4.1 The defence sequence

Host defences are processes that minimize fitness loss when hosts are in the presence of, or are infected by, parasites. Hence, defence is not just immunology but includes a wide range of possibilities, from changes in behaviour, to life history, and morphology. As we will see later, defence comes at a cost to the host’s other fitness components and, for that reason alone, defence is never maximal. As we will also discuss in later chapters, parasites have evolved an astonishingly diverse array of means to counteract the hosts’ defences, rendering these defences less effective.

In this chapter, we look at processes under the control of an individual during its lifetime, rather than on the process of selection that changes the genetic endowment of an evolving population. Such responses are ‘plastic’ in the sense that they respond to changes in the environment; for example, when a host becomes infected by a parasite. Plastic defences can conveniently be ordered along a defence cascade that extends from the time of pre-infection to the processes initiated after an unavoidable infection has occurred (post-infection) (Figure 4.1).

Pre-infection defences can act to minimize exposure to parasites, or reduce the risk of successful entry and establishment of a parasite in or on the hosts by anticipatory defences or barriers. Barriers might be physical, such as an insect cuticle that is difficult to penetrate, or chemical, such as a thin layer of antibiotics on the surface of the body to kill fungal spores on the surface. The latter occurs in a number of ants, where the metapleural gland continuously secretes anti-microbial peptides on to the body surface (Ortius-Lechmer et al. 2000). Post-infection defences include the activation of the immune response, as well as behavioural or life-history changes that might mitigate the damage of infection. The repertoire of behavioural defences (pre- and post-infection) is quite astonishing and is in no way less rich than the immune responses themselves (Moore 2002).

4.1.1 Pre-infection defences

An efficient way to avoid becoming infected is to reduce the probability of exposure to parasites in the first place. A number of behavioural changes are very likely to assume this function, although most of the supporting evidence is by correlation rather than by experiment (Table 4.1).

4.1.1.1 Spatial avoidance

A good way to reduce infection risk is to avoid localities that are associated with parasites or to migrate away from such places. The migration behaviour of reindeer might just do this. For reindeer, flies are a major nuisance and can be lethal if an animal is infested with too many maggots. Observations show that migrating herds have fewer of these flies than those herds that stay in the calving grounds. The migratory behaviour and distance covered by migration has, therefore, been interpreted as a behavioural strategy to reduce parasite load (Folstad et al. 1991; Nilssen and Haugerud 1995).

Changing habitat might also reduce the risk of parasitism. For example, stickleback fish changed habitat preference when the risk of parasitism by blood-sucking
ectoparasites was imminent. These parasites (the branchiuran *Argulus canadensis*) are most prevalent near the bottom of the water column and in vegetation, and fish staying in these areas had a higher prevalence of parasitism. In experimental tanks the sticklebacks preferred to stay near the bottom or near vegetation that provide cover against predators, but only when no parasites were present. The preference for cover decreased when parasites were added (Poulin and FitzGerald 1989a). Habitat shift, therefore, is likely to be a compromise between different risks, such as between predation and parasitism (Decaestecker 2002).

4.1.1.2 Temporal avoidance
Parasites are more common during certain times of the day or in different seasons. This is especially true for parasites that are vectored, as determined by the diurnal activities of mosquitoes. Similarly, the free-living stage of parasitoids is mobile and can show variation in the temporal activity pattern. For example, some ants are heavily parasitized by parasitic flies (phorids). The flies lay eggs on to the neck region of foraging ants and the larva then develop inside the host body. In the South-western US and Mexico the flies are especially active during the day and in the wet season. The host ant, *Pheidole titanis*, correspondingly conducts raids on its prey (termites) mainly outside these risky periods. The success of raids is thus reduced but parasitism also decreases (Feener 1988; Folgarait and Gilbert 1999).

4.1.1.3 Avoiding certain diets
Typically, this problem arises when a high-quality diet is also associated with a high risk of parasitic infection. In an experiment, when having to choose between short and tall swards of grass, sheep avoided the more rewarding tall grass sites when they contained infective stages of the

![Figure 4.1 The defence sequence. Pre-infection defences include reducing the exposure to infection (behavioural avoidance, repelling parasites, change of life history, group life, etc.), anticipatory defences (up-regulation of the immune system, vaccination, change of the chemical milieu or of physical conditions), and the existence of barriers (e.g. skin, cuticle, epidermis, endothelia). Post-infection defences include physiological mechanisms (immune defence, and changes in chemical or physical conditions), and measures to reduce fitness losses (by behavioural changes, medication, changes in life history, social defences, etc.) (tolerance).](image-url)
# Table 4.1 Pre-infection defences. Experimental tests to check for benefits have not been made in every case.

<table>
<thead>
<tr>
<th>Defence element</th>
<th>Example</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Behavioural:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Avoid places</td>
<td>Great tits (Parus major) avoid nests that are experimentally infested with fleas. Fleas reduce reproductive success. Baboons do not return to same sleeping place too often; observed interval of 9 days is likely long enough to kill off most parasites.</td>
<td>Christie et al. 1994; Oppliger et al. 1994; Hausfater and Meade 1982</td>
</tr>
<tr>
<td>Use different habitats</td>
<td>Feral horses avoid habitats where biting flies are common. Water fleas balance risk of fish predation (upper water column) and risk of parasitism by microsporidia (near bottom) by choosing intermediate position in water column.</td>
<td>Powell 2006; Decaestecker 2002</td>
</tr>
<tr>
<td>Migration</td>
<td>Reindeer migratory behaviour reduces parasite load. Fire ants relocate nest more often in areas with high incidence of nematodes.</td>
<td>Nilssen and Haugerud 1995; Oi and Pereira 1993</td>
</tr>
<tr>
<td>Shift activity times</td>
<td>Leaf-cutter ants shift activity towards night time to avoid diurnally active parasitoids.</td>
<td>Orr 1992</td>
</tr>
<tr>
<td>Avoid food</td>
<td>Sheep avoid the more rewarding tall grass when experimentally associated with risk of nematode infection. Rats learn to avoid food taste associated with nematode infections.</td>
<td>Hutchings 2001; Keymer et al. 1983</td>
</tr>
<tr>
<td>Choose mate</td>
<td>Mice can detect the infection status of a matting partner from urine and subsequently avoid mating.</td>
<td>Penn and Potts 1998; Penn et al. 1998</td>
</tr>
<tr>
<td>Join group</td>
<td>Infestation by warble flies (Hypoderma tarandi) in young reindeer is negatively correlated with group size. Shoaling in sticklebacks reduces individual risk of ectoparasites.</td>
<td>Fauchald 2007; Poulin and Fitzgerald 1989b</td>
</tr>
<tr>
<td>Repel parasites</td>
<td>Elephants use tools (twigs) to repel biting flies.</td>
<td>Hart and Hart 1994</td>
</tr>
<tr>
<td>Camouflage</td>
<td>Zebra stripes might be less attractive for biting flies (tsetse) than uniformly coloured dark skin.</td>
<td>Ruxton 1982</td>
</tr>
<tr>
<td>Hygiene</td>
<td>Honeybee workers remove infected larvae to prevent spread of bacterial infection (foulbrood) to others. Some insects defecate away from their daily routines. In social insects, waste can be dumped at locations that are kept separate from the rest of the nest.</td>
<td>Rothenbuhler 1964; Hart and Ratnieks 2001; Weiss 2006</td>
</tr>
<tr>
<td><strong>Physiological:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preventive up-regulation of immune system</td>
<td>Locusts in dense aggregates have higher level of anti-bacterial activity and of the PO-cascade (density-dependent prophylaxis) even when not infected. Pregnant and lactating sifaka (Propithecus verreauxi verreauxi) increase intake of tannins, perhaps because they are effective against helminths. The great apes of Africa are suspected to use leaves as medicines. Many lepidopteran larvae take up plant compounds that are toxic to their parasitoids. Starlings use green vegetation for nest material that has disinfectant properties against ectoparasites. Wood ants collect resins that provide protection against bacteria and fungi. Many birds show ‘anting’ behaviour; they bathe in ant mounds to cover their feathers with formic acid. Anting can also occur with other objects such as limes or mothballs.</td>
<td>Barnes and Siva-Jothy 2000; Wilson et al. 2001, 2002; Wilson 2003; Carrai 2003; Huffman 2003; Krief 2004; Nishida 2002; Clark and Mason 1985; Nelson et al. 1998; Chapuisat 2007; Clayton and Vernon 1993</td>
</tr>
<tr>
<td>Self-medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other:</td>
<td>Multiply mated bumblebee females (Bombus terrestris) have colonies with lower parasite loads and higher fitness.</td>
<td>Baer and Schmid-Hempel 1999</td>
</tr>
</tbody>
</table>
nematode, *Ostertagia circumcincta*, but only if the sheep were not hungry. Hungry sheep, and sheep immunized before the test, did not avoid tall grass (Hutchings 2001). This experimental pattern is also reflected in the foraging behaviour of feral Soay sheep on St. Kilda Island; they avoided feeding on tall tussocks of grass harbouring more parasites than the nearby short grass patches (Hutchings 2002). Parasite-induced changes in diet preferences, therefore, affect how animals most efficiently forage in their environment (Lozano 1991). Nevertheless, consumers might not always avoid a parasite-infected diet or they might not be capable of either distinguishing infected from non-infected prey items, or they cannot avoid consuming infected prey (Wedekind 1996; van der Wal 2000).

4.1.1.4 The selfish herd

When an individual joins a group, then each group member lowers its individual risk of becoming infected, provided parasites do not increase in the same numbers (Hamilton 1971; Rubenstein and Hohmann 1989; Mooring 1992). However, these relationships are not always simple and a number of different factors affect the final result (Wilson 2003). A reverse effect is expected when groups represent a risk of infection, or when alien or infected individuals are joining a group. For example, house finches with outward signs of infection are more often observed foraging alone, although it is not clear whether exclusion from the social group is the cause (Hotchkiss et al. 2004). The risk of infection might be an explanation for xenophobic behaviour observed in primates (Freeland 1976) but good evidence is still lacking (Heymann 1999). When transmission of the parasite is contact-dependent, group size matters; but for highly mobile parasites (e.g. those actively seeking a host), group size is not a good correlate for infection levels (Côté and Poulin 1995).

4.1.1.5 Mating behaviour and mate choice

Parasites can also be contracted from a mate or the mating process itself exposes an individual to parasitism. These and other patterns and consequences for sexual selection will be discussed in more detail in Chapter 6.

4.1.1.6 Self-medication

In principle, animals and plants could use the same strategies as humans do; that is, to vaccinate themselves against a pathogen, to change diet, or to apply anti-parasitic substances so that infections become less likely. Most of the current evidence is anecdotal rather than experimental, however. It is assumed, for example, that the chewing of leaves by chimpanzees (Huffman 1997; Fowler 2007), or the intake of tannin-rich vegetation in Sifakas (*Propithecus*) (Carrai 2003), is a form of self-medication. Experimental evidence shows that, depending on the species of milkweed, where larvae of the monarch butterfly (*Danaus plexippus*) are raised, the effects of the infection by a protozoan pathogen (*Ophryocystis elektroscirrha*) are almost halved (de Roode et al. 2008a). Similarly, wood ant colonies use resins as a product to fight diseases. In experimental tests, the presence of resins increased the survival of larvae and adults when exposed to bacterial and fungal pathogens (Chapuisat 2007).

4.1.1.7 Anticipatory defences

Prominent among the physiological preventive actions are those that involve the immune system. For example, in several insects, individuals that congregate in dense aggregates appear to up-regulate their immune system and to activate the PO-cascade in anticipation of an increased risk of becoming infected; a phenomenon that has been termed density-dependent prophylaxis (Wilson 2003).

4.1.1.8 Genetic defences

In animals (or plants for that matter), where the success of a female depends on the genetic diversity among its offspring, additional options to protect themselves against possible fitness losses due to parasitism exist. For example, social insect females (the queens) can mate multiply with several unrelated males and so reduce the parasite load in their colonies (Baer and Schmid-Hempel 1999). Alternatively, females might increase the recombination rate when producing offspring in anticipation of parasitism. It is as yet not known whether this occurs, however.

4.1.2 Post-infection defences

When exposure or actual infection by a parasite has become inevitable, hosts use a variety of different means to reduce the expected fitness loss or to clear the infection.
Among these, the most obvious response is by the immune system. However, there are also large numbers of behavioural defences or physiological responses not directly associated with the immune system (Table 4.2).

4.1.2.1 Behavioural changes
Infected animals can change their time budget and direct more activities towards defence behaviour. For example, birds start to clean their nest rather than resting when infection is prevalent; ants relocate their nests more often in areas where parasites are common (Schmid-Hempel 1998). In a wider sense, infected hosts might change their life history and, for example, start to reproduce earlier than when uninfected (cf. Chapter 14). This has been documented in snails infected by trematodes. The trematodes castrate the snails as the infection progresses. Therefore, snails accelerate reproduction and in doing so, do leave some offspring, even though their reproductive success is much lower than compared to uninfected snails. This response is triggered even if only a chemical signal associated with the trematode infective stage is present in the water (Minchella 1985).

4.1.2.2 Grooming
Numerous studies have shown that grooming is effective against ectoparasites or helps removing spores before they can infect. In social groups, allogrooming—the grooming of others—is a further option. Allogrooming is known

| Table 4.2 Post-infection defences. Experimental tests to check for benefits have not been made in every case. |
|---|---|---|
| Defence element | Example | Source |
| **Behavioural:** | | |
| Change places | Ant colonies infected by fungi dislocate more often. | Oi and Pereira 1993 |
| Change activity | Great tits reduce sleeping time in favour of increased nest sanitation when infected by ticks. | Christe et al. 1994 |
| Reduce food intake | Infection-induced anorexia might function to combat parasites. | Exton 1997 |
| Self-medication | Many animals eat hardly digestible grass or soil (geophagy, often clay-rich), perhaps to rid themselves of parasites. | Huffman 1997, Mahaney et al. 1996 |
| Behavioural fever | Desert locusts raise their body temperature by change in thermal behaviour, which in experiments reduces infection by fungus. | Elliot 2002 |
| | Experimentally infected desert iguanas change behaviour to increase body temperature and so combat bacterial infections. | Vaughn et al. 1974 |
| | Bumblebee workers infected by parasitoid larvae prefer cold environments. This prolongs their lifespan and reduces the chance for the parasite to develop. | Muller and Schmid-Hempel 1993 |
| Grooming behaviour | Increased grooming is effective against ectoparasites in many animals. | Hart 1991, 1994 |
| Allogrooming | Mutual removal of fungal spores in social groups increases lifespan of termites. | Rosengaas et al. 1998 |
| Cleaning | Specialized cleaner fish attend coral reef fish, most likely to reduce ectoparasite loads. | Poulin and Gutter; 1996; Barber 2000 |
| Change life history | Snails infected with trematodes accelerate reproduction to reduce effects of parasite-induced castration. | Minchella 1985 |
| | Mite-infested Drosophila increase reproductive output to reduce loss due to later parasite effects. | Polak and Starmer 1998 |
| **Physiological:** | | |
| Fever | Increased body temperature is a response to infection that is widespread in actively thermo-regulating animals (endotherms). | Stearns and Koella 2008 |
| Activate immune system | Immune system responds in many ways when a parasite is recognized. | see Chapter 4.2 |
| **Other:** | | |
| Genetically diversify cells | Infected plants increase recombination rate in leaf cells even away from infection site. | Lucht et al. 2002 |
from a very wide range of taxa. For example, honeybees that have become infected by chronic BPV (bee paralysis virus) elicit grooming behaviour in other nest mates. This behaviour is very similar to attacks against intruders or when policing other workers, and so may utilize a behavioural system that is normally deployed in a different context (Waddington and Rothenbuhler 1976; Drum and Rothenbuhler 1985). Cleaning fungal spores from the body of nest mates is a major hygienic behaviour that renders groups of termites and ants less vulnerable to infection (Hughes et al. 2002). In birds, grooming correlates well with exposure to parasites. Parasite load is lower when birds groom, than in non-grooming control groups (Cotgreave and Clayton 1994). In fact, grooming typically occupies a larger fraction of an animal’s time budget.

Allogrooming is pervasive in primates; its hygienic function is obvious. For example, in howler monkeys, hygienic allogrooming is directed to body parts that are inaccessible to self-grooming. Individuals that had left the group had higher loads of ectoparasites, presumably because they lack grooming partners (Sanchez-Villagra et al. 1998). In larger social groups, ectoparasites might spread more easily. It is likely for this reason, that grooming frequency increases with group size, e.g. in primates (Dunbar 1991) or ants (Schmid-Hempel 1998). Allogrooming also has a social function, such as the formation of alliances or to forge bonds between mating partners. A special, inter-specific form of allogrooming is cleaning behaviour, known from specialized cleaner fish or from birds (e.g. oxpeckers) that ride on large ungulates and remove ectoparasites from their host’s body.

