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General Summary

There is a great need to develop novel strategies for parasite control because of the failures of current eradication approaches and the logistical difficulties of implementing them. An interesting aspect of parasitic diseases is that the vector arthropods that transmit the pathogens can mount immune responses against the infection which will kill a large percentage of the parasites. Our group is pursuing research in 4 areas: 1) modification of mosquito vectorial capacity, 2) vector-parasite interactions, 3) immune responses to helminth infection, and 4) the genomics of protozoan parasites.

Research Activities

Early immune responses to chronic infection with a gastrointestinal nematode in mice
Human gastrointestinal (GI) parasitosis is often chronic. However, most murine models of GI parasitic infection terminate after about 2 weeks. In contrast, infection with *Heligmosomoides polygyrus*, a murine GI nematode, persists for about 8 weeks and can serve as a model of chronic infection. To understand the mechanism of chronic infection, early immune responses were examined with flow cytometry and compared with those of acute infection with *Nippostrongylus brasiliensis*. Conventional dendritic cells (DC; $CD11c^{+}B220^{-}$) were analyzed with C-C chemokine receptor type 7 (CCR7), an essential molecule for lymph node recruitment, and MHC class II, an antigen-presenting molecule. Cells positive for CCR7 showed intermediate expression of CD11c and highest expression of class II (DC1: $CCR7^{high}CD11c^{int}$class II^{high}). These DC1 cells showed markedly reduced class II expression from day 4 after *H. polygyrus* infection. On day 8 after *H. polygyrus* infection, the number of CCR7-positive cells, including DC1 cells, was prominently decreased. In the case of *N. brasiliensis* infection, class II expression was reduced on day 5 after infection. Although the number of DC1 cells was decreased on day 8 after *N. brasiliensis* infection, macrophages and newly recruited eosinophils, both of which were CCR7-positive, expressed the same level of class II as did DCs. Concerning $CD4^{+}$ T cells, expression of programmed cell death protein 1, which signals down-regulation, was up-regulated from day 2 after *H. polygyrus* infection. Expression of this protein was slightly up-regulated on day 8 after *N. brasiliensis* infection. These results suggest that reduced ability of antigen presentation and up-regulation of suppressive signals on T cells caused by the parasite are associated with the establishment of chronic GI nematode infections.

Transcriptome analysis of Entamoeba using an ultrafast sequencer
We have been performing transcriptome analysis of *Entamoeba histolytica* and *Ent-*

amoeba invadens, which are parasitic amoebas of humans and reptiles, respectively. Using the oligo-capping method, which guarantees that intact transcripts from the complementary (c) DNA library reach the transcription start site (TSS), we first obtained full-length cDNA sequences. Then, by TSS sequencing analysis, which takes advantage of the oligo-capping method and next-generation sequencing technology, we have determined a massive amount of short (i.e., 36 nt) sequence tags beginning with the TSS. Even though the 5′ untranslated regions were extremely short, the TSSs were not unique to each gene but were clustered. These clusters were distributed unimodally, bimodally, and multimodally, but none showed a flat distribution. We arranged the TSS clusters in order of kurtosis and found that kurtoses of TSS clusters were higher in genes whose TSS tag numbers were larger, i.e., more highly transcribed.

A procedure for permanently stained preparations of cysts of Giardia in stool samples fixed with formalin

Permanently stained preparations of *Giardia* cysts in formalin-fixed stool samples could not be made because: 1) the formalin-fixed stool cannot be smeared on glass; and 2) the formalin-fixed cysts were not stained with solutions used for permanent staining. The first problem was resolved with BD SurePath™ (Becton Dickinson, Franklin Lakes, NJ, USA), a recently developed liquid-based cytology system in which negatively charged cells become attached to positively charged precoated glass slides. Even though the cysts in formalin are not attached to the precoated glass slides, the cysts in water, after being washed with centrifugation in water, are attached. To solve the second problem we applied Kohn's staining with some modifications. Kohn's stain contains a black dye (chlorazol black E) and an alcohol-based fixative called the basic solution. After the basic solution was diluted with water (serially each 10%), the smears were immersed in diluted basic solutions of increasing concentration for more than 10 minutes and then stained with Kohn's stain. As a result, permanently stained preparations were made from formalin-fixed samples: *Giardia* cysts were attached without shrinkage of the cytoplasm, and such structures as the cyst wall, nucleus, karyosome, and flagella were clearly stained.

Molecular dissection of parasitophorous vacuole membrane in malaria liver stage parasites

Malaria is a major global health burden resulting in approximately 1 million deaths each year. The protozoan parasite *Plasmodium* is the causative agent of malaria. This situation has led to attempts to develop novel control and intervention strategies, such as a malaria vaccine. However, these efforts have met with limited success because of the antigenic complexity of the parasite and the differential expression of proteins. Therefore, the next generation of malaria vaccines must include a wide range of activation for the immune response. To clarify the molecular interplay between malaria parasites and the host for future vaccine development, we have focused on malaria liver stage development, especially the parasitophorous vacuole membrane, which is the interface membrane between the parasite and host. Loss-of-function analyses have revealed that protein B9 is specifically expressed in *Plasmodium* liver stages and is important for parasite develop-

ment in the liver. In both rodents and humans *Plasmodium* we show that while *b9* is readily transcribed in sporozoites, B9 protein is present after hepatocyte invasion. Finally we show that B9 is localized on the parasite plasma membrane where it plays a role in establishing and maintaining the parasitophorous vacuole membrane that surrounds the parasite inside the liver cell. On the basis of the structural analyses of the B9 4-cysteine domain and the presence of this domain in several previously identified 6-Cys proteins, we propose that the presence of the 4 positionally conserved cysteine residues are diagnostic for this domain and that B9 belongs to the family of 6-Cys-related proteins. These findings reveal a novel parasite molecular mechanism and suggest malaria liver stage parasites can survive inside host cells.

