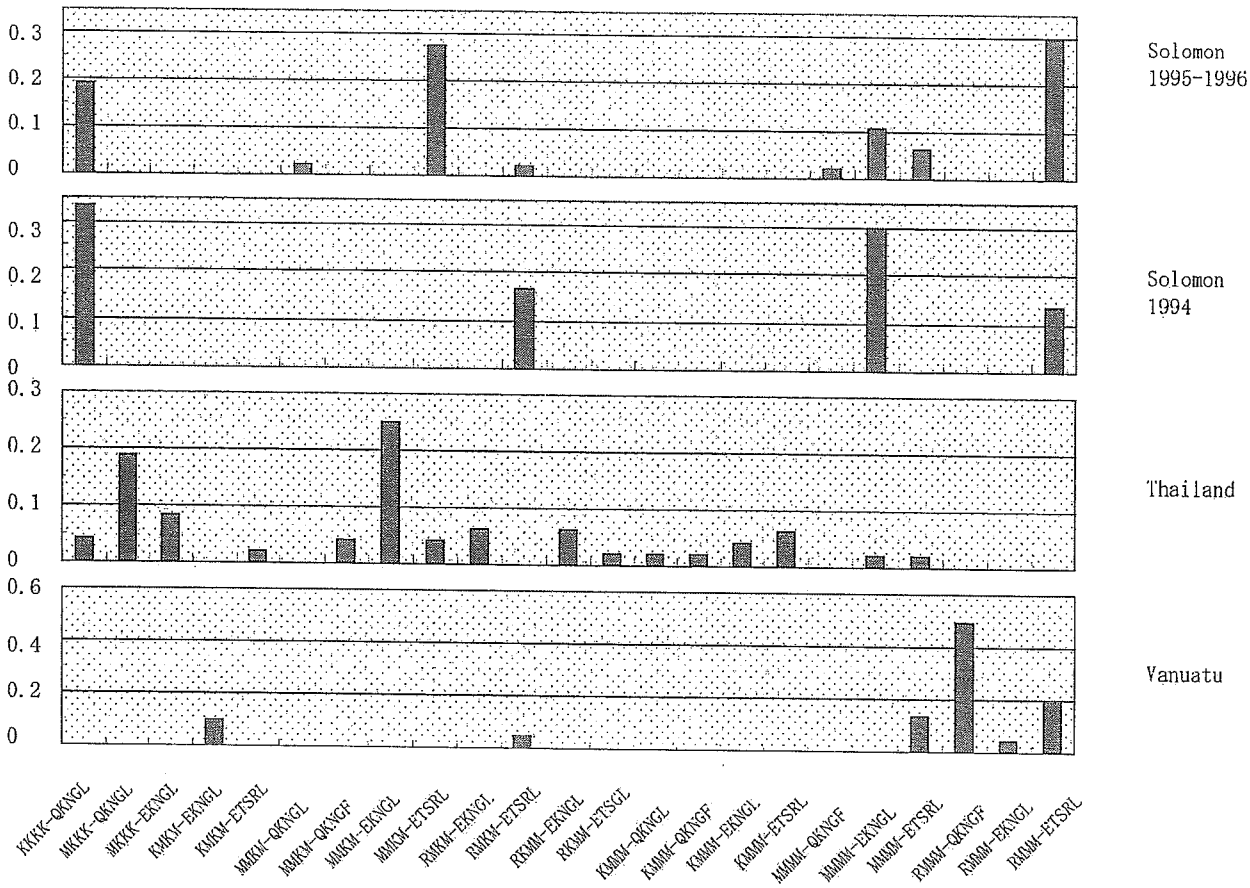


FIGURE 3. Frequency distribution of merozoite surface protein 1 (*msp1*) gene haplotypes in *Plasmodium falciparum* isolates from the Solomon Islands, Thailand, and Vanuatu. The *msp1* haplotypes, which are unique associations of 5′ recombinant types and 3′ sequence types, are indicated along the x-axis.

areas in Africa such as Tanzania.[36] Thus, the question arises as to why linkage disequilibrium is strong in a high-transmission area. We have previously proposed that frequency of transmission is not the sole determinant of the strength of linkage disequilibrium in *msp1*, and that other factors are also intimately involved.[19] We suggest that at least three variables are associated with linkage disequilibrium in *msp1*: 1) the number of alleles prevalent in a local area, 2) the rate of multiple allele infections, and 3) the number of alleles per infection (multiplicity). Effects of those variables were weak or relatively limited in the present samples from Guadalcanal, compared with the samples from Thailand. This suggests a low frequency of out-crossing, resulting in the observed linkage disequilibrium in Guadalcanal. In addition to the three variables, limited gametocyte production among the asexual parasite populations may be added: infections in Guadalcanal are in some way synchronized, so that gametocyte production at any one time may be limited to one of the asexual clones present, whereas in Thailand this is not the case.