4.1.2.3 Fever and chilling

A change in body temperature has many consequences for host metabolism but also for the infecting parasites. Endotherms are capable of actively regulating their body temperature by producing heat and thus can use temperature as a defence mechanism. Metabolically generated fever is a common correlate of infections in mammals or birds, and such physiological fever seems to be a phylogenetically ancient trait (Cabanac 1990). Ectotherms cannot metabolically regulate body temperature but a change of behaviour achieves a similar result. By moving into warmer or sunnier places, the body temperature of insects, amphibians, reptiles, or fish increases. A number of studies have shown that the behaviour of infected ectotherms to move to a warmer environment is widespread. In doing so, they increase their body temperature and, in most cases, prolong survival, reduce infection levels, or increase performance in other respects (Moore 2002). Interestingly, preference for cold places can also be induced by parasitism. In bumblebees infected by larvae of parasitic flies this reduces the development of the parasite and so extends host lifespan (Müller and Schmid-Hempel 1993). A similar case is found in snails (Lefcort and Bayne 1991).

Some flying insects have evolved ways to heat their bodies with their large flight muscles and without actually moving the wings (in bees, for example, above 35°C). In this way, they can regulate their body temperature to some extent. This mechanism is used by honeybees to combat infections through cooperative warming of the colony as a whole. The nest temperature is thus beyond what is tolerated by the parasite (social fever; Starks et al. 2000). The effect of an increase or decrease in body temperature on reducing or clearing the infection is not always simple though. In some cases, the change does indeed benefit the host, e.g. resulting in an extended lifespan of the host (Müller and Schmid-Hempel 1993). In other cases, the temperature change benefits the parasite, which might represent cases of successful host manipulation (cf. Chapter 8). Finally, the most sophisticated defence is the activation of the immune system, which is discussed below. A special section is first devoted to ‘social immunity’, which includes a number of defence strategies not necessarily based on immune defences per se.

4.1.3 Social immunity

Socially living organisms have additional options to defend themselves against parasites and to combat infections (Cremer et al. 2007) (Table 4.3). A few of these have already been mentioned above, e.g. allogrooming. Social defences seem most pronounced in social insects but can also be found in other socially living animals, as allogrooming in social mammals shows. A kind of social immune defence may even occur in corals, where pol-
### Table 4.3 Social immune defences in social insects (after Cremer et al. 2007).

<table>
<thead>
<tr>
<th>Defence element</th>
<th>Defence mechanism</th>
<th>Type 1</th>
<th>Mode 2</th>
<th>Host group</th>
<th>Parasites</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reduce exposure risk:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-overlapping foraging ranges</td>
<td>Territorial demarcation.</td>
<td>Pre</td>
<td>Behav, Spatial</td>
<td>Ants, Termites</td>
<td>All</td>
</tr>
<tr>
<td>Division of labour</td>
<td>Only some individuals forage.</td>
<td>Pre, Post</td>
<td>Behav</td>
<td>All</td>
<td>Fungi, Helminths, Flies</td>
</tr>
<tr>
<td>Avoidance behaviour</td>
<td>Avoid direct contact with parasite.</td>
<td>Post</td>
<td>Behav</td>
<td>Ants, Termites</td>
<td>Fungi, Helminths, Flies</td>
</tr>
<tr>
<td>Select who enters the nest</td>
<td>Avoid cannibalizing infected corpses.</td>
<td>Post</td>
<td>Behav</td>
<td>Ants, Termites</td>
<td>Fungi, Helminths, Flies</td>
</tr>
<tr>
<td></td>
<td>Guard foraging trails.</td>
<td>Pre, Post</td>
<td>Morpho, Behav</td>
<td>Ants</td>
<td>Fungi, Helminths</td>
</tr>
<tr>
<td></td>
<td>Guard nest entrance.</td>
<td>Pre, Post</td>
<td>Behav</td>
<td>Bees</td>
<td>All</td>
</tr>
<tr>
<td><strong>Nest hygiene:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Self-medication</td>
<td>Collect anti-microbial substances.</td>
<td>Pre</td>
<td>Behav</td>
<td>Ants, Bees</td>
<td>Bacteria, Fungi</td>
</tr>
<tr>
<td>Produce chemicals</td>
<td>Metapleural gland secretion.</td>
<td>Pre</td>
<td>Behav, Physiol</td>
<td>Ants</td>
<td>Bacteria, Fungi</td>
</tr>
<tr>
<td></td>
<td>Faecal material.</td>
<td>Pre</td>
<td>Physiol</td>
<td>Termites</td>
<td>Fungi, Helminths</td>
</tr>
<tr>
<td></td>
<td>Venom.</td>
<td>Pre</td>
<td>Physiol</td>
<td>Wasps</td>
<td>Bacteria, Fungi</td>
</tr>
<tr>
<td>Waste management</td>
<td>Removal of dead workers; separated 'graveyards'.</td>
<td>Pre</td>
<td>Behav, Spatial</td>
<td>Ants, Bees</td>
<td>All</td>
</tr>
<tr>
<td>Keeping parasites local</td>
<td>Garbage removal; separated waste dumps.</td>
<td>Pre</td>
<td>Behav, Spatial</td>
<td>Ants</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Cover infectious parasite propagules by material.</td>
<td>Post</td>
<td>Behav</td>
<td>Ants</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Social encapsulation of parasites ('walling').</td>
<td>Post</td>
<td>Behav, Spatial</td>
<td>Bees</td>
<td>Beetles</td>
</tr>
<tr>
<td><strong>Reduce spread within colony:</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Remove parasite</td>
<td>Mechanical removal by grooming.</td>
<td>Post</td>
<td>Behav</td>
<td>Ants, Wasps,</td>
<td>Fungi, Helminths</td>
</tr>
<tr>
<td></td>
<td>Chemical destruction in infrabuccal pockets.</td>
<td>Post</td>
<td>Behav, Physiol</td>
<td>Ants, Wasps</td>
<td>Fungi, Helminths</td>
</tr>
<tr>
<td>Heterogeneous interactions</td>
<td>Behavioural structuring (by age and caste).</td>
<td>Pre</td>
<td>Behav</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td>Social exclusion</td>
<td>Spatial nest compartmentalisation.</td>
<td>Pre</td>
<td>Spatial</td>
<td>All</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Indirect interaction with garbage workers.</td>
<td>Pre</td>
<td>Behav, Spatial</td>
<td>Ants</td>
<td>All</td>
</tr>
<tr>
<td></td>
<td>Pathogen alarm (vibrational displays).</td>
<td>Pre</td>
<td>Behav</td>
<td>Termites</td>
<td>Fungi</td>
</tr>
<tr>
<td></td>
<td>Isolate infected individuals ('walling').</td>
<td>Post</td>
<td>Behav, Spatial</td>
<td>Termites</td>
<td>Helminths</td>
</tr>
<tr>
<td></td>
<td>Remove/cannibalize infected young individuals.</td>
<td>Pre, Post</td>
<td>Behav, Spatial</td>
<td>Bees, Termites</td>
<td>Bacteria, Fungi</td>
</tr>
<tr>
<td>Nest relocation</td>
<td>Abandon infected nest areas.</td>
<td>Post</td>
<td>Behav, Spatial</td>
<td>Ants, Bees</td>
<td>Fungi, Helminths, Mites</td>
</tr>
<tr>
<td>Increase genetic heterogeneity</td>
<td>Multiple mating and/or multiple queens.</td>
<td>Pre</td>
<td>Behav, Genetic</td>
<td>Ants, Bees</td>
<td>All</td>
</tr>
<tr>
<td>Facultative immunity</td>
<td>High recombination rate.</td>
<td>Pre</td>
<td>Genetic</td>
<td>Ants, Bees</td>
<td>All</td>
</tr>
<tr>
<td>Protect queen</td>
<td>Immunity transfer by social interaction.</td>
<td>Post</td>
<td>Behav, Physiol</td>
<td>Termites</td>
<td>Fungi</td>
</tr>
<tr>
<td>Protect brood</td>
<td>No tending by infected workers.</td>
<td>Post</td>
<td>Behav</td>
<td>Bees</td>
<td>Microsporidia</td>
</tr>
<tr>
<td></td>
<td>Application of anti-microbial secretions.</td>
<td>Pre</td>
<td>Behav, Physiol</td>
<td>Ants, Bees, Wasps</td>
<td>Fungi, Helminths</td>
</tr>
<tr>
<td></td>
<td>Feeding of anti-microbial secretions.</td>
<td>Pre</td>
<td>Behav, Physiol</td>
<td>Bees</td>
<td>Bacteria</td>
</tr>
<tr>
<td></td>
<td>Trans-generational transfer of immunity.</td>
<td>Pre, Post</td>
<td>Physiol</td>
<td>Bees</td>
<td>Bacteria</td>
</tr>
</tbody>
</table>
yps die off and are replaced by the regenerating colony, a process that might be induced by the colony and is reminiscent of apoptosis by infected cells in plants. This phenomenon is observed when tissue of foreign corals comes into contact and a histopathic effect develops (Theodor 1970). Common to social immune defences is that each individual gains by the collaborative actions of the society. Vice versa, this system is vulnerable to cheating, since not every individual might contribute equally to the social defence, be this the individual contribution towards heating the nest (social fever) in honeybees or the up-regulation of an individual’s immune system to prevent further spread of a pathogen in the group (herd immunity).

4.2 Defence by the immune system

The immune system has several different functions, including the surveillance against aberrant cells, such as those leading to cancer, or the removal of debris that accumulates during development (e.g. when cells undergo apoptosis during the formation of organs and structures). In the current context, defence against parasites is the

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**Figure 4.2** Generalized scheme of immune defences. Examples are from insects (left) and vertebrates (right). Different parasites are recognized in different ways. In any case, however, a receptor at the cell surface senses the infection, either directly or by intermediates (e.g. by factor Spaetzle). This signal is passed on to the cell's interior by a cascade of adaptor proteins and the signalling cascade. Transcription factors induce the synthesis of effectors, such as anti-microbial peptides. The general structure of signalling pathways is relatively conserved, but the actual signalling proteins show divergence. The figure shows some of the proteins involved. For immunological acronyms, see Glossary. Redrawn from Flajnik and du Pasquier (2008) with permission from Wolters Kluver Health.
relevant function. The immune system can be classified according to different criteria. There is, for example, a cellular and humoral arm of the defence, and the innate and adaptive system. These distinctions are justified based on differences in the underlying mechanisms. However, all arms of the defence are tightly integrated with each other in order to function properly. It is also possible to classify the respective molecules and pathways as belonging to the compartments of recognition, the signalling cascade, or to that of effectors (Figure 4.2).

Any induced response depends on the ability of the immune system to sense an infection. It should recognize non-self and pass on this information to a subsequent signal-processing cascade that can activate the appropriate effectors. Recognition molecules can achieve this. Such recognition molecules are part of a receptor or through intermediate steps activate a downstream receptor, all of which all have some degree of specificity, even as they vary from rather general to very specific. An immune response can also be very fast but sometimes is active only after a considerable delay. Similarly, a response can be very general and be effective against a wide range of challenges, or it can be very specific and attack only a narrowly defined range of pathogens. Moreover, our appreciation of immune systems rapidly changes as receptors of high specificity and a specific immune memory—classically thought to be restricted to the higher vertebrates—have also been discovered in invertebrates.

### 4.3 Basic elements of the immune defence

#### 4.3.1 Humoral and cellular defences

Immune defences can be classified as humoral (based on non-cellular, soluble components in body fluids) or cellular responses (based on cells). These two arms of defence are tightly interconnected, even though they cover different functions and are based on different molecules and processes. Humoral responses include vertebrate serum globulins with antibody activity, collectively called immunoglobulins (Ig) that are grouped into five major classes: IgG, IgM, IgA, IgE, and IgD. Immunoglobulins bind with different specificities to foreign objects (antigens). And even though they may have identical specificities, different immunoglobulins fulfil different functional roles in the defence. Other non-cellular, humoral components are collectively called the ‘complement system’. Complement consists of serum enzymes that are activated by different pathways. Eventually, complement activation leads to the destruction of the invader by direct lysis of the target cell, or by phagocytosis via phagocytic cells, such as polymorphonuclear cells (PMNs) and neutrophils.

The cellular defence involves a large repertoire of different immune cells. In the higher (jawed) vertebrates, the cellular defence also involves T-cells and leads to the elimination of the invader by an activated T-cytotoxic cell or a phagocytic cell. T-cells move to a site where antigens of an invader are presented (as signalled by antigen-presenting cells, APCs) and directly and specifically interact with them (this interaction is also called ‘cognate’). Several major groups of T-cells exist that perform different functions, such as T-helper cells (having CD4 co-receptors), cytotoxic T-cells (with CD8 co-receptors), or regulatory T-cells. Furthermore, B-cells are part of the cellular defence system of the higher (jawed) vertebrates. Even though their main function is to secrete antibodies for the humoral defence, they interact with T-helper cells, become activated and clonally expand. Other important cellular components include monocytes and macrophages; these are recruited to the site of infection in the process of inflammation, which in turn is induced by cytokines (signalling molecules produced by cells for other cells) released by T-helper (and other) cells. In fact, the release of cytokines is a major function of immune defence cells and serves to orchestrate the cellular response. Among the cytokines, cells in response to viral infection produce interferons to alert nearby cells to this threat.

In invertebrates, which have no closed blood circulation system, the cellular response is based on a variety of specialized, freely circulating cells. Such cells, all of haemopoietic origin, can be found in all metazoa (multi-cellular organisms), even though their ontogeny (Figure 4.3) and functions may differ. Altogether, the diversity of invertebrate cells is much lower than the cells known from vertebrates. In insects, for example, the cell populations include plasmatocytes, lamellocytes, and crystal cells. Crystal cells store a precursor molecule
Figure 4.3 Immune cell development (haematopoiesis). (a) Haematopoiesis in insects (*Drosophila melanogaster*). Proliferation and development from the original pro-haemocyte (top) is controlled by PVF2/PVR, Ras/Raf, and the JAK/STAT pathways. With serpent (Srp) and the inhibitory GATA-homologue U-shaped (Ush), new cells develop. Control by GcM transactivators leads to either crystal cells, Lozenge (Lz-) cells, or to plasmatocytes. Lamellocytes, which are important for encapsulation, are specified by the JAK/STAT-pathway. For immunological acronyms, see Glossary. Redrawn from Flajnik and du Pasquier (2008) and Meister (2004) with permissions from Wolters Kluver Health and Elsevier. (b) Haematopoiesis in vertebrates. From the totipotent stem cells, two families of blood cells (myeloid and lymphoid cells) develop. Important immune cells are hatched. Redrawn from Coico and Sunshine (2009) with permission from John Wiley & Sons, Inc.
(PPO) for a key enzyme (phenoloxidase, PO) of the major defence cascade of arthropods that can lead to the release of toxic molecules and eventual melanization. The crystal cells readily rupture and release their contents into the haemolymph when activated. The circulating cells are called ‘haemocytes’ in the arthropods, but ‘coelomocytes’ or ‘amoebocytes’ in annelids, molluscs, and echinoderms (Flajnik and du Pasquier 2008). A more generalized nomenclature (Hartenstein 2006) groups the invertebrate cells into prohaemocytes, hyaline haemocytes (plasmacytes, monocytes), granular haemocytes (granulocytes), and eleocytes (chloragocytes). The circulating cells perform functions such as phagocytosis, encapsulation, nodule formation and clotting, or the production of a range of effector molecules. Activation of the cellular arm of defence is by the recognition of epitopes on the surface of parasites, or via opsonization, whereby particular molecules are deposited on the surface of parasites by the host defence system to mark them for destruction.

4.3.1.1 Phagocytosis

The major cellular defence response is phagocytosis and the melanization–encapsulation cascade in invertebrates. Phagocytosis was one of the first immune defence mechanisms to be discovered around 1900 by Elie Metchnikoff. He pricked larvae of starfish with a thorn and noted cells migrating to the site of the wounding. Phagocytic cells, such as plasmatocytes in insects (cf. Figure 4.3), or ‘professional’ phagocytes, such as a neutrophils or macrophages in mammals, are recruited to the site of an infection by chemical signals. In vertebrates, chemokines (e.g. IL-8) and components of the complement system (C3a, C5a) attract phagocytic cells. Serine proteases and opsonization factors, such as TEP (Thioester-containing protein), attract phagocytes in insects (Flajnik and du Pasquier 2008). The process can furthermore be enhanced by opsonization of the parasite by appropriate molecules. In insects, four classes of molecules are involved in recognition for phagocytosis—complement-like opsonins (TEPs), scavenger receptors, a family of EGF (epidermal growth factor)-like repeat-containing proteins, and the variable Dscam molecules.

