organic and inorganic chemicals and even some metals[8].

Ulrich von Andrian in 2005.

While there are numerous haptens that can induce experimental CHS, many of these agents, including DNFB, are quite hydrophobic, which makes them unsuitable for instillation into the aqueous environment of the bladder. Nonetheless, we chose DFNB because of its potent sensitizing activity when applied to the skin and because it allowed us to rechallenge the bladder with a chemical cousin, 2,4-dinitrobenzene sulfonic acid (DNBS), a water-soluble analogue of DNFB that forms identical covalent adducts and is therefore recognized by DNFB-specific T cells[9].

As expected, instillation of DNBS into the bladder of DNFB-sensitized animals resulted in a vigorous CHS response — a pronounced influx of diverse lymphoid and myeloid leukocytes that were readily detectable in histologic sections of the bladder and could be quantified in single-cell suspensions of dissociated bladder tissue using flow cytometry[10]. The appearance of this DNBS-induced inflammatory infiltrate required prior sensitization with DNFB, indicating that it was a learned response driven by adaptive immune cells. Indeed, the leukocyte infiltrate in DNBS-challenged bladders was dominated by $CD8^+$ T cells bearing activation markers, consistent with our hypothesis that this effect should depend on the recruitment of Ag-specific effector or memory T cells.

Our assumption that the response was T cell dependent was informed by earlier work demonstrating that adoptive transfer of T cells from hapten-sensitized mice to naive congenic hosts is sufficient to confer susceptibility to CHS[11,12]. Moreover, treatment with polyclonal sera or monoclonal antibodies (MAbs) that deplete T cells was shown to abrogate the CHS response[13,14]. However, while the adoptive transfer experiments provided strong evidence that T cells are capable of mediating CHS, they did not rule out a (potentially redundant) role for other immune cells. Moreover, although the question of T cell dependence could theoretically be addressed by antibody-depletion studies, rigorous proof of T cell dependence would require the use of antibodies that are truly selective for T cells. Early depletion studies used serum raised against thymocytes (anti-θ serum) that presumably targeted a plethora of Ags that are not unique to T cells[13]. More recent work had employed a MAb directed against Thy-1 (ref. [14]), a marker that is also not unique to T cells as it is also found on innate lymphoid cells and other cell types. In fact, experiments by the Askenase group had demonstrated that cutaneous CHS responses in immune-competent mice not only involve T cells, but also B1-B cells[7].

The formation of both B and T cells requires the successful assembly of Ag receptors by V(D)J recombination, a process that requires the recombination-activating gene (RAG) complex, which includes the RAG1 and RAG2 proteins. B and T cells are completely absent in mice that lack either RAG protein, whereas the formation of innate lymphoid cells, including natural killer (NK) cells, is RAG independent[15]. Thus, to confirm that the inflammatory response in our bladder CHS model was dependent on T cells, in the same way that contact dermatitis was thought to be mediated by T cells, Mahmoud performed what we expected to be a simple 'checking-the-box' control experiment: he sensitized the skin of RAG-2 'knockout' mice with DNFB and, 5 days later, challenged the bladder with intravesical instillation of DNBS.

Unexpectedly, the inflammatory responses in the bladders of sensitized WT and *Rag2*$^{-/-}$ mice were equivalent in magnitude and were both strictly dependent on prior sensitization. When Mahmoud shared this result with me, my initial response was incredulity (a reaction that was later shared by the peer reviewers of our manuscript). Immunological memory in a mammal without T and B cells — how could this be possible? No obvious mistake or technical error could explain the observation. The results were reproducible even when experiments were performed by blinded observers. Moreover, the test animals' peripheral blood, spleen and inflammatory infiltrate in the bladder were devoid of T and B cells, as expected for RAG-deficient mice. As we were pondering the implications of our observation, other investigators reported that CHS responses could also be elicited in the ear skin of RAG$^{-/-}$ and SCID (severe combined immunodeficiency) mice, suggesting an unknown effector mechanism for CHS that operated independently of the anatomic context and did not rely on T or B cells[16].

