New frontiers in immunology

Workshop on The Road Ahead: Future Directions in Fundamental and Clinical Immunology

Bruce Beutler¹⁺ & Jean-Laurent Casanova²⁺⁺

¹The Scripps Research Institute, La Jolla, California, USA, and ²University of Paris René Descartes, Necker Medical School, Paris, France

The EMBO workshop on 'The Road Ahead: Future Directions in Fundamental and Clinical Immunology' was held at the Centre d'Immunologie de Marseille Luminy in France between 13 and 15 January 2005, and was organized by B. Malissen, J.-P. Gorvel, E. Vivier and J. Ewbank.

Keywords: immunity; infection; tolerance; auto-immunity

immunologists also feel that a central question revolves around the molecular events that initiate or inhibit T-cell activation, and those with a practical bent point to autoimmune diseases as a key challenge. These topics were all discussed at the EMBO workshop 'The Road Ahead: Future Directions in Fundamental and Clinical Immunology'.

Immunity to infection across the animal kingdom

There are nearly ubiquitous microbes but no 'universal' pathogens. Even the most dreaded human pathogens are innocuous in one species or another, and usually in most. For example, human immunodeficiency virus (HIV) is not a pathogen of mice. However, adaptive immunity is a relative constant: the same type of combinatorial system for antigen recognition exists in all mammals. This in itself suggests that inherited disease resistance, or innate immunity, can protect the host against any microbe, and may be the more powerful of the two systems (Beutler & Rietschel, 2003; Casanova *et al.*, 2002).

Classical genetics has yielded much progress in understanding innate immunity; we finally know what most of the innate receptors are and how they signal. Model organisms have played a large part in this story. *Caenorhabditis elegans*, *Drosophila melanogaster*, and even plants such as *Arabidopsis thaliana*, each have mechanisms for resisting infection. J. Ewbank (Marseille, France) discussed specificity in *C. elegans* innate immunity. Here, it seems, innate immunity is discharged by a Toll-like receptor (TLR): much like the situation in *Drosophila* and mammals. However, the single TLR of *C. elegans* is expressed in neurons and confers avoidance behaviour, causing the worms to eschew the pathogenic microbe *Serratia marcescens* (Schulenburg *et al.*, 2004). One does not normally expect host defence to be mediated neurologically, but in this case it seems to be.

B. Lemaitre (Gif-sur-Yvette, France) expanded on innate immune sensing in *Drosophila* spp. and discussed how flies both sense bacterial infection and discriminate between Gram-negative and Gram-positive bacteria (Vodovar *et al.*, 2004). He pointed out that *Drosophila* can serve both as an innate immune model and as part of a host-pathogen interaction model. As in the case of *C. elegans*, the identification of 'natural' fly pathogens is a difficult problem. Proceeding from the observations made in the wild type, two microbes (the Ecc15 strain of *Erwinia carotovora carotovora* and the

EMBO reports (2005) 6, 620–623. doi:10.1038/sj.embor.7400457

Introduction

In science, asking the right questions may be more difficult than finding the right answers. Asking may amount to a feat of invention that requires more imagination than any other step in the scientific process. What are the main questions confronting immunologists? Immunologists have always attempted to elucidate the mechanisms of microbial pathogenesis and immunity to infection. Adaptive

¹Department of Immunology, IMM-31, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, California 92037, USA

²Laboratory of Human Genetics of Infectious Diseases, University of Paris René Descartes—INSERM U550, Necker Medical School, 156 Rue de Vaugirard, 75015 Paris, France

*Corresponding author. Tel: +1 858 784 8610; Fax: +1 858 784 8444; E-mail: bruce@scripps.edu

++Corresponding author. Tel: +1 33 1 40 61 56 87; Fax: +1 33 1 40 61 56 88; E-mail: casanova@necker.fr

Submitted 18 April 2005; accepted 25 May 2005; published online 24 June 2005

L48 strain of *Pseudomonas entomophila*) have been found to induce immune responses spontaneously when fed to flies, whereas most laboratory studies of infection have depended on the rather unnatural procedure of 'pricking' the fly to inoculate (Vodovar *et al*, 2004).