In mammals, phagocytosis can be triggered by a number of different receptors (Greenberg and Grinstein 2002). Following recognition, a specialized machinery, which is similar in insects and mammals, ensures that the particle is internalized (Greenberg and Grinstein 2002; Stuart and Ezekowitz 2008). Two major elements characterize these cellular processes (Figure 4.4). First, there are rearrangements of the cytoskeleton that permit a phagocytic cell to move and change shape. For instance, to engulf a parasite, the cell surface changes curvature and extends pseudopods, which is associated with the formation of structures (the exocyst) that facilitate vesicle fusion with the cell membrane. During internalization of the parasite, large tracts of new membrane must be synthesized by the phagocytic cell and delivered to the surface to compensate for the internalized part. In fact, professional mammalian phagocytes can internalize the equivalent of more than their own surface within half an hour (Greenberg and Grinstein 2002). Second, vesicle formation and trafficking inside the cell are needed, so as to allow the transport and containment of the parasite in a specialized vesicle, the phagosome. Phagosomes undergo a process of maturation during which they may split (fusion) and fuse with other vesicles, notably with endosomes and lysosomes to develop into a phagolysosome; this yields a chemical environment allowing for the degradation and destruction of the engulfed particle or microorganism. The type of phagosome that will develop depends on a number of factors, such as the involved receptors, the kind of particle swallowed, or the nature of the membrane that is utilized. The phagosome is, therefore, a rather complex organelle, where, for example, 600 different proteins are involved in the case of the fruit fly D. melanogaster, of which 70% are orthologues of the mammalian phagosome (Stuart and Ezekowitz 2008).

Eventually, the parasite is also killed by oxidative mechanisms that produce reactive molecules in a process called ‘respiratory burst’ or ‘oxidative burst’. In this defence effector process, cells rapidly release reactive oxygen species (ROS; super oxides, hydrogen peroxides) and nitric oxide that are toxic to microorganisms. The oxidative burst is especially prominent in the hypersensitive responses of plants, but also occurs in insects and mammals. The process is affected by NADPH-oxidase generating superoxides, which spontaneously interact with other molecules to produce highly reactive free radicals. These mechanisms are very ancient, as they are
Figure 4.4 The mechanism of phagocytosis. (a) This general scheme shows how a foreign particle (such as a parasite) is delivered from the surface to the interior of the cell, and into a phagosome. The recognition of a parasite by receptors triggers the orderly progression of events where, first, the cytoskeleton acts to form pseudopodia that help engulf the particle. By fusion with endosomes and lysosomes, the new vacuole matures into a phagosome with an acidic and hydrolytic milieu that helps destroying the parasite. (b) Left: In insects (Drosophila melanogaster), specific phagocytosis is associated with recognition by the variable receptor, soluble Dscam. In this yet conjectural scheme the opsonized parasite is recognized and eventually phagocytosed by other haemocytes. Right: In mammals, the recognition can be through immunoglobulins (B-cell receptor, BCR). The opsonized particle is recognized by Fc receptors (FcR) and subsequently phagocytosed. Redrawn from Stuart and Ezekowitz (2008) with permission from Macmillan Publishers Ltd.
conserved across phyla. In vertebrates, phagocytosis also stimulates the adaptive immune system.

In addition to phagocytosis, cellular defence also includes endocytosis. With endocytosis, macromolecules (instead of entire parasitic cells) are engulfed by specialized cells, such as macrophages. Endocytosis can be unspecific by simple membrane fold (pinocytosis) or based on the binding of the macromolecules by specific receptors, followed by the internalization of the foreign material. In either case, the vesicle containing the macromolecules fuses with endosomes (containing acidic components) and with lysosomes (containing degrading enzymes, as in phagocytosis), which leads to destruction of the ingested material.

4.3.1.2 Melanization, encapsulation
Melanization is a cellular defence that is characteristic for invertebrates, primarily arthropods. The proPhenoloxidase (PPO)-cascade is central to this process. The cascade is triggered following the recognition of a parasite by various binding proteins (such as LPS-binding proteins, PGRP, GNBP). This releases serine proteases that cleave the pro-form, PPO, into the active enzyme PO (which in turn can also activate the pro-form). As the cascade starts running, cytotoxic intermediates are produced, such as phenols, quinones, and reactive oxygen species (Nappi and Vass 1993; Nappi and Ottaviani 2000; Sugumaran et al. 2000; Nappi and Christensen 2005), which can directly kill pathogens. The PPO-cascade also stimulates several other immune reactions, and is part of a more general cascade leading to phagocytosis; for example, in crustaceans. Regulatory proteins (serpins) play a major role controlling and localizing the melanization process.

In insects, the reservoir of the precursor molecule, PPO, is stored in the circulating crystal cells, which represent around 5% of all plasmatocytes. Their activation does not depend on the major immune signalling pathways, such as Toll, Imd, or JAK/STAT (Lemaitre and Hoffmann 2006). The molecules of the PPO-cascade are present across many phyla, but its major role as a defence cascade is mainly found in arthropods. The cascade as such is absent from vertebrates (Flajnik and du Pasquier 2008).

To immobilize and kill larger invaders, such as eggs or larvae of parasitoids, invertebrates defend themselves with encapsulation, which constitutes another major cellular defence response (Lemaitre and Hoffmann 2006). An invader is detected by the circulating plasmatocytes that attach to the foreign surface. Signals then recruit large numbers of lamellocytes (cf. Figure 4.3) to the site of infection. The lamellocytes form a multi-layered capsule around the invader that eventually melanizes into a tight capsule sealing the parasite from the rest of the host body. The PPO-cascade is again central to this process. It cross-reacts with clotting, and eventually leads to melanization that is visible from the outside by the darkening of the encapsulating cells.

4.3.1.3 Clotting, nodule formation
These responses represent further important cellular defences. For example, in vertebrates, local blood clotting is a process concurrent to the activation of complement and inflammation, yet interacting with those cascades. The resulting blockage of the blood flow draining from the site of infection helps to reduce the spread of infecting microorganisms to the rest of the body (Markiewski et al. 2007). Due to its potential to form thrombi, to spread systematically, and to disable blood flow, blood clotting also carries a risk of inflicting secondary damage to the host. It is, therefore, also under the control of inhibitors in the form of anti-coagulation systems (Markiewski et al. 2007). The open circulatory system of arthropods poses additional challenges, as invading microorganisms can enter the haemocoele and then easily spread to the entire body without additional barriers. Clotting is, therefore, an important response of the arthropod immune system, too. Furthermore, clotting does not have the same risk in arthropods, as they lack the highly evolved vesicular system that can be completely disabled by extensive blockage. The formation of a matrix of cells during clotting additionally facilitates wound sealing and healing (Theopold et al. 2004). Nodule formation in insects is a further cellular defence response during which invading microorganisms, such as bacteria, become trapped inside an aggregation of haemocytes (Ratcliffe and Rowley 1979). Biochemically, this aggregation process is regulated by eicosanoids, which are metabolically active derivate of certain fatty acids. Eicosanoids are important as modulators of local cell activities in most organisms.
4.3.1.4 Inflammation

This is a complicated but orchestrated early response by the immune system of vertebrates during which the vascular system changes, such that blood flow to a site of infection is increased and cells are recruited that aid in defence against an invader and in the subsequent healing process. Typically, this is visible from the outside as swelling and an increased reddening of the skin at the site of infection. During acute inflammation, blood vessels at the site of infection become permeable, such that blood plasma and leukocytes can infiltrate the surrounding tissue at a site of infection. There is a rapid influx of a certain class of blood cells, the granulocytes, typically neutrophils. Subsequently, monocytes infiltrate the tissue and develop into (inflammatory) macrophages, which also interact with local, resident macrophages in the affected tissue. The inflammation response is orchestrated by pro-inflammatory cytokines released from tissue cells at the site of infection. Their function is to attract more leukocytes (white blood cells). These blood cells and the proteins contained in blood plasma, e.g. from the complement system, act to contain and eliminate an infection before it can spread further. The sequence to terminate the process seems to be initiated soon after inflammation begins by the granulocytes that enter the infected tissue. Inflammation ends when granulocytes are removed and the macrophages have been drained by the lymphatic system (Serhan and Savill 2005).

4.3.2 Innate and adaptive (acquired) immunity

4.3.2.1 Innate immune defence

Innate immunity has a limited number of receptors for the recognition of parasite molecules. The latter typically represent molecular motifs that are conserved across a range of microbial taxa (Kimbrell and Beutler 2001). These motifs are components that seem difficult to change; for example, deep structural elements of the parasite's cell wall (Beutler 2004). Innate immunity is furthermore characterized by becoming active with a short delay, in contrast to adaptive immunity. Innate defence, therefore, plays an important role as a first and generalized defence and is especially prominent in physical barriers, such as skin or mucosa. In vertebrates, the innate immune system also contributes to the activation and orchestration of the adaptive response. Traditionally, host molecules that can recognize general features of a parasite have been named PRRs (pattern recognition receptors) and the features that they recognize PAMPs (parasite-associated molecular patterns). However, these terms have been coined in classical studies of microbial pathogens and now appear unnecessarily restrictive. Rather, host molecules just recognize parasite molecules, or molecules from any other microbe, harmless or symbiotic. Hence, PAMPs are not different in kind from any other epitope that an immune system might recognize and PRR is a term that is synonymous with a recognition molecule more generally. It is thus more instructive to look at the different recognition molecules and what they recognize in the first place. Recall that innate immunity is based on, both humoral and cellular defences, as will be highlighted below.

4.3.2.2 Adaptive (acquired) immunity

Any immune defence is obviously ‘adaptive’ in the sense used in evolutionary biology. However, immunologists have coined this term to indicate that the immune system of an affected individual responds increasingly specifically to an ongoing infection during the life of an individual. For this purpose, the adaptive immune systems acquire information about the ongoing infection and adapt their response to the particular parasite. As a consequence, an adaptive immune response is not only more specific, but also inevitably delayed, as compared to the innate response. Response times are measured in days, rather than in minutes to hours as with innate defences. This adaptive system has evolved later in the history of life than the innate systems (which are very old). Both systems, however, interact closely and an adaptive response would not be possible without a previous innate response. The distinction between innate and adaptive immunity also becomes more and more blurred as new insights into mechanisms of immune defences of invertebrates are gained. Also note that there are actually two versions of the adaptive immune system: the one of the jawless vertebrates (agnatha; known from hagfish and lampreys), and that of the jawed vertebrates (gnathostomes; the higher vertebrates). These versions differ
in several of the key mechanisms and also in the kinds of proteins that are used.

A hallmark of adaptive immunity in the higher (jawed) vertebrates is the presence and clonal expansion of highly variable lymphocytes (B- and T-cells) with antigen-specific functions, as well as the formation of memory cells. Memory means that, while there is a standard immune response for the primary challenge, a second or further encounter with the same parasite induces the secondary response, which is faster and stronger. These terms have been formulated, based on observations in vertebrate defences. However, similar to the antiquated usage of PAMPs or PRRs above, invertebrates are now also known to possess a ‘memory’ that, although necessarily based on different mechanisms, is characterized by a more efficient response upon a second encounter with the same parasite (Sadd and Schmid-Hempel 2006) and also seems quite specific (Roth et al. 2008).

Prime components of the humoral arm of adaptive immunity are the secreted antibodies that circulate in the bloodstream and bodily fluids. They recognize and directly bind to specific, parasite-associated motifs, the antigens. Antibodies of jawed vertebrates are secreted by the B-cells. B-cells not only secrete antibodies, which are immunoglobulins by their nature, but the respective immunoglobulins are also present as cell-bound receptors on the surface of the B-cells. They can also bind directly to antigens. T-cells, by contrast, are unable to bind directly to the antigens of parasites. Rather, T-cells recognize and bind to antigenic peptides that are presented to them by other, specialized cells of the immune system, the antigen-presenting cells (ACPs, such as macrophages or dendritic cells). These cells display parasite-derived peptides by means of the MHC-complex. Furthermore, the activation of T-cells requires a co-stimulation of a co-receptor.

Adaptive immunity is delayed but also highly specific because the lymphocytes that match the current infection must first undergo clonal expansion to generate a large enough population of cells that are specifically tuned towards the ongoing infection. B- and T-cells are restricted to jawed vertebrates, and presumably VLR-A and VLR-B have the same role in lampreys. As cell lines of jawed vertebrates undergo clonal expansion, the appropriate types of B- and T-cells multiply and mature. This process involves a cascade of events, including the release of cytokines from specifically activated T-cells that can in turn activate B-cells with the matching specificity of their cytokine receptors. This stimulates the proliferation of B-cells with fitting antibodies, as well as the diversification in the kind of immunoglobulins produced (class switching).

An adaptive immune system depends on some important organs, the most prominent of which are the lymphatic organs. In these organs, lymphocyte development, differentiation and proliferation take place (cf. Figure 4.3b). In jawed vertebrates, primary lymph organs are the sites where B- and T-lymphocytes mature into cells that can recognize antigens or the appropriate peptides. In mammals, B-cells mature in bone marrow, whereas in birds this happens in an organ called the Bursa of Fabricius. T-cells start their maturation in bone marrow but then migrate to fully mature in the thymus glands. The thymus gland grows during foetal development but eventually stops growing at sexual maturity and recedes in size during adulthood. The secondary lymphatic organs are located in the periphery and harbour the mature, functional lymphocytes. Secondary lymphatic organs include the lymph nodes, the spleen, the tonsils, or Peyer’s patches in the small intestine. Different kinds of secondary lymphatic organs are in fact found at various sites of the body, in mucosa lining the digestive, respiratory or in the genital-urinary tracts. At these sites, e.g. in the lymph nodes of mammals, the activation of the B- and T-cells takes place, aided by close spatial proximity of lymphocytes with antigens and with other lymphocytes that become easily trapped in these organs. The architecture of the lymphatic system varies among organisms; but most species do not have lymph nodes (Boehm and Bleul 2007).

4.3.3 Signalling cascades
The immune system is a network that is tightly regulated to generate an appropriate response in the right place and at the right time. In the general scheme (Figure 4.2), a receptor will convert a recognition event into a signal that is passed on to subsequent elements of a regulatory
A given regulatory cascade is inhibited and stimulated by a variety of concurrent processes that act in parallel or are part of overlapping molecular cascades. The cascade eventually releases a transcription factor that leads, for example, to the production of anti-microbial peptides, or to a product that stimulates division of immune cells, or that triggers apoptosis. Although the signalling cascades are different in different organisms, the general structure of the signalling pathways is nevertheless much less diverse than the recognition steps. On the other hand, the signalling proteins themselves rapidly diversify and show large genetic divergence (Waterhouse et al. 2007). The characteristics and complexity of immune signalling are here illustrated with examples from extra-cellular innate immune receptors in plants, insects and mammals (Figure 4.5).

Cytokines are produced by almost all cells of the immune system. Their function is to regulate and orchestrate the immune response, but some also act as effectors. Cytokines that signal between leukocytes have been named interleukins (ILs) and play an important role in defence; around 30 different types have been identified (Coico and Sunshine 2009). Cytokines can be secreted (IFN-γ, IL-2) or remain membrane-bound (TNF-α, TNF-β). Generally, as a molecule, they do not persist for very long, in line with their function as regulators. Their

Figure 4.5 Examples of signalling cascades for extra-cellular parasites. The cascades lead to the generation of transcription factors that induce the production of effector proteins, or proteins that induce cell death, phagocytosis, and so forth. The schemes are simplified for clarity. (a) In plants, recognition can be via an LRR-receptor. Signal transduction occurs via a kinase and the MAPK-pathway to activate WRKY transcription factors. Redrawn with permission from Ausubel (2005). (b) Two characteristic pathways of insects (Drosophila). Left: Toll does not recognize parasites directly, but is activated by a cleavage product (from pro-Spaetzle) that is produced after a pattern recognition receptor (such as GNBP, PGRP) has bound to a parasite epitope (PAMP). Toll consists of a peptide-receptor and several signal-transducing proteins (adaptor proteins TIR, MyD88, Pelle) that eventually activate the Cactus-Dorsal/DIF transcription factor. The Toll-pathway results in the production of anti-microbial peptides (e.g. drosomycin). Right: In the Imd-pathway, the receptor (PGRP-LC) directly recognizes parasite epitopes (from Gram-negative bacteria). Signal transduction is by various proteins (RIP, IMD) and, via two pathways (DREDD, IKK-complex), activates the Relish transcription factor. At the same time, the JNK-pathway is activated. The Imd-pathway results in the production of anti-microbial peptides (e.g. diptericin). Redrawn with permission from Ferrandon et al. (2007). (c) Toll-like receptors (TLRs; here TLR4) of mammals have an extra-cellular LRR and can sense microbial epitopes (PAMPs). Signal transduction has similarities with the Toll-receptor in insects (transducing proteins). Downstream signalling leads to the activation of transcription factors IRF-3, NF-κB, and the p38-protein kinase and JNK-pathways. IκB is an inhibitory protein that binds to transcription factors of the NF-κB type. For immunological acronyms, see glossary. Redrawn from Akira et al. (2006) with permission from Elsevier.
effect on cells depends on the target cells having cytokine receptors that are specific to some cytokines rather than others. The combination and concentration of different cytokines recognized by a cell ultimately decide its activity in response. Consequently, some cytokines also act antagonistically, such as the ones inducing either T₇₁ or T₇₂-cell development.