At this stage, Jackie O'Leary, a clinical fellow in hepatology at Massachusetts General Hospital, joined our group and set out to explore the mechanism by which CHS responses occurred in RAG-deficient mice. Since this phenomenon had also been observed in the skin[16], Jackie opted to explore the classic ear skin model, whereby mice were sensitized by painting hapten on the shaved dorsal skin and, 5 days later, one ear was painted with a lower concentration of the same hapten while the opposite ear was exposed to diluent. The CHS response was assessed one day later by measuring the difference in thickness between the hapten-challenged ear and the contralateral control ear, a measure of inflammation-induced tissue swelling. Consistent with our earlier findings and regardless of the genetic background or the specific gene deletion, mice that were devoid of T and B cells readily mounted a dermal CHS response to DNFB[10].

Puzzled by these unexpected findings, I mentioned our observations to our neighbor, Klaus Rajewsky, who astutely asked a simple and critical question: is this apparent CHS response Ag specific? Does it reflect a truly adaptive immune response? Extrapolating from Burnet's classic clonal selection theory, which was devised to explain antibody specificity but can also be applied to T cells (and perhaps also memory NK cells)[17], immunological memory, the quintessential feature of adaptive immunity, arises when a pre-existing population of lymphocytes that share a single, unique Ag receptor are stimulated by 'their' specific Ag to proliferate, and future encounters with the same (but no other) Ag produce an enhanced and/or qualitatively distinct recall response.

Is Ag specificity possible in the absence of V(D)J recombination, the cardinal molecular mechanism that generates the vast repertoire of B and T cell clones in higher vertebrates? To explore this question, Jackie immunized RAG-deficient or SCID mice with DNFB or one of two other contact sensitizers, oxazolone and picryl chloride, and then challenged the animals with either the same or a different hapten. CHS responses were elicited to each of these three haptens, but only when the same hapten was used for sensitization and challenge. Moreover, vigorous recall responses were observed even when sensitized animals were allowed to rest for several months prior to challenge. Thus, although T and B cells are both capable of mediating adaptive immunity, neither appeared to be indispensable to develop long-lived Ag-specific immunologic memory in our CHS model.

These findings prompted us to search for immune cells other than B and T cells that could mediate hapten-specific recall responses. We soon began to suspect that NK cells could play a role because these (presumably) innate lymphocytes are present in both RAG-deficient and SCID mice, and, after local hapten challenge, NK cells were prominently recruited to the exposed bladder or ear skin in a sensitization-dependent fashion. Moreover, the CHS response was abrogated both after antibody-mediated depletion of NK cells in RAG-deficient mice and in mouse strains with combined genetic mutations that lacked both B and T cells as well as functional NK cells. Thus, NK cells appeared to be required to elicit CHS responses in the absence of T and B cells.

These observations raised the question of whether NK cells — and which NK cell subset(s) — were sufficient to elicit CHS. At the time, most experiments in mice were conducted with splenic NK cells, which are relatively easy to obtain from single-cell suspensions of excised spleens. However, mice also harbor a sizeable NK cell population in the liver, and there were reports that hepatic NK cells are functionally and transcriptionally distinct from their splenic cousins[18,19]. Thus, we conducted adoptive transfer experiments by isolating NK cells from both the spleen and liver of DNFB-sensitized RAG-deficient donors and injecting them into separate groups of naive recipients. Subsequent challenge with DNFB resulted in a vigorous CHS response in recipients of hepatic, but not splenic, NK cells, indicating that memory NK cells are confined to the liver.