The mouse has come into its element as a genetic tool with the advent of improved methods for creating and detecting mutations. B. Beutler (La Jolla, CA, USA) reported the results of mutagenesis work in mice whereby he has achieved approximately 20% phenotypic saturation. On the basis of this work, he has calculated that about 50 genes in total have non-redundant functions in TLR signalling. More globally, about 300 genes are required for robust resistance to mouse cytomegalovirus (MCMV; Beutler *et al*, 2005). Among the new genes identified, one encodes a protein of previously unknown function, marked by a mutation called *3d* that is required for nucleic-acid sensing (through TLRs 3, 7 and 9) and for exogenous antigen presentation: particularly cross-presentation and CD8 priming. *3d* homozygotes are highly susceptible to a variety of microbes, and particularly to infection by MCMV. Although crucial for innate perception, the TLRs are dispensable for adaptive immune competence. Beutler and colleagues have identified a separate pathway that causes a powerful CD8 response to a specific antigen expressed in the context of a dying cell. This pathway may be one embodiment of the 'danger model' offered by P. Matzinger (Bethesda, MD, USA), as discussed below.

By no small measure, the methodical deletion of TLR family members has led to the elucidation of the biochemical pathways as we now understand them. T. Mak (Toronto, Canada) discussed his identification of a new and unanticipated role for interferon regulatory factor 5 (IRF5) in connecting TLRs and nuclear factor- κ B binding (NF- κ B) dimers (Takaoka *et al*, 2005).

Many genes were found to be mutated in human patients following the comparison of their immunological phenotype with that of a mutant mouse strain. Intriguingly, there is much less resemblance when the infectious phenotypes of mice and men are compared with each other (Casanova & Abel, 2004). J.-L. Casanova (Paris, France) reported that human patients with mutations in the interleukin-12 (IL-12)-interferon- γ (IFN- γ) circuit were mostly vulnerable to mycobacteria, whereas the corresponding mutant mice were vulnerable to most intracellular microbes tested, including several viruses, bacteria, fungi and parasites. Similarly, patients with complete interleukin-1 receptor-associated kinase 4 (IRAK4) deficiency, who do not produce pro-inflammatory cytokines in response to TLR and interleukin-1 receptor (IL-1R) agonists, suffer mostly from invasive disease caused by *Streptococcus pneumoniae*. A few infections were caused by *Staphylococcus aureus*, and even fewer by Gram-negative bacteria. Finally, a patient with herpes simplex virus encephalitis (HSE) was shown to respond poorly to TLRs 3, 7, 8 and 9, in terms of interferon- α/β (IFN- α/β) and the production of other cytokines. The disease-causing gene *HSE1* is not any of the known genes involved in the TLR signalling pathway and may be the orthologue of *3d*.

As noted earlier, there are no universal pathogens. F. Barre-Sinoussi (Paris, France) noted that HIV infection occurs only in primates, and pathogenic and non-pathogenic models exist in many primate species (Kornfeld *et al*, 2005). A distinction can be made between HIV-1 and HIV-2 pathogenesis, and progressors (individuals who progress to clinical disease) versus long-term non-progressors. The natural history of these diseases suggests that susceptibility to acquired immunodeficiency syndrome (AIDS) does not correlate with acute viral load. Rather, a lack of chronic immune activation is associated with resistance in non-pathogenic infections.

Natural killer cells and innate immunity

Natural killer (NK) cells are peripheral cytotoxic lymphocytes that are devoid of T and B receptors for antigens. They were discovered and are characterized by their natural cytotoxicity against tumour cells lines. In humans, the NK cells in blood are CD3-negative, CD16-positive and CD56-positive lymphocytes. In the mouse, they are most commonly defined as NK1.1-positive, CD3-negative lymphocytes. Their role in host defence remains poorly defined in humans, but recent studies have clearly indicated that they are crucial for innate anti-viral immunity in the mouse.