Antipathogen responses and structural homeostasis maintain midgut wall of malaria vector mosquito

The midgut of disease vectors is the primary and most important physical and immune barrier against pathogens. To clarify the conformation of midgut cells and the function of each cell type, which had been poorly understood, the barrier function of the midgut of mosquitoes (*Anopheles stephensi*) infected with a rodent malaria parasite (*Plasmoidum berghei*) was analyzed with confocal microscopy. When parasites were crossing midgut epithelial cells, the cells invaded by parasites were extruded from the midgut wall, indicating active exclusion of cells damaged by parasites. Meanwhile, in a number of midgut cells containing parasites, phosphorylation of c-Jun N-terminal kinase (JNK), a component of the stress response pathway, was enhanced and showed capsular structures strongly stained with active JNK antibody. This finding suggests a mechanism excluding invaded and damaged cells through the activation of JNK and the encapsulation of pathogens from the midgut wall. Furthermore, midgut stem cells, which undergo mitosis and differentiation, were identified with a 5-ethynyl-2′-deoxyuridine DNA synthesis marker. A number of these midgut stem cells entered mitosis during the 24 hours after blood sucking by the mosquito. Our findings suggest that structural homeostasis mechanisms, such as the exclusion of invaded cells and the division of stem cells, maintain the mosquito midgut wall after pathogen invasion.

Genetic dissection of intermediate host and tapeworm interaction

The dwarf tapeworm, *Hymenolepis nana*, which belongs to the order *Cyclophyllidea*, is the most common cestode of humans. Its intermediate hosts are arthropods, in particular, beetles. Once the intermediate host ingests tapeworm eggs, oncospheres immediately hatch and pass through insect gut wall. Cysticercoids develop within the hemocoel, where they survive without loss of infectivity until the intermediate host is ingested by a definitive host. To examine the interaction between the tapeworm and the intermediate host, we employed a reverse genetic approach with the red flour beetle, *Tribolium castaneum*, in which a robust systemic RNA interference (RNAi) response is observed, as a model system to explore host responses to tapeworm infection. Adult knock-down phenotypes in *T. castaneum* were induced by injection of double-stranded RNA (dsRNA) into late instar larvae. We performed RNAi screening targeting several gene transcripts of the Toll and the immune deficiency pathways, which are major signaling pathways of

the humoral immune response in insects. Reduction of Toll pathway function, which was induced by RNAi-mediated silencing of *MyD88*, *Dif1*, and *Dif2*, in addition to JAK/STAT and JNK components, increased the burden of cysticercoids. On the other hand, RNAi-mediated knockdown of immune deficiency pathway components, *dredd* and *imd*, had no significant effect on the cysticercoid load. Our findings suggest a pivotal role of specific pathways, such as the Toll signaling pathway, in regulating resistance to tapeworm infection.

Publications

Saiki E, Nagao K, Aonuma H, Fukumoto S, Xuan X, Bannai M, Kanuka H. Multivariable analysis of host amino acids in plasma and liver during infection of malaria parasite Plasmodium yoelii. *Malar J.* 2013; **12:** 19.

Bando H, Okado K, Guelbeogo WM, Badolo A, Aonuma H, Nelson B, Fukumoto S, Xuan X, Sagnon N, Kanuka H. Intra-specific diversity of Serratia marcescens in Anopheles mosquito midgut defines Plasmodium transmission capacity. *Sci Rep.* 2013; **3:** 1641.

Nei Y, Obata-Ninomiya K, Tsutsui H, Ishiwata K, Miyasaka M, Matsumoto K, Nakae S, Kanuka H, Inase N, Karasuyama H. GATA-1 regulates the generation and function of basophils. *Proc Natl Acad Sci U S A.* 2013; **110:** 18620-5.

Takeishi A, Kuranaga E, Tonoki A, Misaki K, Yonemura S, Kanuka H, Miura M. Homeostatic epithelial renewal in the gut is required for dampening a fatal systemic wound response in Drosophila. *Cell Rep.* 2013; **3:** 919-30.

Obata-Ninomiya K, Ishiwata K, Tsutsui H, Nei Y, Yoshikawa S, Kawano Y, Minegishi Y, Ohta N, Watanabe N, Kanuka H, Karasuyama H. The skin is an important bulwark of acquired immunity against intestinal helminths. *J Exp Med.* 2013; **210:** 2583-95.

Aonuma H, Badolo A, Okado K, Kanuka H. Detection of mutation by allele-specific loop-mediated isothermal amplification (AS-LAMP). *Methods Mol Biol.* 2013; **1039**: 121-7.