Because hepatic NK cells are heterogeneous and composed of multiple subsets that express discrete combinations of surface markers, we performed additional adoptive transfer experiments with carefully purified NK cell subsets, which showed that only a fraction (~10%) of hepatic NK cells are capable of exerting memory responses. In the C57B6 and C57B10 backgrounds, the most potent memory NK subset expressed Ly6C-I, inhibitory receptors that recognize self–MHC-I (ref. [20]). Previous work had suggested that, as compared to other NK subsets, these self-reactive NK cells mediate more potent effector activities in response to a variety of stimuli[21]. Memory-capable NK cells also obligatorily express the surface marker Thy1, which is found on approximately half of all liver NK cells[10]. In a later study, we also showed that hepatic memory NK cells require CXCR6, a chemokine receptor whose ligand, CXCL16, is constitutively expressed by liver sinusoidal endothelial cells and is essential for memory NK cell survival. Consequently, in the absence of CXCR6, NK cell memory was abolished[22]. However, none of the memory-associated markers we identified were truly unique to hepatic memory NK cells or showed differential expression between memory NK cells with different Ag specificities; many were also found on subsets of splenic NK cells or on other leukocytes that did not confer detectable CHS responses upon adoptive transfer. Thus, it appears unlikely that any of the surface molecules we identified are directly involved in the cognate recognition of recall Ags. In fact, the nature of the Ag receptor(s)

employed by memory NK cells remains a mystery to this day.

The publication of our findings proved quite challenging. Reviewers raised numerous objections and some obliquely implied that the story may have been a result of observer bias (a concern that we sought to address even prior to submission of our manuscript by conducting CHS studies with blinded experimenters). To be fair, our peers' skepticism was hardly surprising. After all, our experiments called into question a long-held central tenet of modern immunology, which posed that Ag-specific memory in higher animals depends exclusively on the clonal response of T and B lymphocytes that express Ag receptors generated by RAG-dependent V(D)J recombination. However, at the time, this paradigm was already in need of revision due to seminal work by Max Cooper and colleagues, who had shown that lymphocytes in jawless fish express a highly diversified repertoire of Ag receptors called variable lymphocyte receptors (VLRs) that do not rely on RAG proteins and are genetically and structurally different from the immunoglobulin (Ig)-based Ag receptors in mammals and other vertebrates[23]. Since the first vertebrates that evolved ~525 million years ago were similar to today's agnathans, it is conceivable that these common ancestors of all vertebrates relied on a VLR-based immune system prior to the subsequent emergence of jawed fish, ~25 million years later, that first evolved RAG proteins and modern Ig-based Ag receptors[24].

In the meantime, NK cell memory has been independently confirmed not only in murine models of hapten-induced CHS[25–27], but also in several infection models[22,28,29]. Moreover, NK cell memory can arise in response to viral Ags and vaccines[22,28,30,31]. Indeed, vaccination of *Rag*-deficient mice with recombinant influenza A Ag markedly prolongs the animals' survival upon subsequent challenge with live influenza virus[22]. Similarly, NK memory has been shown to arise in conventional mice in response to mouse cytomegalovirus (MCMV) and vaccinia virus[28,30] and in humanized mice and non-human primates in response to a variety of Ags, including HIV[31,32]. Indeed, recent work indicates that NK cell memory is also evident in humans[32–35].

While the phenomenon of NK memory is now widely accepted, the mechanism(s) that confer(s) Ag specificity to select NK cells in most settings are still unresolved. One exception is the formation of murine NK memory to MCMV. In a seminal study in 2009, Sun and Lanier[30] reported that the immune response to MCMV in C57B6 mice depends on a population of NK cells that recognize a viral MHC-I-like protein through a germline-encoded activating pattern receptor, Ly49h. Remarkably, the antiviral response by Ly49h$^+$ NK cells closely resembles that of antiviral T cells. Following exposure to MCMV, Ly49h$^+$ NK cells undergo clonal proliferation followed by a period of contraction and, ultimately, form long-lived memory cells that are more potent on a per cell basis than naive Ly49$^+$ NK cells in mediating antiviral immune responses[30]. A genetic deficiency in Ly49h renders mice largely defenseless against MCMV challenge, suggesting that this genetically encoded defense mechanism arose in response to recent evolutionary pressure. In this context, it is more difficult to explain why murine NK cells display specificity for haptens and other non-pathogenic Ags. Moreover, a notable difference between the Ly49h-dependent memory NK cells and hapten-specific NK cells is the fact that the former are not restricted to the liver and do not require CXCR6.