4.3.3.1 Plants
The pathway of signalling elements downstream of the receptors is not yet well understood for plants. However, a recognition event mediated by extra-cellular receptors (e.g., sensing a component of bacterial flagellin) activates a MAPK-dependent cascade that leads to the release of WRKY-transcription factors, which induce the production of effector proteins (Figure 4.5). Recognition by these receptors also induces cell death (apoptosis) and the so-called hypersensitive response.

In plants, the recognition of intra-cellular parasites by two major intra-cellular receptors typically activates two different pathways (characterized by the NDR1- and EDS1-genes). These pathways seem to be different from the ones induced by the extra-cellular receptors (Ausubel 2005). In the signalling cascade induced by the different R-gene receptors, the protein encoded by the RAR1-gene seems to act as a non-redundant convergence point, whereas the genes SGT1a, SGT1b code for a convergence point for additional pathways (Holt III et al. 2003). Additional signalling pathways in plants are based on a variety of different compounds, such as salicylic acid, jasmonic acid, ethylene, or nitric oxides. These pathways also have points of mutual interactions (Ausubel 2005). Transcription factors, such as WRKY, are unique to plants and, vice versa, the transcription factors found in insects or mammals are not present in plants. Hence, it appears that the plant immune system, despite some similarities and the presence of similar protein families, is not entirely homologous. The signalling system, in particular, seems to have evolved independently and thus represents a case of analogy rather than homology.

4.3.3.2 Insects
The best-studied insect for this purpose, Drosophila melanogaster, shows several characteristic pathways in its immune system, notably the Toll-, Imd-, and JAK/STAT-pathways (Figure 4.5). The Toll-pathway responds to signatures from fungi and bacteria, the Imd-pathway primarily to Gram-negative bacteria (and fungi), and the JAK/STAT plus several other pathways appear to be involved in defence against viral infections (Box 4.1). These latter pathways are, therefore, an example of sensing intra-cellular parasites. Signalling pathways of insects and mammals share similarities and, therefore, are likely homologous in many of their elements. For example, the insect Toll-pathway resembles the mammalian signalling cascades involved in the IL-1R pathway (interleukin-1 receptor) and TLR (Toll-like) receptors. The insect Imd-pathway is similar to the mammalian TNFR (tumour-necrosis factor-receptor)-pathway. However, aphids seem to lack the Imd-pathway altogether (Gerardo et al. 2010), which illustrates the diversity of immune pathways in different organisms, and in the huge group of insects, in particular.

4.3.3.3 Mammals
TLRs form a family of proteins that are involved in innate immune defences of vertebrates (Leulier and Lemaitre 2008). Signal transduction (as in other cases) occurs by a conformational change of the TLR-molecules; this recruits adaptor proteins such as MyD88 or TIRAP and leads to signalling along a cascade (Figure 4.5) (Akira 2003). The adaptor, MyD88, is a key element for all TLRs (except TLR3). It recruits kinases (IRAK) and so passes on the signal to downstream elements. Eventually, key transcription factors, such as NF-κB or IRF (interferon response factor), are activated, which initiates the transcription of anti-microbial peptides (e.g. defensins) or pro-inflammatory cytokines. Several ligand receptors (TLR4, IL-1R) can feed to the same convergent element (MyD88), and different TLRs have slightly different signalling architectures of their intra-cellular adaptor proteins (Kopp and Medzhitov 2003). For example, TLR3 can serve in the recognition of viruses by sensing double-stranded RNA (dsRNA); its signalling cascade is very similar to the one depicted in Figure 4.5 for TLR4, except that it does not use MyD88. In addition, the signalling cascades of other kinds of intra-cellular viral receptors also feed to important kinases, such as IKK or TBK, and eventually activate...
Viruses are very common parasites of all organisms. In the vertebrates, interferon-based processes, MHC-complexes, and T-cells are involved. However, no such mechanisms and cells are present in invertebrates and plants. Instead, an ancient mechanism is responsible for the general anti-viral defence: RNAi-silencing. Viral activities typically lead to the production of dsRNA (double-stranded RNA) in the host cell. This dsRNA is recognized and degraded or blocked from being translated into a protein.

**Box 4.1 Anti-viral defence of invertebrates**

Viruses are very common parasites of all organisms. In the vertebrates, interferon-based processes, MHC-complexes, and T-cells are involved. However, no such mechanisms and cells are present in invertebrates and plants. Instead, an ancient mechanism is responsible for the general anti-viral defence: RNAi-silencing. Viral activities typically lead to the production of dsRNA (double-stranded RNA) in the host cell. This dsRNA is recognized and degraded or blocked from being translated into a protein.

**Figure 1** Anti-viral defence by RNAi-silencing (mina, saran-paths) in insects (*D. melanogaster*) and nematodes (*C. elegans*). In both cases, dsRNA from viral activity is sensed. In the mina-pathway of *Drosophila*, pre-miRNA is attacked by the slicing enzyme Dicer (Dcr1) and cut into siRNA that binds to Argonaute (AGO1). This presumably blocks further RNA-translation. In *Caenorhabditis*, AGO1 is replaced by ALG. In the siRNA-pathway of *Drosophila*, Dicer (Dcr2) binds directly to viral dsRNA and cuts it into siRNA. This is attacked by AGO2 from the RISC-complex. Subsequently, the RNA is degraded or translation may be repressed. In *Caenorhabditis*, Dcr1 is present. In the further process, the RNA is cut by the help of RDE and handed over to SAGO to be degraded. R3D1, R2D2, RDE are co-factors of Dicer. RRF-1 and EGO-1 are RNA-dependent RNA-polymerases that produce more siRNAs from stabilized templates. Several factors can block the processes; B2, 1A are virus-derived RNAi-suppressors acting on dsRNA/Dicer. For immunological acronyms, see glossary. Redrawn from Ding and Voinnet (2007) with permission from Elsevier.
Three classes of RNAi-mechanisms can be distinguished (Ding and Voinnet 2007; Kemp and Imler 2009):

1. **Micro-RNA-pathway.** This starts with the recognition of RNA in the form of stem loops (primary, pre-miRNA transcripts) that are processed in the cytoplasm (insects) or in the nucleus (plants). Stem loops are generally important during development in animals and plants. After binding to Dicer (an enzyme that cleaves RNA), small pieces of RNA (saran) are generated that can be recognized and bind to an Argonaut (AGO1)-complex, which probably leads to blocking of translation. The pathway seems to be present in most animals.

2. **Small interfering RNA-pathway.** Dicer binds directly to dsRNA and cuts it into siRNAs, which can be recognized by Argonaut to form the RISC-complex (RNA-Induced Silencing Complex) that is able to cut further RNA resembling the template of the bound viral RNA. By as yet unknown processes, cytokines are produced at the same time, which are able to signal the infection status to other cells via the Domeless receptor that activates the JAK/STAT pathway, and that eventually induces gene transcription (Beutler et al. 2007).

3. **Pi-RNA pathway (piwi-associated RNA).** This pathway is independent of Dicer and involves further Argonaut proteins. piRNAs are involved in controlling mobile genetic elements in the germ line, including viral elements. The path is found in flies and vertebrates (Ding and Voinnet 2007; Kemp and Imler 2009).

Different groups of organisms differ in the proteins that take part in these pathways. For example, the micro-RNA-pathway of insects contains two forms of Dicer, in vertebrates only one Dicer seems present, and in plants (Arabidopsis) four Dicer-proteins are known. Similarly, organisms differ in the kinds of co-factors that are needed for Dicer to function. Further central elements are the Argonaut proteins that exist in different forms in plants, insects, and nematodes. Generally, Argonauts are enzymes that specifically target RNA-templates and slice them into pieces. As core elements of the RISC-complex they are guided towards complementary RNA-sequences.

Viruses in turn have evolved various suppressors of the RNAi-silencing mechanisms (VSR, viral suppressor of RNA-silencing). For example, FHV (flock house virus) and DCV (Drosophila C-virus) of Drosophila have suppressors for the dsRNA/Dicer step (B2 and 1A, respectively; Figure 1). Probably for this reason, Dicer2, Argonaut-2, and co-factor proteins (R2D2) show signs of fast evolution (Kemp and Imler 2009).

In addition to the RNAi-mechanism, a systemic response is induced in plants (Ding and Voinnet 2007) and Drosophila (Kemp and Imler 2009) as defence against intra-cellular parasites. In plants, a Dicer-dependent signal moves through inter-cellular bridges (and even via long-distance spread in the phloem) to ‘immunize’ neighbouring cells by inducing the production of RNA from a given template and thus activating the RNAi-machinery ahead of actual infection (Ding and Voinnet 2007). Around 150 genes, differing according to infection by bacteria or fungi, become activated in the Drosophila systemic response.

**Vago**, a candidate effector gene is an inducible peptide in the fat body, where viruses often seem to reside. **Varo** is activated by the specific sensing of the viral dsRNA-infection with Dicer (Kemp and Imler 2009). Dicer-2 contains an amino-terminal domain (Dead/H-box) in both, Drosophila and mammals, which does not bind itself but seems to change the conformation of the RNA-molecule; this seems to
facilitate the further processing of RNA into the RISC-complex where actual slicing takes place. The Dead/H-box is interesting, since this domain is similar and phylogenetically close to the helicase domain of a mammalian receptor family (RLR) that induces interferon gene expression (an anti-viral response).

A further mechanism of anti-viral defence in *Drosophila* is the opening of ATP-sensitive K+-channels that is associated with increased resistance. Furthermore, the Imd/Jnk, JAK/STAT and Toll-pathways all seem additionally involved in anti-viral defences at least in mosquitoes when challenged by Parvovirus (Fragkoudis et al. 2009). Hence, the anti-viral defences are quite varied but the RNAi-silencing mechanism is a centrepiece in plants and invertebrates.

the same transcription factors to induce pro-inflammatory cytokines and interferons. Immune signalling in mammals is understood best and, as the example shows, the respective cascades show complexity, redundancy, and convergence to key elements at various stages.

### 4.3.4 Proteolytic cascades

Proteolysis is a process of degradation of proteins by specialized enzymes, the proteases. Proteolytic cascades are triggered by the binding of soluble host-recognition proteins to a corresponding parasite motif, and result in the production or activation of proteases. The best studied such cascade is complement that is highly conserved throughout animals and can respond very early to infection (Nonaka 2001; Nonaka and Kimura 2006). In the jawed vertebrates, complement has several functions, notably the opsonization of pathogens to mark them for phagocytosis, the initiation of inflammatory responses, and the direct killing, by lysis, of pathogens.

Complement are soluble proteins of the blood serum that ‘complement’ the cellular components. In humans, complement consists of around 30 different proteins. It is activated by one of three different pathways, the so-called ‘classical’, ‘lectin’, and ‘alternative’ pathways (Figure 4.6). After infection, the normally circulating precursors are activated by cleavage to become active components. In the process, the C1, C4, C5, and particularly the C3-protein, are the key players that, after cleavage, become active forms of various long and short fragments.

In the *classical pathway* (only found in jawed vertebrates), the binding of an antibody (lg) to an antigen triggers the cascade. In the process, the C1-protein (i.e. the C1q fragment) can bind to an antibody that has docked on to an antigen. This activates C2 and C4 proteins by cleavage, which in turn activates the most abundant key C3-protein (C3 can also activate itself). An activated C3-protein (C3b) can bind to an invading pathogen and mark it for phagocytosis; hence, C3b functions as an opsonin. But in the cascade, C3 can also activate C5. Another fragment (C3a) can stimulate basophils and mast cells to release compounds that affect the vascular system in the vicinity, e.g. to increase blood flow. The C5-protein is cleaved and stimulates the formation of a so-called membrane attack complex (MAC) that involves further C-proteins. The complex is able to bind and puncture bacterial cell walls. The channel formed leads to a loss of cellular contents and so destroys invaders. The attack complex can also lead to the destruction of own, aberrant cells. With the *alternative pathway*, the triggering of the cascade happens independently of the binding of an antigen, and C3 acts rather as a non-specific, releasing protein. In the process, the active C3b-protein is spontaneously generated. Normally, C3b is quickly inactivated by binding to the surface of its own cells. But in the presence of microbial pathogens, the C3b-protein binds to a certain protein (factor B, properdin) on the pathogen’s surface, which, through different steps, also leads to the formation of the attack complex. The alternative pathway is phylogenetically old. With the lectin pathway, a cascade similar to the classical pathway is triggered by a lectin that binds to mannose on microbial cell walls (Figure 4.6).

More generally, the complement has many different functions as it bridges innate immune defences (e.g. inducing cell lysis by the attack complex, or triggering the release of histamines from mast cells to induce
inflammation) and adaptive immunity (e.g. inducing the production of antibodies). It is, therefore, also a crucial component of vertebrate innate immunity. Evolutionarily, the complement is older than the adaptive immune system of vertebrates, as key components are already found in deuterostomes, such as sea urchins and hemichordates (Nonaka 2001; Zarkadis et al. 2001). Some elements are also known from early arthropods or corals (C3-like genes), and others from sea anemones, suggesting that this defence cascade is at least one billion years old (Flajnik and du Pasquier 2008). These early components seem to have been lost and were presumably replaced by thioester-containing proteins (TEPs; as is known from insects, e.g. Drosophila, Anopheles; Blandin

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**Figure 4.6** The complement cascade in mammals. It is activated by either one of three pathways that respond to different signals. The complement proteins C1 to C9 are variously cleaved, or form complexes. Eventually, the cascade converges to generate either opsonins (with fragment C3b and others), induces inflammation (with fragments C3a, C5b), or forms the membrane attack complex (MAC) that attaches to microbial surfaces and eventually kills the pathogen. Redrawn from Coico and Sunshine (2009) with permission from John Wiley & Sons, Inc.
and Levashina 2004) that function as opsonins in similar ways as C3 in the vertebrate complement.

4.3.5 The deployment of effectors

Ultimately, the immune system must do something to eliminate or control an invader. Effectors can be specialized cells, such as professional phagocytes or cytotoxic T-cells that release components to kill infected cells. Some other cellular mechanisms have been mentioned above, e.g. melanization, clotting, and so forth. However, there are also a variety of non-cellular effectors that are deployed as a result of activating different cascades. The most notable of these effectors are the anti-microbial peptides (see below) that are able to kill an invader by a variety of mechanisms.

4.4 Immune defence protein families

Immune systems are based on a limited set of protein (super-) families. However, members of these families show a large degree of diversification and specialization. During evolutionary history, members of the same family have been put in the service of many different immune defence functions (Flajnik and du Pasquier 2008). This suggests that the general structure of these protein families makes them valuable and flexible enough for key functions such as recognition and binding (Table 4.4).

4.4.1 Immunoglobulin-superfamily (IgSF)

The molecules made of IgSF (immunoglobulin-superfamily) domains represent a vast number of different molecules with a broad range of functions, from cell adhesion in the nervous system to molecules directly binding to antigens, presenting antigens, or being co-stimulators, in addition to their ‘classic’ antibody role as immunoglobulins or T-cell receptors. The immunoglobulin superfamily is the most numerous and important group of defence molecules. The surface of vertebrate lymphocytes, for example, can show more than 30 different types of IgSF receptors (Barclay 2003). Immunoglobulin domains can associate with other proteins, such as LRRs or fibronectins, to form mixed proteins (chimaeras) that assume an additional variety of functions.

Structurally, molecules of the Ig-superfamily can be made up of one to several different domains. Each domain is made up of strands (β-strands), which are usually connected to one another by disulfide bonds, and form two sheets in the three-dimensional structure of the molecule. This arrangement results in the formation of one or several folds within each domain (the Ig-folds). Consequently, different immunoglobulins are characterized and classified by which domains (V, variable; I, intermediate; C, constant) they are made of. The genetic coding of these molecules is quite complex because different gene segments can encode various parts of the β-strands. For example, in T-cell receptors the V-gene exon encodes for strands in both sheets, while the J-segment encodes only one strand in one of the two sheets of the V-domain. Within each of the regions, each of the various domains, V, C, and I, show diversification across the organisms. The V domain alone, or in combination with other V domains, acts to recognize antigenic epitopes and is therefore the most prominent part. The binding specificity of this domain results from different underlying amino acid sequences that lead to different three-dimensional structures of the folds.