Immunity to MCMV depends largely on NK cells. K. Kärre (Stockholm, Sweden) first proposed and validated the model of missing self-recognition for NK cells (Kärre, 2002). According to this model, NK cells detect and eliminate cells that fail to express normal self-markers, such as major histocompatibility complex (MHC) class I molecules. This model predicted the existence of inhibitory MHC class-I-specific receptors, which were later identified by other investigators. At the meeting, Kärre discussed missing self-recognition by NK cells in health and disease. Class I molecules each 'educate' NK cells for missing self-recognition. Some MHC class I molecules are more crucial to this process than others, and the more MHC molecules missing, the lower their collective 'educating impact'.

A further complexity of NK cells was discussed by A. Moretta (Genova, Italy). NK cells may contribute to dendritic cell (DC) maturation either by direct stimulation of DCs or by killing certain DCs. DCs have a crucial role in immunity by priming T cells for antigen-specific responses. NK cells acquire the ability to kill autologous immature (but not mature) DCs. Lysis of immature DCs (iDCs) occurs through activation of the NKp30 receptor (for which the ligand is unknown), and Moretta and colleagues have previously shown that transforming growth factor- β 1 (TGF- β 1) mediates the downregulation of both NKp30 and the NK-cell activating receptor NKG2D (Moretta *et al*, 2004). By killing iDCs, NK cells might potentially promote the maturation of iDCs and remove DCs that have not undergone maturation, effectively shaping the innate response and the adaptive response that follows.

Surprisingly, immunity to malaria involves NK cells as well. E. Vivier (Marseille, France) presented recent data on the role of NK cells in immunity to infection, using a human model of immunity to *Plasmodium*. He described studies in which he was able to show that malarial merozoites (the erythrocytic phase of the infection) can induce CD25 and CD69 expression on NK cells. *P. falciparum*-infected erythrocytes induce NK cell activation, and in particular, IFN- γ secretion. The level of activation was equivalent to that induced by K562 tumour cells. All *Plasmodium* strains cause induction, which indicates that a conserved component of the pathogen, or a consistent alteration induced by the pathogen, must be detected. The NK signalling pathways involved have yet to be discovered (Vivier *et al*, 2004).

G. Griffiths (Oxford, UK) described the way cytolytic T lymphocytes (CTLs) kill target cells by releasing cytotoxic proteins that are stored in preformed secretory lysosomes. Selective killing is assured by vectorial movement of these organelles along microtubules leading to focused secretion in the immunological synapse. Some of the proteins controlling these secretion steps are defective in patients with inherited disorders associated with albinism and/or haemophagocytosis, such as Chediak-Higashi syndrome, Griscelli syndrome, Hermansky-Pudlak syndrome and familial lymphohistiocytosis. Key regulators such as adaptin binding protein 3 (Abp3), RabGTPase,

Rab27a (a small GTP-binding protein), Lyst and Munc13-4 act in an orchestrated way to deliver the 'kiss of death' (Stinchcombe *et al*, 2004).

The T cell and the question of tolerance

T lymphocytes originate from the bone marrow but acquire their T-cell phenotype in the thymus, where α/β T-cell receptors for antigens are subject to positive and negative selection, which ensures self-tolerance and self-MHC restriction. They are divided into CD4 α/β T cells (helper T cells), which recognize antigens as peptides embedded into MHC class II molecules, and CD8 α/β T cells (CTLs), which recognize antigens as peptides loaded onto MHC class I molecules.

It is now widely understood that negative selection in the thymus is an imperfect process, and that some autoreactive T cells survive the process. A. O'Garra (London, UK) compared two types of regulatory T cell (Tregs). The first type, the interleukin-10 (IL-10)-secreting regulatory T cell, was discovered some time ago (O'Garra *et al*, 2004). The second type, the CD4⁺CD25⁺ regulatory T cell, has come to light more recently, with the discovery that FoxP3 is required for their development. These Tregs represent about 5%–10% of the peripheral T-cell population, and their absence—due to FoxP3 mutations, for example—leads to the autoimmune scurfy phenotype in mice and to the immunodeficiency, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome in humans. IL-10-producing regulatory cells do not produce FoxP3, and notably, Tregs make no IL-10. The IL-10-producing regulatory cells are also induced by immunization, and mediate tolerance after immunization: perhaps more so than FoxP3-dependent Tregs. Moreover, IL-10-deficient mice develop the autoimmune phenotype later than FoxP3-deficient mice, and different tissues are affected. Hence, it may be argued that two separate T-cell lines have regulatory functions, and do so under a separate set of conditions.