The ultimate significance of NK memory in the mammalian immune system (other than the response to MCMV) is still unclear. A rigorous assessment of this question will require the availability of new biological tools and a molecular understanding of the mechanism(s) employed by NK cells to detect, remember and respond to specific Ags. ❐

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References

1. Morales, A., Eidinger, D. & Bruce, A. W. *J. Urol.* **116**, 180–183 (1976).
2. Taniguchi, K. et al. *Clin. Exp. Immunol.* **115**, 131–135 (1999).
3. Ratliff, T. L., Shapiro, A. & Catalona, W. J. *Clin. Immunol. Immunopathol.* **41**, 108–115 (1986).
4. Redelman-Sidi, G., Glickman, M. S. & Bochner, B. H. *Nat. Rev. Urol.* **11**, 153–162 (2014).
5. von Andrian, U. H. & Mackay, C. R. *N. Engl. J. Med.* **343**, 1020–1034 (2000).
6. Asherson, G. L. & Ptak, W. *Immunology* **15**, 405–416 (1968).
7. Askenase, P. W. *Clin. Exp. Immunol.* **125**, 345–350 (2001).
8. Elmas, Ö. F., Akdeniz, N., Atasoy, M. & Karadag, A. S. *Clin. Dermatol.* **38**, 176–192 (2020).
9. Phanuphak, P., Moorhead, J. W. & Claman, H. N. *J. Immunol.* **112**, 849–851 (1974).
10. O'Leary, J. G., Goodarzi, M., Drayton, D. L. & von Andrian, U. H. *Nat. Immunol.* **7**, 507–516 (2006).
11. Marchal, G., Seman, M., Milon, G., Truffa-Bachi, P. & Zilberfarb, V. *J. Immunol.* **129**, 954–958 (1982).
12. Bellavia, A. et al. *Int. Arch. Allergy Immunol.* **101**, 402–407 (1993).
13. Asherson, G. L., Allwood, G. G. & Mayhew, B. *Immunology* **25**, 485–494 (1973).
14. Biedermann, T. et al. *J. Exp. Med.* **192**, 1441–1452 (2000).
15. Jung, D. & Alt, F. W. *Cell* **116**, 299–311 (2004).
16. Boehncke, W.-H. et al. *Exp. Dermatol.* **14**, 70–80 (2005).
17. Burnet, F. M. *Aust. J. Sci.* **20**, 67–69 (1957).
18. Vermijlen, D. et al. *J. Leukoc. Biol.* **72**, 668–676 (2002).
19. Vermijlen, D. et al. *Eur. J. Immunol.* **34**, 2529–2540 (2004).
20. Hanke, T. et al. *Immunity* **11**, 67–77 (1999).
21. Kim, S. et al. *Nature* **436**, 709–713 (2005).
22. Paust, S. et al. *Nat. Immunol.* **11**, 1127–1135 (2010).
23. Pancer, Z. et al. *Nature* **430**, 174–180 (2004).
24. Flajnik, M. F. & Kasahara, M. *Immunity* **15**, 351–362 (2001).
25. Majewska-Szczepanik, M., Paust, S., von Andrian, U. H., Askenase, P. W. & Szczepanik, M. *Immunology* **140**, 98–110 (2013).
26. Rouzaire, P. et al. *Eur. J. Immunol.* **42**, 80–88 (2012).
27. van den Boorn, J. G. et al. *Immunity* **44**, 1406–1421 (2016).
28. Gillard, G. O. et al. *PLoS Pathog.* **7**, e1002141 (2011); erratum https://doi.org/10.1371/annotation/b29086ef-e08d-444c-8113-18a6dd429a7c (2011).
29. Habib, S., El Andaloussi, A., Hisham, A. & Ismail, N. *PLoS ONE* **11**, e0153223 (2016).
30. Sun, J. C., Beilke, J. N. & Lanier, L. L. *Nature* **457**, 557–561 (2009).
31. Reeves, R. K. et al. *Nat. Immunol.* **16**, 927–932 (2015).
32. Nikzad, R. et al. *Sci. Immunol.* **4**, eaat8116 (2019).
33. Foley, B. et al. *Blood* **119**, 2665–2674 (2012).
34. Milush, J. M. et al. *Retrovirology* **10**, 158 (2013).
35. Stary, V. et al. *Sci. Immunol.* **5**, eaba6232 (2020).