4.4.2 Leucine-rich repeats (LRRs)

Leucine-rich repeats (LRRs) are found from plants to all metazoan animals. There is a minimum of six families in this superfamily that differ in length and the motifs that make up the repeats. Because these motifs are different among families, they probably have evolved independently. LRRs consist of anything between 2 and 45 motifs, each 20–30 amino acids in length. They characteristically bend into an arc-shaped form where the inner, concave part is well-suited for interactions with other proteins or carbohydrates. Structurally, LRRs often are flanked by cystein-rich domains. LRRs can be extra- or intra-cellular components; they can be soluble in cytosol or trans-membrane structures. Testifying to their extraordinary versatility, LRRs mediate protein–protein interactions and function as molecules of cell-adhesion, signal transduction, DNA-repair, in the recombination machinery, in addition to their many
roles in the immune system. In immune defence, they are involved in the recognition of antigens, act as a cytokine or parasite recognition receptor (like the vertebrate TLR; Akira 2004; Akira et al. 2006), or control the motility of lymphocytes (in vertebrates) and haemocytes (in insects). LRR-proteins can also associate with other domains intra- and extra-cellularly. In these chimaera molecules, the LRR-part functions as a recognition protein.

4.4.2.1 Toll and Toll-like receptors (TLRs)

These molecules are LRRs, too, and important enough to deserve special mentioning. Toll became first known as receptors involved in insect development. Now, they are known as important gateways that respond to signals from an infection and activate the important Toll-pathway of the invertebrate (insect) immune defence (cf. Figure 4.5). Toll does not directly recognize the parasite, and is therefore not a receptor for the parasite but binds to a signalling factor, the ligand Spaetzle, that results by cleavage from the upstream processes of recognition (e.g. as mediated by PGRPs). Across all animals, Toll is structurally very similar. The extra-cellular component of Toll, able to recognize Spaetzle, consists of LRR; the intra-cellular segment has a TIR-domain (Toll-interleukin-1 receptor) that, together with other transducing proteins (MyD88), activates the signalling cascade. This internal cascade seems conserved across major phyla, whereas the outer, extra-cellular part varies and works differently (i.e. direct parasite recognition, or binding to a signal peptide). The Toll-pathway eventually leads to the production of antimicrobial peptides. While the number of different Toll in invertebrates is limited, the group of TLRs is very varied and specific in vertebrates. They have evolutionarily diversified independently of the Toll and Toll-like protein diversification in the invertebrates. Remarkably, in echinoderms (sea urchins), hundreds of different TLRs are found. In vertebrates, the TLRs form a single protein family with a large diversity of approximately 20 members (Flajnik and du Pasquier 2008).

4.4.3 Lectins

Lectins are characterized by being able to bind to carbohydrates, such as sugars, that often are important surface elements of microbial parasites (e.g. the variable glycoprotein surface molecules of African trypanosomes). C-type lectins (requiring calcium for binding) of animals are involved in the activation of complement (via the lectin pathway; Figure 4.6), which is triggered when mannose-binding protein (MBP) binds to a microbial parasite (e.g. bacteria, protozoa, fungi). The C-type lectin family share structural similarities in their hydrocarbon-recognition domain. C-type lectins are also involved in the functioning of dendritic cells and the formation of the immunological synapse (Figure 4.10), as well as in many other non-immunological processes. Lectins can be membrane-bound or in soluble form, and they have sometimes altered recognition capacities. Mouse NK(natural killer)-cells, for example, have C-type lectins that act as receptors on the surface membrane (and by which MHC expression is controlled in the body). Here, lectins have evolved to recognize peptides rather than sugars. In all, C-type lectins are found in many phyla, and their diversity and polymorphism within any given population is huge (Flajnik and du Pasquier 2008).

4.4.4 Other important families

There are numerous other families of defence molecules that show different structures from the ones mentioned above or that are composed of domains coming from different families. Molecules involved in recognition or as effectors are particularly interesting (Coico and Sunshine 2009).

4.4.4.1 Tumour necrosis factor family (TNF)

This is a group of cytokines and receptors that are involved, among other things, in the process of cell apoptosis. Some TNF also function as secreted pro-inflammatory cytokines (TNF-α). Some TNFs can also be membrane-bound and function as cytokine receptors. The TNF-receptor (TNFR) family is variously characterized by differences in their cytoplasmic tails, i.e. the end of the molecule that reaches inside the cell. The tails can be Death receptors, decoy receptors, or activating receptors; the extra-cellular parts of the TNFRs are quite conserved, ligand-binding domains. Activation of a TNF-receptor by binding to a cytokine leads to an
intra-cellular signal through adaptor proteins (TRAF, TNF-receptor-associated factor). One of the most prominent TNFR is the CD40 co-receptor of antigen-presenting cells, such as macrophages.

4.4.4.2 Cytokine receptor families
The class I cytokine receptors typically have two polypeptide chains: a cytokine-specific and a signal-transducing unit. Most cytokines use these kinds of receptors. Class II cytokine receptors are structurally similar, although the single domains show difference in their detailed composition. Class II receptors are used by interferons that signal the presence of viral infections.

4.4.4.3 Chemokine receptor family
These belong to a superfamily of receptors that are characterized by a unique, snake-like ('serpentine') shape in their extra-cellular part. Chemokine receptors not only bind to chemokines, but also directly to parasites. For example, Plasmodium vivax (malaria) binds to a chemokine receptor known as the Duffy blood group antigen, which is highly variable. HIV-virus binds to CR4-receptors on various immune cells.

4.4.4.4 PGRP, GNBP
Peptidoglycan-recognition proteins (PGRPs) come in various types—and their somatic diversity can be enhanced by alternative splicing. They are found in invertebrates on the surface of blood cells, or as soluble forms. When infected by bacteria, PGRBs are up-regulated and can bind to the corresponding microbial ligands. They activate two important immune defence pathways, the Toll- and Imd-pathways, as well as other (proteolytic) cascades, which results in the production of anti-microbial peptides, melanization, or in the activation of phagocytosis. Homologues of PGRPs are found in soluble form in vertebrates, too, and have anti-microbial activity. Gram-negative-binding proteins (GNBPs) can bind to Gram-negative bacteria, and fungi, but also to Gram-positive bacteria (e.g. in Drosophila). In each case, they are recognizing β-1,3 glucans and so activate the immune defence. Hence, their true function is not recognizing Gram-negative bacteria as the (misleading) name suggests, but recognizing β-1,3 glucans, wherever they occur. PGRPs and GNBPs play an important role as recognition molecules that ultimately activate appropriate defence pathways (Flajnik and du Pasquier 2008).

4.4.4.5 NOD and other intra-cellular sensors
This group comprises molecules that also contain LRR in prominent functions. Hence, it is not a family in the strict sense. Nevertheless, they are important for defence inside cells. In fact, parasites that manage to get into host cells (with viruses a typical example) pose a special problem for the host defence. Their presence has to be recognized amidst a multitude of intra-cellular processes and own molecules. An important family of intra-cellular receptors is the NOD/NLR/CATERPILLAR (CLR) group. The proteins of this family are very varied in structure, but in all cases a leucine-rich-repeat (LRR; see above) forms the C-terminal of the molecule and provides specificity for binding to a parasite. These proteins are also involved in regulating the immune defence. The family is well-represented in plants (Jones and Dangl 2006) and probably is responsible for the specific defences against pathogens (Dangl and Jones 2004). NOD-like receptors appear to have diversified enormously in groups such as bony fish and sea urchins (Zhang et al. 2010).

4.4.4.6 Scavenger receptors (SRCR)
This is a group of conserved proteins found on cell surfaces and in soluble form. Their core function is to ‘scavenge’ and remove oxidized or otherwise modified lipids to prevent damage. However, SRCR are also involved in immune defences, for example, in phagocytosis (e.g. the receptor eater in Drosophila).

4.4.4.7 Down syndrome cell adhesion molecules (Dscam)
Dscam is a protein made of IgSF and fibrinogen domain. The IgSF domain provides many isoforms in the binding region (several tens of thousands). These isoforms are generated by alternative splicing of the transcripts. Alternative splicing of Dscam is known for insects and crustaceans, but it remains unclear how widespread this process is beyond these groups. Dscam is, on one hand, important as an organizer (an axon-guiding protein) in insect neuronal development; on the other, Dscam is also involved
in arthropod immune defence, notably in phagocytosis (Watson et al. 2005). Dscam is present, both as a membrane form and as soluble form that might be cleaved from the membrane (in shrimps, it may be secreted). Dscam was one of the first known instances of invertebrates possessing highly variable receptors, similar to the variable antibodies found in vertebrates. The relevance of Dscam for variable defences is still under scrutiny, however.

4.4.4.8 Fibrinogen-related protein (FREP)
Discovered in molluscs (the snail Biomphalaria), FREPs are up-regulated after infection by trematodes (*Schistosoma*) (Adema et al. 1997). FREPs are made of IgSF and fibrinogen-like domains; they bind to ligands on the parasite surface. To date, however, it is unclear what role they play in immune defences.

4.4.4.9 Variable domain chitin-binding proteins (VCBPs)
These are made up of two tandem Ig domains of variable type (V) at the N-terminal end, and a chitin-binding domain (CBP) at the C-terminal end. This latter domain shows similarity to chitinase. VCBPs are probably effector molecules and typically are found in the gut. VCBPs show variation among individuals in a population with polymorphism and variation in the contributing loci.

4.4.4.10 Anti-microbial peptides (AMPs)
AMPs are among the most important effectors of the immune system of invertebrates and vertebrates alike. They typically protect surfaces, such as mucosa lining the gut, respiratory or urinary tracts, and, in vertebrates, are delivered to the site of infection by the lymphocytes. Several hundreds of AMPs from many organisms are currently known (Table 4.4). They are grouped into different families, the best known of which are the defensins. Across organisms, the amino acid sequences in regions important for AMP-translation or intracellular trafficking are fairly conserved, indicating common constraints on their production and delivery (Zasloff 2002). In general, AMPs have poor specificities and will therefore be effective against a wide range of pathogens, even those that the organism normally never encounters (Casteels et al. 1993). It is believed that microbes, in turn, might be unable to adapt to AMPs, since these target important structural elements (such as components of the cell wall) that cannot be easily changed by selection (see below). Alternatively, or in extension of this, any one individual host usually produces a cocktail of different peptides when infected (Zasloff 2002). Because several different AMPs are deployed in any one infection, the various AMPs likely act synergistically on a parasite and the variation in the composition of the AMP-‘cocktail’ might be of great significance to combat a diversity of parasites (Yan and Hancock 2001; Ganz 2003; Rosenfeld et al. 2006; Riddell et al. 2009). Such variable, synergistic action might similarly prevent that microbes can easily adapt and evade the effect of AMPs.

AMPs are highly effective against microbes, i.e. bacteria, fungi, or protozoa, and kill these pathogens in a variety of ways (Zasloff 2002; Brogden 2005). One effect is to induce pore formation in parasite membranes, which leads to cell death. In this process, the AMP is attracted to a pathogen’s surface, e.g. a bacterium. After attachment, the peptides can insert themselves into the parasite’s membrane and form pores in a number of different ways (Brogden 2005). It appears that, in addition to pore formation, AMPs also kill in other ways. For example, some AMPs have other targets inside the parasite cell as well. Some peptides (e.g. buforin II, apidaecin) can indeed permeate the parasite’s cell membranes by utilizing appropriate transporters, and accumulate in its cytoplasm where anti-microbial activities proceed, for example, by the inhibition of nucleic acid or protein synthesis or by impeding enzyme activities (Brogden 2005). Why certain AMPs are effective against a given parasite is not well understood. However, it is clear that a range of factors are important, such as the size of the peptide, its three-dimensional structure, electrical charge, or its hydrophobic properties (Brogden 2005). Anti-microbial peptides are produced from larger precursor molecules. In some cases, the germ line encodes multiple copies of the same AMP, presumably to increase the speed at which a large number of peptides can be transcribed and synthesized. Furthermore, anti-microbial peptides seem to be a category of molecules that, together with polypeptide venoms, kinocidins (special chemokines), and others, form an arsenal of evolutionarily ancient defence molecules (Yeaman and Yount 2007).
Table 4.4 Anti-microbial peptides. The large diversity is classified according to their secondary structure (based on Brogden 2005).

<table>
<thead>
<tr>
<th>Peptide class</th>
<th>Characteristics</th>
<th>Examples in invertebrates</th>
<th>Examples in vertebrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5) Anionic, cationic fragments</td>
<td>Fragments of larger proteins. Role in immune defence unclear.</td>
<td>Casocidin I, from casein (humans).</td>
<td>Domains from α-lactalbumin (cattle), from haemoglobin, lysozyme, ovalbumin (humans).</td>
</tr>
</tbody>
</table>
4.5 The generation of diversity in recognition

Host-parasite co-evolution is based on antagonistic interactions. Such a system tends to become diversified over evolutionary time. This is because a host evolves new defences, but then the parasites adapt and evolve new attack-strategies; and the same is true the other way round. Hence, how defence systems generate diversity in their capacities to recognize or attack parasites is a central theme in immune system evolution. The diversification within a few major families of molecules, as discussed above, is one example of how suitable molecules are evolving and diversifying towards new variants and, sometimes, new functions (Du Pasquier 2006).

In any immune defence, recognition of a parasite is the first step. We can expect that there is strong selection on the host, due to the diversity of parasites, to cope with a huge set of motifs that need to be recognized. The diverse repertoire of antibodies found in the vertebrates is a prime example of an evolutionary solution to the problem of diversity. However, modern research suggests that diversity in recognition is not just the privilege of the higher (jawed) vertebrates. Across the immense kingdom of chordates, invertebrates, and plants, very different solutions of how to diversify the repertoire of recognition have evolved. In principle, diversity can be encoded by the diversity of genes in the germ line (the gene-sequences). Such diversity will change as the population evolves under the effects of selection. Alternatively, and in addition, diversity can be generated somatically, that is, during the lifetime of an individual and out of a given repertoire of genetic elements.

Distinguishing self from non-self against a background of highly diverse motifs is therefore a prime task. Three major strategies to recognize non-self and distinguish it from self can be outlined (Medzhitov and Biron 2003):

1. Recognition of microbial non-self. This is the most universal strategy, certainly among the animals. It relies on the detection of conserved motifs that reveal the presence of a pathogen (e.g. the PAMPs, that is, epitopes on the surface of microbes).

2. Recognition of missing self. This strategy is followed by NK-cells in higher vertebrates, which respond to the absence of a signal that identifies a cell as self. Cells lacking this signature are treated as non-self and destroyed. This is especially effective against intra-cellular parasites that otherwise might hide from the immune defences.

3. Aberrant activities. This strategy is especially found among plants (the ‘guard hypothesis’ (Holt III et al. 2003)). Rather than recognizing, for example, the surface of a pathogen, this mechanism detects the aberrant consequences of pathogen activity in the host body. Examples are the effect of virulence factors that change normal processes and are therefore recognized by the plant’s receptors. A similar strategy might be the surveying of the commonly associated and symbiotic/communalistic bacterial fauna within the gut of an animal. When a pathogen enters this ‘ecosystem’, a change in the normal functioning occurs that is detected by the immune system, as perhaps found in Anopheles gambiae (Meister et al. 2009).

4.5.1 Polymorphism in the germ line

Genetic polymorphism can involve the presence of several variants of the same gene in a population (polymorphism in the narrower sense), or the presence of different genes at different loci (polylocism). As it is immediately obvious, the number of possible genes is still much lower than the enormous diversity of parasites and the epitopes they possess. Genetic variability is basic to all defence systems, but additional somatic processes are important as well. As the above discussion of some major families of immune defence proteins also has shown, families and their members diversify over evolutionary time (Du Pasquier 2006; Flajnik and du Pasquier 2008). Some of this diversity is maintained in the different lineages and leads to a richer (or poorer) repertoire of immune genes. Major factors underlying these changes are the standard processes of mutation and selection, leading to a birth-death process in different gene families. Furthermore, gene conversion and gene duplication, followed by the
subsequent divergence of the copies, are important processes in immune gene evolution. Duplications can lead to clustered genes within the same neighbourhood of the genome, but translocation to new sites is also possible and observed.

Examples of important genetic polymorphisms are lectins in nematodes. In the genome of *Caenorhabditis elegans* around 180 different C-type lectins have been found. Although the functions are not known for most of them, such variation maintained in the population seems important for immune defences (Nicholas and Hodgkin 2004; Du Pasquier 2005; Du Pasquier 2006). Similarly, recognition and opsonization molecules, such as PGRPs, GNBPs, and TEPs, are genetically polymorphic in arthropods (insects, crustaceans), as are the recognition segments of their TLRs (the LRRs). In early chordates (the lancelet, *Amphioxus*), VCBPs, lectins, and recognition segments of TLRs, are encoded by polymorphic genes. In jawless (variable lymphocyte receptors, VLRs) and in the higher (jawed) vertebrates, many important elements such as immunoglobulins, TLRs, complement factors, or the major histocompatibility complex are encoded by polymorphic genes, which are furthermore subsequently diversified by somatic processes. As a result, different families of immune genes can be enormously diversified and contain a large number of genes, some of them species-specific, some being orthologues in congeneric species or within larger groups (Figure 4.7).