C. Benoist (Cambridge, MA, USA) argued that, regardless of the receptor and its location, all that the immune system recognizes is the difference between self and non-self. Failure to discriminate is evident in genetically transmitted type I diabetes, in which both central and peripheral tolerance defects apply. Benoist pointed out that mutations in the autoimmune regulator (*AIRE*) gene reveal the overall importance of central tolerance. *AIRE* controls the thymic expression of genes normally expressed in the periphery. Evidence that central tolerance is defective in a specific example of autoimmunity comes from fetal thymic organ culture, in which the deletion of autoreactive clones that recognize a model diabetogenic antigen, in the presence or absence of the relevant peptide, is impaired in the non-obese diabetic (NOD) environment (Zucchelli *et al*, 2005). Moreover, there is a high frequency of double-positive clones, which implies a problem with negative selection. By monitoring the frequency of double-positive cells in thymic organ culture as a phenotype, Benoist found a locus on chromosome 1 that was influential in the absence of added peptide, and ascertained an even stronger linkage to chromosome 3 in the presence of peptide.

A different point of view was voiced by Matzinger, who maintained that self/non-self discrimination is not the key issue in adaptive immunity at all; rather, the context in which an antigen is presented (dangerous versus non-dangerous) is the primary determinant of whether a response occurs (Matzinger, 2002). Matzinger's danger model, like the 'pattern recognition receptor' hypothesis, leaves open the questions of which receptors detect danger, how danger is defined and what molecular pathways elicit adaptive activation. The

identification of these receptors—if they exist—and the pathways they serve, looms as an important challenge.

Some decisions concerning tolerance are evidently made beneath the level of the receptor. B. Malissen (Marseille, France) discussed the activation pathway that is initiated by TCR ligation. When TCR encounters an antigen presented by an MHC protein, the tyrosine kinase Zap70 is activated, the linker for activation of T cells (LAT)—a raft-anchored molecule—is phosphorylated, and a platform for further molecular recruitment is created (Ardouin *et al*, 2005). Malissen likens LAT to "a protein fishing line"; flexible and extended in the cytosol to allow a large capture radius. In effect, it organizes other proteins into a form of signalosome. Remarkably different phenotypes emanate from different *LAT* mutations, several of which have been made as knock-ins by gene targeting. If *LAT* is knocked out, or if the last three tyrosines (7/8/9) are mutated, there is total ablation of conventional T cells. Conversely, the Tyr5Phe substitution causes splenomegaly and large lymph nodes to develop by six weeks of age, which are associated with a normal or small thymus, and ultimately, a massive accumulation of Th2 effectors in the periphery.

DCs have the unique capacity to present antigen and prime T cells. S. Amigorena (Paris, France) presented a dynamic analysis of T-lymphocyte fate in intact lymph nodes using bi-photon microscopy. He showed that during the induction of T-cell immunity, T cells establish long-lasting contacts with DCs 15–20 h after immunization (Hugues *et al*, 2004). By contrast, during the induction of peripheral T-cell tolerance, the contacts are transient, which suggests that the formation of stable, long-lasting DC–T-cell conjugates is required for T-cell priming.

Cellular microbiology: microbial variations

The cell biology of infection by several intracellular bacteria was reviewed. J. Galan (New Haven, CT, USA) discussed *Salmonella enterica*, a Gram-negative bacillus that has evolved the capacity to invade non-phagocytic epithelial cells of the digestive tract. Like other enteric bacteria, *Salmonella* delivers virulence proteins into the target cell through the type III secretion system. These factors subvert the cytoskeletal machinery of the host cell by promoting actin rearrangements and altering vesicle transport to trigger invasion (Patel & Galan, 2005). On contact, the bacterial factors SopE/E2 and SopB induce RhoGTPase activation, leading to actin remodeling, whereas SipA promotes F-actin polymerization and prevents its disassembly. Once internalized, the bacterial factor SopB contributes to the creation of a spacious vacuole.

P. Cossart (Paris, France) discussed *Listeria monocytogenes*, a Gram-positive bacillus responsible for gastroenteritis and, more rarely, septicemia and meningitis. *L. monocytogenes* invades non-phagocytic epithelial cells of the intestinal tract. Entry to the cells is mediated by internalin (InlA), cell lysis is controlled by listeriolysin (LLO), and the motion in and between cells by ActA. A central function of ActA is to be a nucleation-promoting factor and activate the Arp2/3 complex, responsible for one of the two main actin-nucleating activities, which leads to actin polymerization and actin-based mobility of *Listeria* in infected cells (Gouin *et al*, 2005).

Brucella suis is able to infect both animals and humans (J.-P. Gorvel, Marseille, France). Its virulence depends on survival and replication properties in different cell types, in which *Brucella* controls the maturation of its vacuole to avoid innate immune responses and to reach its replicative niche associated with the

endoplasmic reticulum (ER). In this process, two sets of genes, those of the VirB type IV secretion system and the cyclic β -1,2-glucan (C β G) synthase of *Brucella abortus*, have critical roles (Lapaque *et al*, 2005). C β G synthase has a central role in circumventing host-cell defence by acting in lipid rafts found on host-cell membranes. C β G-deficient mutants failed to prevent phagosome–lysosome fusion and could not replicate. However, fusion between the ER and the *Brucella*-containing vacuole (BCV) depends on the VirB type IV secretion system, rather than C β G.

S. Girardin (Paris, France) discussed a new class of mammalian pattern recognition molecules. In contrast to TLRs, Nod1 and Nod2 are cytosolic molecules that achieve microbial detection in this cellular compartment. Nod1 and Nod2 both detect peptidoglycan, a macromolecule found in bacterial cell walls (Girardin & Philpott, 2004). In the peptidoglycan polymer, Nod1 detects (GlcNAc)-MurNAc-L-Ala-D-Glu-mesoDAP, a motif found principally in Gram-negative bacteria, whereas Nod2 detects (GlcNAc)-MurNAc-L-Ala-D-Glu, the minimal peptidoglycan signature found in all bacteria. Girardin further showed that TLR2 is not a sensor of highly purified peptidoglycan, but instead efficiently detects bacterial contaminants often co-purified with peptidoglycan. Therefore, Nods and TLRs have complementary, rather than overlapping, recognition properties.

Recognizing that autoimmunity is the evolutionary cost of an adaptive immune system, J. Howard (Cologne, Germany) proposed that the innate system carries risks as well. The p47 GTPases provide an example of this. Six members of the p47 GTPase family are among the most IFN- γ -inducible proteins, and are TNF- α -independent proteins recruited to nascent phagosomes (Martens *et al*, 2004). Some of the p47 GTPase knockouts (interferon-inducible GTPase, IGTP; interferon-inducible protein 1, LRG47; and interferon-inducible protein, IRG47) show susceptibility to *Toxoplasma gondii*. LRG47 alone is required for the containment of various intracellular bacteria and protozoa. LRG47 is a Golgi protein, but there is no precise understanding of the mechanism by which it confers resistance. It comes as a great surprise to find that humans do not have the p47 GTPase system at all. Dogs have several p47s, and fish have a complex p47 system, so why have humans dispensed with it?

Conclusion

As discussed by R. Perlmuter (Seattle, WA, USA), the pathway to practical implementation of basic immunological discoveries is a difficult one. Indeed, when successes are recorded (as with TNF blockade in rheumatoid arthritis and a handful of other diseases), we may still lack a precise understanding as to why we have succeeded. That being said, most immunologists rightly remain focused on basic questions, and there is no denying that some fundamental (and very stubborn) immunological questions have recently given ground to persistence and to impressive new technologies. Exceptions to the new understandings have already emerged, and perhaps we should be glad of this, as it is the exceptions that drive advancement. When TLR signalling does not provide an adjuvant effect, what does? Given what we understand about NK function, how are these cells educated for tolerance? When we speak of 'danger' as a condition that incites an adaptive immune response, what exactly does this mean in molecular terms? The primary molecular defects that lead to autoimmunity are mostly unknown, and may not fit into biochemical pathways of

immune response as they are drawn at present. Moreover, additional innate response pathways may await discovery, and it is absolutely certain that many molecular details remain to be added to those that are known. So there are many questions, but there are also many tools to choose from when addressing them.