![Figure 4.7 Diversity of immune gene repertoire. The graph shows the number of genes belonging to different gene families, as found in two species of mosquitoes (Ag: *Anopheles gambiae*, Aa: *Aedes aegypti*) and *Drosophila melanogaster* (Dm). Black: orthologous genes in all three species. Dark grey: orthologous genes for mosquitoes. Light grey: genes only found in one species. Redrawn from Waterhouse et al. (2007) with permission from AAAS.](image-url)
4.5.2 Somatic generation of diversity

Further diversification of recognition molecules happens in many different ways during the lifetime of an individual. While the germ-line information thereby stays the same, the diversification occurs in the somatic cells (Figure 4.8). The prime example is the generation of variable receptors in the immune system of jawed vertebrates, as discussed in the next section. Some other processes leading to somatic diversification are discussed first.

4.5.2.1 Alternative splicing

This is known from a number of different recognition proteins, from plants to the higher animals, but also for some effectors, such as human defensins and likely many others (Du Pasquier 2006). The Down syndrome cell-adhesion
molecule (Dscam; see above) of insects and crustaceans is another compelling example for this process (Figure 4.8a). Dscam is a protein that can be expressed so as to form a repertoire of highly variable immune defence receptors, primarily involved in phagocytosis. The binding site is in the IgSF domain. Estimates for Drosophila suggest that around 36,000 different isoforms are possible, of which only a limited set (perhaps ten to a few dozen isoforms) is expressed by one haemocyte (Watson et al. 2005; Dong et al. 2006; Du Pasquier 2006). The generation of
isoforms is based on differential processing of the RNA-transcripts from the germ-line encoded gene cassettes. As each cassette is transcribed into mRNA, the transcripts are spliced and reassembled in different ways by still poorly known processes. The isoforms can bind to different pathogens and might thus function in analogy to vertebrate antibodies. Perhaps, the Dscam-repertoire might change according to the kind of infection (Dong et al. 2006); this infection-tailored expression is not yet unambiguously demonstrated, however. Further examples of proteins that undergo alternative splicing are LRR-domains in mammals, or the scavenger receptors (SRCRs) in sea urchins, where thousand of isoforms are expressed based on 150 different genes (Du Pasquier 2006).

A related mechanism is RNA-editing. In this process, a specific nucleotide of the mRNA is changed catalytically. In contrast to alternative splicing, therefore, the transcripts are not cut and reassembled in different ways, but the information that the mRNA carries is altered in some places. This mechanism seems to be very old, as it has been found in single-celled organisms and may occur in some higher organisms, too (Du Pasquier 2006).

4.5.2.2 Somatic rearrangement, copy choice
In strict immunological terms, ‘rearrangement’ refers to the RAG-mediated recombination events found in the jawed vertebrates (see below). But processes with a similar outcome are also known from jawless vertebrates; that is, processes that affect how DNA-segments somatically come together in new combinations. The main example in jawless vertebrates (hagfish and lampreys, e.g. Petromyzon marinus) is the variable lymphocyte receptors (VLRs) (Figure 4.8b). In the process, VLRs are assembled near-randomly from gene sequences at different locations in the genome. Structurally, VLRs consist of invariant N- and C-terminal ends that flank a region of variable and highly diverse LRRs elements. This variable region functions as an antigen-binding region and shows specificity towards bacterial infections. The variability in this region is generated in a very unusual way that probably works as follows.

The genes encoding for the N- and C-terminal ends of the molecule reside in a cassette each on the genetic sequence. As the lymphocyte matures, the stretch between the two ends is filled with single genetic elements encoding for LRRs; these LRR-cassettes are located upstream or downstream from the two ends and copied into this space. Hence, as the lymphocyte matures, LRR-sequences from different locations are pasted into the space between the two ends by a complex and near-random process. The insertion of the LRR-elements is due to small homologous regions where copies can insert. The entire copying mechanism is similar to the onset of a gene-conversion mechanism but the transfer of genetic material is incomplete; this process has been termed ‘copy choice’. This indicates that an evolutionarily early solution for the diversification of recognition molecules might have used mutation and gene conversion before RAG-dependent recombinatorial arrangements had emerged. In the lamprey, different lymphocytes end up with different combinations of the copied LRR-cassettes. Lymphocytes thus have a monotypic expression but as a population vary in their binding specificities, analogous to the lymphocyte repertoire of the higher (jawed) vertebrates. Estimates are that as many as $10^{14}$ different specificities could be generated by this system (Flajnik and du Pasquier 2008).

The RAG-dependent somatic rearrangement is found in immunoglobulins (Ig), T-cell receptors, TLRs, and the major histocompatibility complex of the jawed vertebrates. In the jawed vertebrates, notably the extant sharks, rays, and chimera, lymphocytic receptors are somatically diversified by processes of somatic recombination based on the RAG-1 and RAG-2 genes that activate the enzyme recombinase, as well as by somatic hypermutation and gene conversion (Figure 4.8c). This will be discussed in more detail in the context of the variable receptors of the higher vertebrates below.

4.5.2.3 Somatic (hyper-) mutation, gene conversion
Somatic mutation to diversify recognition molecules is an old evolutionary process and plays an important role for lymphocytes of vertebrates. Gene conversion is a large mutation event caused by asymmetric recombination that leads to the transfer of a gene sequence from one genomic set to another; the receiving sequence is thereby altered. Gene conversion is mediated by a key enzyme, the activation-induced cytidine deaminase (AID) (Figure 4.8c). Gene conversion leads to the diversification
of immunoglobulins as, for example, in B-cells of rabbits (Winstead et al. 1999), and birds (V-region of light chain; McCormack et al. 1991; Flajnik 2002). Furthermore, AID seems an ancient all-purpose enzyme that is involved in somatic hypermutation, gene conversion, class switching, and RNA-editing (Papavasillou and Schatz 2002; Honjo et al. 2004; Odegard and Schatz 2006; Schatz 2007).

Discovered in molluscs (the snail Biomphalaria), fibrinogen-related proteins (FREPs) are quite diverse. Many isoforms are encoded by different genetic regions (around 11 are known) and undergo alternative splicing. In addition, mutation and/or gene conversion somatically further diversifies the region of the IgSF domains (Zhang et al. 2004). FREPs are made up of one or two tandem Ig-domains at their N-terminal end, and of the fibrinogen-like domain at the C-terminal end. The variable part of the FREPs is found in both domains but only the N-terminal Ig-domain is somatically diversified. In the process, the DNA coding for this domain of somatic cells (haemocytes) appears to be changed by mutation (or gene conversion), and therefore produces an alternative isoform. It is not clear though whether this region actually binds to the parasite, although such diversification is a hallmark of immune recognition molecules.

4.5.3 The structure of immunoglobulins of B- and T-cells

This account is influenced by our understanding of mammalian lymphocytes, but there is considerably diversity across many other vertebrate systems. Their full representation of which can be appreciated from several reviews (Du Pasquier 2006; Flajnik and du Pasquier 2008).

4.5.3.1 B-cells

Immunoglobulins (Ig) are among the most remarkable recognition molecules. They are membrane-bound or secreted. A membrane-bound form is present on the surface of B-cells. When it recognizes an antigen (i.e. an epitope on a foreign cell or material), the B-cell is activated, whereby a so-called Igα/Igβ-heterodimer plays an important role. The secreted forms are also known as antibodies; they are produced by plasma cells, which are the differentiated B-cells located mainly in the bone marrow. The structure of immunoglobulins has several features that make them uniquely suited to act as recognition molecules. On one hand, the three-dimensional structure of the Ig-molecule with its folds allows specificity in the binding to an antigen. The so-called variable region determines the specificity. On the other hand, this variable region is constructed and generated in a way that allows the production of a huge number of different binding specificities. The population of the different immunoglobulins in a given individual, therefore, represents an enormous range of different specific recognition capacities.

The basic structure of an immunoglobulin (Coico and Sunshine 2009) invariably consists of polypeptide chains: four chains in the case of antibodies/B-cell receptors. The amino acids of these chains are encoded in the germ line with variability through polymorphism and polylocism. But the sequence of amino acids can be further modified beyond what is given by the germ line, by somatic mutation and other processes. Nevertheless, the four chains of an antibody are always made up of two identical so-called light chains, and two identical heavy chains, forming a symmetrical globular molecule that is usually sketched in a Y-shape, as shown in Figure 4.9. In mammals, the heavy chains come in five different classes (the isotypes): the IgA, IgM, IgG, IgE, and IgD forms. These classes differ, for example, for birds (classes IgA, IgM, IgY), amphibia (IgM, IgD, IgY IgX), or bony fish (IgM, IgD, IgZ) (Flajnik and du Pasquier 2008). Ig-classes can be further divided into sub-classes, for example, into IgA1, IgA2, and the IgG-sub-classes. The difference among classes is based on differences in the amino acid sequence of the constant regions (towards the C-end of the molecule) of the heavy chain (Figure 4.9a); the different constant regions are each encoded by different genes. An individual mammalian host has all five classes but the proportions of these five classes vary among different species. The different classes fulfil different functions in the immune system, even though the immunoglobulin molecule, as such, may have the same specificity for recognizing an epitope. For example, IgM is involved in activating the complement, whereas IgG can easily agglutinate and precipitate antigens. In both cases, however, the epitopes actually recognized by the immunoglobulin
**Figure 4.9** Structure of immunoglobulin (Ig) receptors. (a) Antibody secreted by B-cell. Two light chains (L, grey) and heavy chains (H, black) form the backbone of the Ig. The chains are interrupted by within-chain disulfide bridges (dotted lines) that lead to the formation of Ig-fold domains (‘loops’). The N-terminal loop of each chain is variable (V); variability is additionally increased by three hypervariable regions (complementary-determining regions, CDRs; white rectangles) in each loop, which are the prime binding elements. Together, the V-domains of the L- and H-chains form the antigen-binding site and determine the binding specificity of the antibody. Towards the C-terminal end, the chains have further (less variable) ‘constant domains’ (C); they determine the isotype (class) of the Ig. The chains are held together by further disulfide bridges. The hinge region allows the molecule to change the spread of the distal ends, so as to accommodate binding sites with a given geometry. The amino acid sequence determining the binding region of the molecule (area of dotted line) is varied by somatic V(D)J-gene rearrangement in addition to further processes (hypermutation, gene conversion, etc.).

could be the same. Hence, the variable region determines the binding specificity, whereas the constant region of the heavy chain decides the biological function (Figure 4.9a). Consequently, the Ig-isotype forms vary in their abundance in different tissues and at different times of the unfolding immune response.

The heavy chain also carries a variable region (towards the N-end) that, together with the N-terminal part of the constant region and the light chain, forms the binding region (Figure 4.9a). Within the heavy or light chains, loops are formed by disulfide bridges, which leads to so-called immunoglobulin fold domains. The folds create two domains on each light chain, and four to five domains on each heavy chain, with straight pieces of the chain between them. The shape of the first such domain (V, counting from the N-end of the molecule) is determined by the highly variable amino acid sequences in this region, with the different versions being part of the repertoire of any one host individual. In particular, this region (on light and heavy chains) also contains three hypervariable subdomains (the complementary-determining regions) that are primarily responsible for the binding to the antigen.
The parts of the heavy and light chains that make up this first variable domain are labelled as V_H and V_L regions, respectively. The following, second and subsequent domains, are much less variable and are therefore labelled, C_H and C_L (‘constant’) regions, respectively. The hinge region of the molecule is located at the ‘kink’ in the Y-shaped sketch (Figure 4.9a), between the second and third domain of the heavy chain (C_H1, and C_H2). Its function is to allow the molecule to shift shape, i.e. to provide flexibility when an antigen binds into the pockets of the distal domains, or when the molecule ‘stretches out’ to bind to two epitopes on a microbial surface separated by a certain distance.

The shape and therefore the binding specificity of any Ig-molecule depend on its amino acid sequences and how it thus folds. As mentioned above, the sequence is most variable towards the N-terminal end of the heavy and light chains, notably in three hypervariable regions. How is this diversity generated? One source of variability is germ-line encoded. The light chain is encoded by a variable (V) and a small, joining (J) genetic domain (both derived from a common pre-sequence that is split and

Figure 4.9 (Continued) (b) Structure of the T-cell receptor complex. The T-cell receptor (TCR) consists of α- and β-chain with a roughly similar structure as antibodies. TCRs typically bind with their variable region (Vα, Vβ, hatched area) to a peptide that is presented by the MHC-complex. The variable region also has three hypervariable regions (white rectangles). The C-terminal region anchors the molecule in the cell surface. The T-cell receptor complex furthermore contains the signal transduction complex, CD3, assembled from γ, ε, and δ-chains, plus the two ζ-chains connected by disulphide bridges (dotted lines). The Ig-folds of CD3 are invariable. The TCR only binds to a peptide; binding activates the signal transduction complex that generates the signal. Each such transducing polypeptide chain has several adaptor molecules at their intra-cellular end (white ovals). The T-cell surface furthermore contains co-receptors (such as CD4, CD8) that bind to the MHC-molecule (not shown here for clarity). Redrawn from Coico and Sunshine (2009) with permission from John Wiley & Sons, Inc.
rearranged). The variable part of the heavy chain is also encoded by genes for a V- and J-region, plus by genes for a third domain, the D-region. Together, the V(D)J-domains determine the variable region of the molecule. To give a rough indication, humans have around 50 genes for the V-region, 6 genes for the J-region, and some 20 genes for the D-region for the heavy chain. For the light chain, there are 30 to 40 genes for the V-region and perhaps 1 to 5 genes for the J-region (Coico and Sunshine 2009). There are two types of light chains (κ and λ) in mammals, but three types in other groups, such as shark or amphibians, whereas birds have only λ-chains. Both of the κ and λ-chains are present in an individual host; they are encoded by genes located on different chromosomes and by slightly different numbers of genes. As the B-cells mature and the chains are synthesized, the two (three) domains are rearranged to generate a novel genetic sequence for the molecule, a process that is referred to as V(D)J-recombination. This novel genetic sequence defines specificity for the antibody. The combination process is under the control of the recombination-activating genes (RAG1, RAG2) that are typical for the jawed vertebrates. (Interestingly, the RAG-process exists in echinoderms but seems not to be involved in immunity). Given the number of genes, there could be approximately 50 × 20 × 6 = 6000 different VDJ-combinations in the heavy chains, and 40 to 200 VJ-combinations in the light chain. As the two chains can by themselves associate in different ways, the total number of combinations is in the order of 10^6 different specificities—all derived from around 150 genes. But this enormous number is still below the estimated 2.5 × 10^7 specificities known to circulate in the blood and lymph system of a human body.

In fact, there are additional processes that generate diversity:

1. **Junctional diversity** results from the fact that the VJ-pre-form of the genetic sequence is split to generate the later V and J forms. This split is not always exactly at the same position. When the segments are subsequently re-joined, these split variations lead to alterations of the amino acid sequence in the respective fragments because the reading frame has changed (Hsu et al. 2006).

2. With **N-region diversity**, a small number of amino acids can be inserted in the joining regions of V and D, and of D and J in the heavy chain.

3. **P-diversity.** Here, palindromic (P-) nucleotides are added or deleted at the junction of gene segments (Wuilmart et al. 1977; Di et al. 2009). The DNA polymerase TdT (terminal deoxynucleotidyl transferase) plays an important role in this process.

4. **Somatic hypermutation** affects the genes coding for the V-regions of light and heavy chains and thus the binding specificity. These mutational events are stimulated by binding to antigens and happen over the lifetime of the B-cell; they are around 10^5-times more frequent than the background mutation rate in the germ line. This is the key process for ‘affinity maturation’ during which the B-cells increase their binding capacity towards a prevalent antigen. Inevitably, mutations are more frequently found in older cells and, therefore, are more important for the secondary response, i.e. when the host encounters the same antigen again. At any rate, however, the genetic sequence of the actually synthesized chains deviates from what is encoded in the germ line.

5. **Class switching** occurs after gene rearrangement (determining specificity), and as the heavy chain (isotype) undergoes alternative splicing in the constant region. Class switching does not affect the variable region and thus the binding specificity of the immunoglobulin, but it yields different classes of the immunoglobulin, e.g. IgM and IgD -forms, that can be expressed by the same B-cell. Class switching yields, for example, IgG-isotypes, which thus become the most common class in the circulatory system, and IgM in mucosa. In the process, the entire stretch of genes for a given VDJ-combination is recombined with a new set of genes for the constant region. The new recombinated genetic sequenced is then transcribed and the molecule is synthesized.

Class switching occurs when antigens for the respective VDJ-specificity are present and cytokines from T-cells...
stimulate the process. In this way, specificities for a recognized parasite can be transferred to other classes of immunoglobulins that activate additional cascades. By contrast, B-cells that have not yet been stimulated (the naive cells) by binding to a parasite’s epitope produce immunoglobulins of unknown specificity, called ‘natural’ antibodies. Their presence characterizes a kind of background specificity that circulates independent of any infection. Natural antibodies are typically immunoglobulins of type IgM, which have relatively broad binding affinities.

It has been found that other groups of jawed vertebrates may heavily use other processes to diversify their VDJ-specificities. In birds and rabbits, for example, gene conversion leads to somatic diversification of B-cell specificities. In the process, a stretch of DNA is copied into a receptor strain, and only this gene is converted into a new sequence. As we begin to understand more of the immune system of vertebrates, it becomes apparent that there is a remarkable diversity in how variation in the immunoglobulin recognition molecules is generated.

4.5.3.2 T-cells

The other major category of lymphocytes is the T-cells. T-cells cooperate with B-cells and fight parasites that have managed to infiltrate host cells, e.g. viruses, bacteria, but also many protozoa. T-cells mature in the thymus. T-cells have T-cell receptors (TCRs) that are antigen-specific IgSF-receptors, with each clonal line of T-cells having a different specificity, analogous to the situation in the B-cells. The population of T-cells in a single host thus represents a large repertoire with estimates in the range of 10^{11} different specificities. Diversity is generated by the same V(D)J-gene rearrangement as for the immunoglobulins described above. TCRs (T-cell receptors) are always membrane-bound and belong to the immunoglobulin superfamily (IgSF). Activated T-cells therefore do not secrete these Ig-molecules, as would be the case in B-cells. Also, TCRs never change by hypermutation or class switching as do the B-cells after they have recognized an antigen; yet, TCRs in sharks are different because mutation occurs in the corresponding \( \gamma, \delta \) -chains (Flajnik and du Pasquier 2008). Nevertheless, TCRs show some similarities with the immunoglobulins of B-cells, although they consist only of two polypeptide chains (\( \alpha \) and \( \beta \)), again linked by disulfide bridges. These bridges generate folds that form a binding pocket for the molecule to be recognized (Figure 4.9b). Each chain (\( \alpha, \beta \)) has a variable region (V), located more distally, and a less variable region (‘constant’, C) located closer to the T-cell surface. The whole TCR is anchored in the membrane and its short tails reach into the cell’s interior, the cytoplasm. Similar to B-cells, the variable region, furthermore, contains three hypervariable domains that together determine the shape of the binding pocket and thus the specificity of the receptor. TCRs preferentially recognize small protein fragments, the peptides (that are between approximately 8 and 17 amino acids long), and which are presented by another class of immune cells, the antigen-presenting cells (APCs) in the major-histocompatibility complex (MHC), as we will see below. In fact, TCRs typically recognize the MHC-peptide complex in contrast to other Ig-molecules that can directly bind to parasite surfaces. Because the organization of the genes coding for these regions is similar to the immunoglobulins of the B-cells, it is likely that the two recognition molecules evolved from the same ancestral form.

On the surface of T-cells, however, the TCRs are always expressed in a receptor complex, together with another receptor, CD3, and with two simple polypeptide chains (the \( \zeta \)-strains) (Figure 4.9b). Structurally, CD3 also consists of two polypeptide chains that have one loop forming a binding pocket. Moreover, the CD3 molecule has long tails that reach into the cell’s interior; long tails also characterize the \( \zeta \)-strains (Figure 4.9). These additional elements (CD3, \( \zeta \)-strains) are co-stimulated in a recognition event by the TCR-complex; they pass on the signal into the cell’s interior (signal transduction). As a result, the T-cell becomes activated. T-cell surfaces also have a range of other expressed molecules, some of which are acting as co-receptors (CD4, or CD8), others as adhesion molecules (CD2, LFA-1) or cytokine receptors (CCR7)(Coico and Sunshine 2009). Across different groups of vertebrates, we again find considerable diversity in the chains that make up these molecules are. The recombinatorial arrangement is not known from the jawless vertebrates, similar to the situation in the immunoglobulins (Igs) sof B-cells (Flajnik and du Pasquier 2008).
4.6  The diversity of immune defences

4.6.1  Defence in prokaryotes

It is amazing, but even prokaryotes, such as bacteria and Archaea, have an ‘immune system’ that defends them against viral (phage) attacks or nucleic acids that invade the cell. This defence is based on the so-called clustered, regularly inter-spaced, short palindromic repeats (CRISPRs) (Horvath and Barrangou 2020). These palindromic repeat genetic sequences are regularly separated by spacer regions. The number of repeat-spacer tandems is mostly around 50 units (but up to several hundreds); and more than one such complex locus (up to 18 different ones) can be present in any one bacterium. These different CRISPR-loci have highly variable and diversified spacer sequences, even when compared among closely related strains. Furthermore, the spacer regions show homology to phages/viruses and plasmids. The CRISPR-loci are defence elements that are targeted towards nucleic acids in a sequence-specific manner. For this purpose, the sequence of phages infecting a population is integrated as a spacer element into the bacterial CRISPR-loci. Later, the transcripts of these spacer elements is deployed by the bacterial cell, most likely, to interfere with phage conjugation and transformation, and for interfering with invading nucleic acids more generally (Horvath and Barrangou 2020). The range of possible target sequences is large because these loci are hypervariable. Viruses have evolutionarily countered this defence system by mutational escape strategies that can neutralize the CRISPR-system.

4.6.2  Defence in plants

Plants face the same challenge as any other organism and have evolved an effective immune system that can protect them against a variety of pathogens, such as viruses, bacteria, fungi, or nematodes. The characteristic immune defence is the hypersensitive response consisting of rapidly induced cell death at, and around, the site of infection. Sacrificing own cells helps to prevent the spread of the infection into neighbouring tissues; a strategy that is also followed by animals with the same modular architecture, such as corals. Although such apoptosis occurs in animals, too, plants can exploit this possibility much more efficiently due to their modular body architecture. To put it simply, the shedding of an infected leaf or a twig is not a problem for a plant, as it can easily be replaced without compromising vital functions, but shedding an infected liver is impossible for an animal.

The typical plant defence, the hypersensitive response, is triggered when the presence of an effector molecule from a pathogen (the ‘avirulence factor’, avr) leads to an interaction with a specific plant receptor protein encoded by the plant resistance genes. In only very few cases, however, has the actual molecular interaction been analysed (Holt III et al. 2003). These avirulence factors are pathogen products that might help the parasite extract resources or to penetrate the host cell walls. Typically, therefore, the presence of these products cannot be avoided or shielded by the pathogen. Recognition and activation of the signalling cascade leads to a range of responses, including an increase in ion fluxes, an oxidative burst, the production of nitric oxide and anti-microbial peptides, and cell apoptosis. Over twenty different plant resistance genes have been characterized that belong to five different classes (Cohn et al. 2001).

Plants have a large number of intra-cellular, cytoplasmic receptors that can trigger the hypersensitive response. The model plant species, *Arabidopsis thaliiana*, for example, has around 140 receptors, and rice (*Oryza*) has more than 500 families of NBS-LRR receptor proteins; not all of these are necessarily involved in defence (fish have hundreds of LRRs, too). The plant receptors typically do not directly recognize parasite molecules, such as pieces of peptidoglycan, but respond to something that is associated with the expression of pathogen-specific (avirulence) factors (indirect recognition; Holt III et al. 2003). Moreover, it appears that these parasite signals are not recognized themselves, but instead their effects on the plant, especially on the plant’s own signalling components that do represent the plant’s ‘self’. This explains why plants can respond to a large variety of pathogens with a limited number of variable receptors encoded by the germ line (Ausubel 2005).
4.6.3 Defence in invertebrates

4.6.3.1 Nematodes
Studies in the model organism, Caenorhabditis elegans, suggest that nematodes do not possess a cellular immune defence. At least three different pathways are involved in C. elegans immune defence: the TGF-β (transforming growth factor) pathway, the insulin/insulin-like growth factor pathway, and the p38 MAPK (mitogen activated protein kinases) pathway (Pradel and Ewbank 2004). C. elegans is able to recognize different kinds of pathogens and to respond accordingly. Recognition is through variable lectins, with variation encoded in the germ line; they are maintained as a polymorphism in the population (Nicholas and Hodgkin 2004; Du Pasquier 2005). In addition to these immune defences, C. elegans also shows elaborate avoidance behaviours guided by the recognition of bacterial products, and thus reduces the risk of infection in the first place (Schulenburg and Ewbank 2007).

4.6.3.2 Molluscs
In the molluscs, the snail Biomphalaria glabrata, the intermediate host of schistosomes (trematodes) that cause bilharzias in humans, has been studied in some detail. It is possible that fibrinogen-related highly polymorphic proteins (FREPs) play a role as variable receptors (Zhang et al. 2004).

4.6.3.3 Insects
Studies in Drosophila and the mosquito, Anopheles, have been helpful to understand insect immune systems. The insect immune system shows several characteristic pathways, notably the Toll-, Imd-, and JAK/STAT-pathways (cf. Figure 4.5b). Insects also have an astonishingly diverse range of different receptors that serve different purposes, from being involved in phagocytosis (e.g. Dscam, Eater), cytokine regulation (Toll, Domeless), and in directly recognizing microbes (PGRP, GNBP) (Ferrandon et al. 2007).

Contrary to earlier beliefs, insects (and likely many other invertebrates, too) can specifically respond to different infections. Furthermore, insects (and, so far, crustaceans) show the phenomenon of immune memory, that is, individuals challenged by a given parasite have better chances to deal with a second challenge of the same kind (Sadd and Schmid-Hempel 2006). The response can be very specific and can differentiate between two strains of the same bacterial parasite (Roth et al. 2008). It should be remembered that Drosophila and Anopheles are not paradigmatic for all insects. For example, honeybees (Apis mellifera) have only around one-third of the immune genes as compared to other insects (Evans et al. 2006), and aphids seem to lack an important pathway altogether (Gerardo et al. 2010).

A recurrent problem for the immune system is to detect parasites lodged inside the host cell. In mammals, for example, some of the TLRs recognize non-self that is outside the cell (e.g. TLR-4), while others (TLR-3) can sense non-self inside a cell. The latter are correspondingly located on endosomal membranes or are present in the cytosol (e.g. NOD-like receptors). In plants, a variety of R gene-encoded receptors respond to changes in cell properties. In insects, the sensing of intra-cellular parasites, such as viruses, seems to happen via several pathways (Box 4.1).

4.6.3.4 Sea urchins
Judging from the genome of the purple sea urchin (Strongylocentrotus purpuratus), these animals have a surprisingly complex immune system. For example, more than 220 TLR-genes have been identified (Hibino et al. 2006). The TLR-proteins consist of an extra-cellular LRR-domain, a trans-membrane region, and an intra-cellular Toll/IL1R (TIR)-signalling domain that elicits the cascade inside the cell. The LRR-domain is especially variable and the signature from the DNA sequences suggests a highly dynamic evolutionary turnover in these genes. Furthermore, a large set of over two hundred cytoplasmic-recognition proteins (NACHT domain and leucin-rich, NLR, proteins resembling plant LRRs) have been found, encoded in the germ line of sea urchins. A set of some 200 genes for scavenger receptor cystein-rich (SRCR) proteins can form more than 1000 SRCR domains by alternative splicing (Du Pasquier 2006; Hibino et al. 2006). The extraordinary richness of genes that code for different immune receptors and their close relatedness among each other seems to be the result of an evolutionarily recent expansion in the number of genes (Flajnik and du Pasquier 2008).
4.6.4 Early vertebrates

4.6.4.1 Cephalochordates
In the lancelet, *Amphioxus*, variable TLRs, lectins, NLRs, and proteins of the immunoglobulin superfamily, i.e. the variable region-containing chitin-binding proteins (VCBPs), have been found in sequencing projects. This variation seems to be maintained at the level of genetic polymorphism in the population, rather than by somatic modification. The involvement of these molecules in immunity has not yet been clarified.

4.6.4.2 Urochordates (tunicates)
With the genomic sequence and the identified genes, the tunicate, *Ciona intestinalis*, is a good milestone for the evolution of adaptive immunity. So far, no evidence for the presence of the MHC-complexes, T-cell receptors, the typical dimeric immunoglobulins of vertebrates, nor RAG-genes or the AID-enzyme, has been found in *Ciona*. On the other hand, there are many genes associated with innate immunity, such as genes coding for components of the complement, TLRs, and genes coding for elements of the intra-cellular signal transduction pathway; these genes are very diverse, compared to the vertebrates. However, there is evidence for molecules that have an extra-cellular C-type lectin or immunoglobulin (as a receptor), and intra-cellular motifs (ITAM, ITIM) that can both activate and inhibit signal transduction. Their general similarity to the vertebrates suggests an early evolution of elements that later might have become co-opted for the regulation of MHC-activity (Azumi et al. 2004). The *Ciona* genome also demonstrates that the evolution of the adaptive immune system happened at some point on the way from urochordates to the (jawless) vertebrates.

4.6.4.3 Jawless vertebrates
As was discussed before, somatically diversified lymphocytes as the basis for receptor diversity are the common theme, starting from the lampreys and hagfish (the jawless fish, agnatha), the cartilaginous fish (the chondrichthyes), and to the other higher vertebrates. The diversification is based on the somatic rearrangement of segments that code for the immunoglobulin domains in T- and B-cell receptors. While the rearrangement process in lamprey and hagfish occurs in a different way, for all the higher vertebrates (jawed vertebrates) the rearrangement is mediated by the recombination activating genes (RAG1, RAG2).

4.6.5 The jawed (higher) vertebrates
One of the most prominent features of the higher (jawed) vertebrates is the adaptive immune system, where diversity is generated by recombinatorial arrangement of recognition molecules mediated by the RAG-genes, as described above. In addition, the main carriers, the B- and T-cell lymphocyte populations of a single individual, undergo clonal expansion when stimulated by recognizing an antigen, or more generally, the corresponding epitopes. Any single, infecting parasite can have thousands of such epitopes and almost the entire surface of a parasite presents many overlapping molecular domains that might be recognized as epitopes.

The process of cell maturation and differentiation (haematopoiesis, cf. Figure 4.3) leads to variable receptors. As mentioned above, the B-lymphocytes of mammals develop from stem cells in the bone marrow and end up as immune cells circulating in the blood and in the lymphatic system. As cells are produced, a huge diversity of different binding specificities is generated. Some of those might react against own tissue. Hence, before they enter the defence repertoire, B-cells first undergo a process of negative selection against self-reactive variants by being exposed to self-molecules. If self-reactive, the cells either induce apoptosis and die, or they can become tolerant to self by further gene arrangements (receptor editing). Furthermore, some cell lines no longer express self-reactive antibodies on their surface (they become aneroid). These are processes that yield central tolerance. Self-reactive B-cells at the periphery can be silenced by apoptosis or become aneroid in the processes leading to peripheral tolerance. Mature cells secreted antibodies that can recognize parasite epitopes (Figure 4.10a).

As the cells of these different B-cell populations circulate in the host’s body, one or a few of the cells might also manage to directly bind to epitopes of an infecting parasite (Figure 4.10b). When B-cells bind epitopes and an interaction with T-helper cells occurs (see below),
Figure 4.10  Simplified scheme of defence against extra-cellular parasites by the vertebrate adaptive immune system. (a) Antibodies are secreted by B-cells and circulate to bind on epitopes of the parasite. ‘Naïve’ B-cells secrete ‘natural’ antibodies (IgM). Soluble IgM are pentameric molecules. (b) The same Ig (B-cell receptors) are also membrane-bound on B-cells and recognize the same epitopes. If a B-cell binds it is stimulated to secrete more antibodies. (c) B-cells can also become activated for clonal expansion. This depends on a parallel process, where, firstly, parasite-derived molecules (antigens) are recognized and internalized by antigen-presenting cells (APCs). Inside the APC, the antigens are degraded into pieces (peptides) that can be recognized and bind to MHC-class II proteins. The MHC-peptide complex is then presented on the surface to passing T-helper cells. If the T-helper cell has the matching specificity for the peptide, as determined by its TCR-receptor complex (cf. Figure 4.9), and the appropriate co-receptor to recognize the MHC-class II molecule (CD4 in this case), the T-helper cell binds to the APC. Meanwhile, the B-cell must also have encountered the same antigens that are similarly internalized and processed to peptides, and eventually presented with the MHC-class II complex on the B-cell surface. The same matching T-helper cell that is activated by the binding to the APC can, therefore, also bind to the B-cell. This yields a signal to the B-cell that becomes activated to proliferate for clonal expansion and affinity maturation. In the process, antibodies (IgA, IgG) binding more specifically to the inducing antigens are produced and secreted instead of IgMs. In the further course of the response, some B-cells might develop into memory cells that can be-reactivated more quickly and specifically on a secondary challenge by the same parasite. The binding of APCs and B-cells with T-cells thus leads to the formation of an ‘immunological synapse’ that contains many molecules and signals.
the B-cells are simulated to divide. As a result, clonal expansion of this particular B-cell population unfolds. Eventually, a large number of specifically binding cells is built up. Furthermore, B-cells that happen to bind more strongly (because their receptors match the epitopes better), divide more rapidly, and by somatic changes (hypermutation) the cell population fine-tunes its binding specificities further (affinity maturation). Affinity maturation thus leads to a rapid accumulation of highly specific B-cells and their antibodies, with affinities (i.e. the binding strength towards a single epitope) towards the stimulating antigen approximately 30,000 times higher than in the beginning. This change results from the substitution of amino acids with a consequent change in the shape of the binding pocket. The particular parasite epitopes that have triggered this process will thereby generate a response that is dominated by these few attracting epitopes: this phenomenon is called ‘immunodominance’, which is mediated by the T-helper cells (see below). Macroscopically, we can see that the immune system adapts to an infecting parasite by recruiting more and more cells that specifically target this infection. An important further consequence of this expansion is that some of these activated B-cells develop into memory cells that maintain the trace of the former infection and, upon exposure to the same parasite, can produce larger numbers of matching antibodies much faster than on the first encounter (Figure 4.10c).