REFERENCES

- Ardouin L, Rolink AG, Mura AM, Gommeaux J, Melchers F, Busslinger M, Malissen M, Malissen B (2005) Rapid *in vivo* analysis of mutant forms of the LAT adaptor using Pax5–Lat double-deficient pro-B cells. *Eur J Immunol* **35**: 977–986
- Beutler B, Rietschel ET (2003) Innate immune sensing and its roots: the story of endotoxin. *Nat Rev Immunol* **3**: 169–176
- Beutler B, Crozat K, Koziol JA, Georgel P (2005) Genetic dissection of innate immunity to infection: the mouse cytomegalovirus model. *Curr Opin Immunol* **17**: 36–43
- Casanova JL, Abel L (2004) The human model: a genetic dissection of immunity to infection in natural conditions. *Nat Rev Immunol* **4**: 55–66
- Casanova JL, Schurr E, Abel L, Skamene E (2002) Forward genetics of infectious diseases: immunological impact. *Trends Immunol* **23**: 469–472
- Girardin SE, Philpott DJ (2004) Mini-review: the role of peptidoglycan recognition in innate immunity. *Eur J Immunol* **34**: 1777–1782
- Gouin E, Welch MD, Cossart P (2005) Actin-based motility of intracellular pathogens. *Curr Opin Microbiol* **8**: 35–45
- Hugues S, Fétter L, Bonifaz L, Helft J, Amblard F, Amigorena S (2004) Distinct T cell dynamics in lymph nodes during the induction of tolerance and immunity. *Nat Immunol* **5**: 1235–1242
- Karre K (2002) NK cells, MHC class I molecules and the missing self. *Scand J Immunol* **55**: 221–228
- Kornfeld C *et al* (2005) Antiinflammatory profiles during primary SIV infection in African green monkeys are associated with protection against AIDS. *J Clin Invest* **115**: 1082–1091
- Lapaque N, Moriyo I, Moreno E, Gorvel JP (2005) *Brucella* lipopolysaccharide acts as a virulence factor. *Curr Opin Microbiol* **8**: 60–66
- Martens S, Sabel K, Lange R, Uthaiar R, Wolf E, Howard JC (2004) Mechanisms regulating the positioning of mouse p47 resistance GTPases LRG-47 and IIGP1 on cellular membranes: retargeting to plasma membrane induced by phagocytosis. *J Immunol* **173**: 2594–2606
- Matzinger P (2002) The danger model: a renewed sense of self. *Science* **296**: 301–305
- Moretta L, Bottino C, Pende D, Vitale M, Mingari MC, Moretta A (2004) Different checkpoints in human NK-cell activation. *Trends Immunol* **25**: 670–676
- O'Garra A, Vieira PL, Vieira P, Goldfeld AE (2004) IL-10-producing and naturally occurring CD4⁺ Tregs: limiting collateral damage. *J Clin Invest* **114**: 1372–1378
- Patel JC, Galan JE (2005) Manipulation of the host actin cytoskeleton by *Salmonella*—all in the name of entry. *Curr Opin Microbiol* **8**: 10–15
- Schulenburg H, Kurz CL, Ewbank JJ (2004) Evolution of the innate immune system: the worm perspective. *Immunol Rev* **198**: 36–58
- Stinchcombe J, Bossi G, Griffiths GM (2004) Linking albinism and immunity: the secrets of secretory lysosomes. *Science* **305**: 55–59
- Takaoka A *et al* (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. *Nature* **434**: 243–249
- Vivier E, Nunes JA, Vely F (2004) Natural killer cell signaling pathways. *Science* **306**: 1517–1519
- Vodovar N, Acosta C, Lemaitre B, Boccard F (2004) *Drosophila*: a polyvalent model to decipher host–pathogen interactions. *Trends Microbiol* **12**: 235–242
- Zucchelli S, Holler P, Yamagata T, Roy M, Benoist C, Mathis D (2005) Defective central tolerance induction in NOD mice: genomics and genetics. *Immunity* **22**: 385–396