T-cell receptors can bind to various targets, but primarily to small fragments (the peptides). One class, the T-helper cells, have the co-receptor CD4 on their surface (the CD4+ T-cells). As mentioned above (cf. Figure 4.9), both the actual T-cell receptor complex (TCR) and the co-receptor CD4 must be stimulated to activate the cell. T-helper cells engage with the antigen-presenting cells (APCs) that circulate in the blood and lymphatic system. APCs can take up parasite-derived fragments, process them into suitable peptides, and present them with the help of the major histocompatibility complex (MHC) on their surface, specifically through the MHC class II for extra-cellular parasites (Figure 4.10c). Passing T-helper cells with the corresponding specificity, bind to the presented MHC-peptide complex and so become activated. The activated T-helper cells subsequently bind to the receptors of a B-cell with the matching specificity and so activate the B-cell. For this, the B-cell must also independently have bound to circulating antigens, e.g. a suitable protein fragment. These become internalized in the B-cell and are then digested to peptides. The peptides become entangled with MHC class II molecules of the B-cell and are subsequently presented on the cell surface (Figure 4.10c). If an activated passing T-helper cell with the same specificity recognizes these peptides, it binds to the complex and sends a signal to the B-cell. This leads to B-cell stimulation and cell division, that is, to clonal expansion of this specific B-cell line. Hence, the expansion of a given B-cell clone requires binding of the B-cells to (an epitope) of an antigen, the processing of this protein and subsequent presentation of the MHC-peptide complex on the surface, and the presence of a T-helper cell that was activated (via APCs) by the same peptides and so provides the additional signal for stimulation. B-cell activation happens in specialized organs, that is, in the lymph nodes of mammals, to where the B-cells with their load migrate and find a matching T-helper cell. Lymph nodes have a high density of T-cells and so the frequency of contacts is much higher than if the B-cell had to find its T-helper cell somewhere else in the body.

The CD4+ T-helper cells can furthermore differentiate into various subsets, notably the T_\text{H}1 and T_\text{H}2-cells (Coico and Sunshine 2009). This difference is important as one subset inhibits the development of the other set. Differentiation is affected by cytokines that are released mainly from the dendritic cells, mast cells, and NK-cells. T_\text{H}1-cells require the cytokine IL-12 to develop, which is typically generated in response to bacterial and viral infections. T_\text{H}1-cells subsequently release the key cytokine, IFN-\gamma, which inhibits T_\text{H}2-cells but also activates NK-cells and macrophages. T_\text{H}2-cells, by contrast, are stimulated by the cytokine IL-4. This cytokine is produced in response to parasitic worm infections. They subsequently produce IL-4 and IL-5 that stimulates B-cell growth, promotes IgE and IgG synthesis, but suppresses T_\text{H}1-cells. Because of the mutual suppression of these T-cell populations, their differential regulation in response to different infections has been implicated in various
autoimmune diseases, notably the development of asthma (Coico and Sunshine 2009).

In contrast to B-cells and antibodies that patrol the exterior environment in the blood and lymphatic system, the T-cells also fight parasites inside host cells (Figure 4.11). Inside the infected host cell the proteins of an invading parasite are processed and cut into small peptides by the host cell’s own proteases. These proteases are part of the proteasome, large intra-cellular protein complexes that degrade damaged own proteins, or foreign proteins (such as antigens) to fragments (the peptides). The transporter of antigen presentation (TAP) moves these peptides from the cytosol to the endoplasmic reticulum. Then, molecules encoded by MHC class I genes attach to these peptides. The resulting MHC-peptide complex is subsequently transported to the cell surface, where it is presented to passing T-cells that have the CD8 co-receptor (CD8⁺ T-cells). This binding activates the T-cell and transforms it to become a ‘cytotoxic T-cell’ that starts to destroy the host cell that has signalled as being infected. Hence, CD8⁺ T-cells recognize infected cells by the presentation of a MHC-peptide complex that signals non-self.

In humans, there are around $10^{11}$ T-cells that are patrolling the body. These T-cells develop and mature in the thymus. The T-cell receptors (TCRs) vary in what they recognize and can bind to, such that different clonal populations of T-cells differ in their binding specificities. The developing T-cell clones are ‘screened’ and those that react too strongly with self are eliminated, aided by a critical enzyme (AIRE; Mathis and Benoist 2007). Those that do not self-react will thus be selected. Out of the entire range of T-cells that are generated in the thymus, only a minor fraction (estimates are less than 1%) are allowed to mature and become part of the patrolling immune cell population (Müller and Bonhoeffer 2003). Like in the case of the B-cells, this leads to central tolerance, which is dominated by elimination of unwanted cells early in development. Self-reactive T-cells that nevertheless make it to the periphery will subsequently become inactivated in a process of ‘peripheral tolerance’. Similar to B-cells, the host retains a memory of an earlier encounter and, when a second exposure to the same epitopes occurs, the matching T-cells can multiply more rapidly.

**Figure 4.11** Simplified scheme of defence against intra-cellular parasites (such as a virus) by the vertebrate adaptive immune system. The proteasome (large intra-cellular protein complexes) degrades the proteins derived from a parasite. The resulting peptides are transported to the endoplasmic reticulum by TAP (transporter of antigen presentation), where they are recognized and bind to MHC-class I proteins. The MHC-peptide complex is then presented on the cell surface where it is recognized by a passing T-cell with a matching TCR (cf. Figure 4.9) and co-receptor (CD8 in this case). If binding occurs, the T-cell becomes activated and transforms into a cytotoxic T-cell (CTL) that starts to destroy the (infected) host cell.
The interaction with MHC class I molecules is achieved by short peptide motifs that are around 8–10 amino acids long, and with MHC class II molecules through motifs of perhaps 13–17 amino acids (Janeway et al. 2001). Although these lengths are not easy to estimate, they generally seem to be surprisingly short. Yet, even with these lengths and given that there are 20 different amino acids, an enormous number of combinations are possible: with 8 amino acids we have $20^8 = 25.6 \times 10^9$ different peptide sequences, and with 17 amino acids we get $20^{17} = 1.3 \times 10^{22}$ different sequences, a combinatorial space that covers almost all realistically possible peptide variants (Burroughs et al. 2004).

Looking at the genetics, in humans, the MHC class I molecules are encoded by only three, tightly linked genetic loci (but two loci in mouse, one in chicken, eleven in the axolotl). In a heterozygous human individual, there are thus only six alleles that code for all the variation in the MHC class I molecule, or $2^3 = 8$ different genotypes (MHC diversity is entirely coded in the germ line, with no somatically generated variation). Therefore, each single MHC I molecule must be able to present a large number of different peptide sequences in order to be effective. In the host population as a whole, an extensive polymorphism of the MHC I alleles is maintained. In human populations, for example, the estimate is around 180, 350, and 90 alleles each for the first, second, and third MHC class I-locus (Marsh et al. 2000). Similar constraints apply to the MHC class II molecules, although their binding is more flexible. Estimates are around 250, 50, and 90 alleles for the corresponding three loci.

### 4.7 Evolution of the immune system

#### 4.7.1 Recognition of non-self

During the evolution of organismic diversity, the mechanisms of allore cognition, to distinguish self from non-self, have diversified. A fundamental process of allore cognition is intolerance to foreign tissue, e.g. the rejection of grafts. This distinction is already present in very early metazoa, such as sponges. Colonies of these organisms often compete for space on the substrate. When they come in contact with one another, allore cognition takes place that leads to destruction of foreign tissue. This histocompatibility (‘tissue compatibility’) response is not based on the same MHC-system as in vertebrates, however (Flajnik and du Pasquier 2008). Nevertheless, sponges and cnidaria already possess a complex histocompatibility system with polymorphic genes, perhaps based on only one locus in sponges, but on a larger genetic region in cnidaria (Flajnik and du Pasquier 2008). In the cnidarian, *Hydractinia symbiolongicarpus*, the genetic complex responsible for allore cognition was identified. A key gene sequence codes for a trans-membrane receptor that is highly polymorphic; the molecule has three extra-cellular domains that are similar in sequence to IgSF domain (Nicotra et al. 2009).

Among the early chordates, the bryozoans and tunicates show allore cognition, with partial tolerance for kin-related tissue as in *Celleporella hyaline* (Hughes et al. 2004b). Fusion or rejection of foreign tissue has been studied intensively in *Botryllus schlosseri*. This depends primarily on a highly polymorphic locus (FuHC-locus; fusion/histocompatibility locus) that has several hundred alleles (Flajnik and du Pasquier 2008). Allograft rejection was also the original observation that led to the discovery of the vertebrate MHC-mechanism, notably from allografts given to pilots suffering from severe burns during the Second World War. Despite the widespread occurrence, it is not clear what caused the early evolution of intolerance to foreign tissues. Several factors might have been important, either alone or in combination. For example, the rejection of foreign tissue provides advantages in competition for space and resources, as in sponges or bryozoa. Furthermore, it prevents inbreeding as in the rejection of own pollen in plants. Allore cognition and rejection also prevents parasitism, of course, and this might indeed have been the major original function.

#### 4.7.2 The evolution of adaptive immunity

The adaptive arm of the immune system of vertebrates is characterized by lymphocytes, specialized lymphoid tissues, antigen receptors, MHC, and by processes such as somatic recombinatorial rearrangement of genetic information, somatic hypermutation, and gene conversion.
So, when did these components appear in evolutionary history? Unfortunately, cells, molecules, and immunological mechanisms do not fossilize as readily as do skulls or bones. But the evolutionary events have left traces in the genome and in the organization of immune systems of organisms living today. The evidence for the evolutionary steps in the immune system, therefore, comes from comparative immunology that has made enormous progress with the advance of genome sequencing projects (Flajnik and du Pasquier 2008) (Figure 4.12).

Based on comparative studies in jawless fish (extant lampreys and hagfish), the VLR-receptors are highly diversified. As mentioned above, this diversity is based on mechanisms such as somatic hypermutation and gene conversion, where the AID-enzyme plays a key role (Schatz 2007; Guo et al. 2009). Later, the involvement of recombination-activating genes (RAGs), together with the presence of other enzymes in jawed vertebrates, has led to an almost unlimited variation in the receptors from a small number of genes in the germ line. It is now accepted that RAG-genes came into the evolutionary line leading towards extant species by horizontal transfer and transposition from bacteria (Thompson 1995). Given that sea urchins have RAG-genes too, but which are not involved in immunity, this transfer seems to have happened much earlier than the origin of the modern adaptive immune system (Flajnik and du Pasquier 2008). Note that the activity of RAG-genes induces somatic rearrangements that not only are beneficial, but could also become dangerous to self. Hence, any incorporation of horizontally transferred RAGs must have prompted the evolution of other processes that helped to control the negative effects of generating undesirable new cell specificities.

The first adaptive immune system based on RAG-activities appears in the early sharks, some 450 million years ago (Figure 4.12). Virtually all elements were in place at that time, such that the step towards adaptive immunity has also become known as the ‘immunological big-bang’. However, closer scrutiny suggests that some elements were already present for considerably longer and might have performed functions not related to immunity, such as the RAG-genes in sea urchins. Other elements had evolved gradually before and were then newly recruited for the immune function, e.g. DNA-polymerases (Flajnik and du Pasquier 2008). Hence, rather than a big-bang, the elements of the machinery seem to have been stitched together from existing mechanisms and molecules, although this happened rather fast on the evolutionary scale. The same picture arises for the other key process of adaptive immunity, somatic hypermutation. This machinery was also present before the RAG-process emerged, such that mutation and gene conversion provided the original means of somatic diversification before the even more powerful recombinatorial rearrangement evolved. This event affected the already existing Ig-superfamily (IgSF) genes. The precise nature of this ancestral IgSF-set is not clear but putative genes could be related to ancestral forms found in early chordates, such as the sea squirt, Ciona (Urochordata) (Flajnik and Du Pasquier 2004; Flajnik and du Pasquier 2008). With the amphibians, the process of Ig class-switching evolved, allowing for a further fine-tuning of the response in different tissues and various infections. Specialized centres for B-cell maturation finally evolved in mammals (Flajnik and Du Pasquier 2004).

The MHC is another important element of adaptive immunity. A single-copy MHC gene class III-region is found in the lancelet (Cephalochordata), whereas all higher animals have multi-copy MHC-regions. MHC class I and class II molecules are only present in the jawed vertebrates, with the latter perhaps preceding the former in the course of evolution. The early MHC presumably contained important genes of the innate immune defences that only functioned properly in this highly organized form. A putative gene duplication event may have relaxed this selection pressure for the new group and led to it being coerced for the adaptive arm of the immune defences subsequently (Flajnik and du Pasquier 2008). The C1-domain found in proteins of the Ig-superfamily is found in antigen receptors of lymphocytes, as well as in the MHC class I and class II molecules. This domain apparently evolved in the early jawed vertebrates, together with the advent of the full adaptive immune system; a characteristic is its interaction with co-receptors, which is so important to activate T-cells. Similarly, the variable (V)-domains are of interest, since they accomplish recognition of an antigen by forming appropriate folds (Figure 4.9). Protein domains yielding a typical such
V-fold is found in all metazoa (from sponges to vertebrates), some involved in immune functions but many others with functions unrelated to immune defences.

These patterns reinforce the general picture that the evolution towards a full system of adaptive immunity involved pre-existing molecules and mechanisms with the right properties to be recruited for new functions. Alongside, new elements were added, such as when RAG-genes now targeted new molecules that lend themselves to generate new recombinatorial rearrangements.
Hosts defend themselves before infection by changes in behaviour, in diet, habitat choice, body walls, and so forth. Post-infection defences can also include changes in behaviour or life history. But immune responses are a major defence system of all organisms.

Immune systems have a humoral and cellular arm, and an innate and adaptive arm. Signalling cascades ensure that a recognized infection eventually leads to a suitable response, such as the production of anti-microbial peptides. Proteolytic cascades, such as complement, are another prominent feature of immune defences.

Immune systems are based on a limited number of protein families, such as the immunoglobulin superfamily (IgSF), leucine-reach repeats (LRRs), lectins, variable domain chitin-binding proteins, or anti-microbial peptides. Molecules within these families are divergent among organisms and sometimes have diversified very fast.

A high diversity of molecules that recognize infections is essential in view of the large number of different parasites. A limited amount of diversity is encoded in the germ line as genetic polymorphism. However, there are a range of processes that generate diversity somatically, such as gene conversion, rearrangement, somatic hypermutation, alternative splicing, RNA-editing, copy choice, and so forth. Different groups of organisms use different such mechanisms. Jawless vertebrates also have an adaptive immune system but based on different mechanisms of generating receptor diversity.

Immunoglobulins of B-cells are important as secreted antibodies and membrane-bound receptors and consist of characteristic heavy and light peptide chains. The variable part of the molecule determines the binding specificity; the constant part determines the class of the Ig-molecule. Variability in the specificity is generated by a number of processes, notably RAG-dependent rearrangement and somatic hypermutation. During B-cell affinity maturation, specificity and class can be fine-tuned towards the actual infection and the tissue where the infection takes place. T-cell receptors are always membrane-bound. Variability in T-cell specificity is generated by similar processes. B- and T-cells cooperate to stimulate clonal expansion of appropriate cell lines, or to destroy infected cells.

A major task of immune defence is to distinguish self from non-self. Among all organisms, the respective defence systems show divergence, as well as similarities, but the basic capacities have evolved very early in the history of life. Members of protein families are sometimes highly diversified, sometimes coerced for other functions, or the same function can be ensured by different mechanisms and molecules. The adaptive immune system evolved somewhere between the urochordates (tunicates) and the jawless vertebrates.