Since epidemiologic settings vary substantially between and within geographic areas, a direct comparison of multiplicity of infection must be cautious. Multiplicity tends to decrease with age in areas highly endemic for malaria and it is lower in asymptomatic carriers than in individuals with clinical malaria.[5,37] Age-dependent acquisition of strain-specific immunity, which is mounted after repeated infections of different genotypes, is considered to contribute to the reduction of multiplicity. In contrast, in low-transmission areas multiplicity does not always correlate with age or the presence of clinical malaria. In the present study, isolates were collected from both symptomatic patients and asymptomatic carriers. We were unable to find a relative reduction of multiplicity of *msp1* 5′ recombinant types in asymptomatic partially-immune adults in rural areas (areas B and C) in Guadalcanal because our samples from rural villages were limited in number and had biased distribution among ages (primarily school children) and parasite density. We therefore do not consider that our results exclude the presence of age-dependent reduction of multiplicity in asymptomatic semi-immune carriers in the Solomon Islands. Nevertheless, multiplicity in area A (Honiara City) was significantly lower than in areas B and C. Acquired strain-specific immunity might not be so intense to significantly reduce multiplicity in areas B and C. Low multiplicity in area A, compared with areas B and C, may simply be due to limited mosquito biting or relatively easy accessibility to anti-malarial drugs. Our Thai samples were from clinical cases, and thus are comparable with hospital samples (area A) in Guadalcanal. Multiplicity in Thailand was significantly higher than in Guadalcanal, despite a lower transmission level in Thailand. Samples from Vanuatu were from asymptomatic carriers and thus comparable to those from

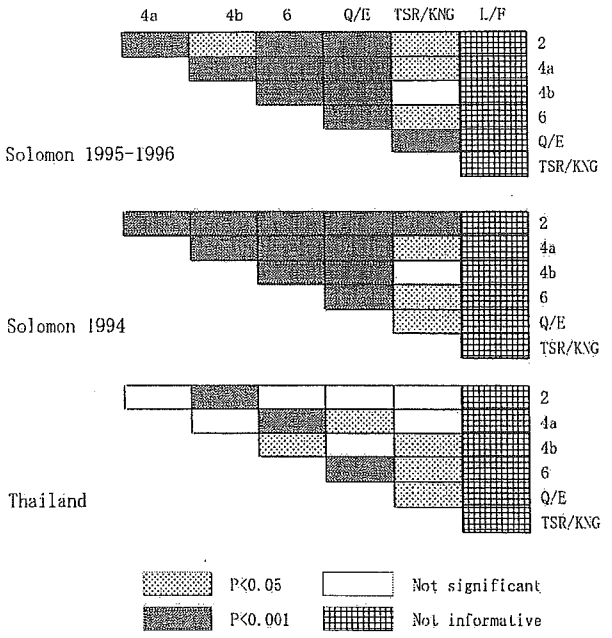


FIGURE 4. Linkage disequilibrium in the *Plasmodium falciparum* merozoite surface protein 1 gene in populations from the Solomon Islands (area A = 1995–1996; area B = 1994) and Thailand. Pairs of polymorphic blocks 2, 4a, 4b, and 6, and three polymorphic residue sites (Q/E, TSR/KNG, and L/F) in block 17 were subjected to the $R^2$ test.

rural villages in Guadalcanal (areas B and C). Multiplicity in Vanuatu was nearly 1.0 and significantly lower than in Guadalcanal, where transmission is more stable and higher than in Vanuatu. These results indicate that the level of multiplicity in the Solomon Islands was intermediate between Thailand and Vanuatu, reinforcing the idea that multiplicity is not always associated with the intensity of malaria transmission.

The present study also showed temporal variation in the distribution of *msp1* haplotypes in area B in Guadalcanal between the 1994 samples and the 1995–1996 samples. The present finding that all isolates from the two populations have the chloroquine-resistant *pfcrt* alleles indicates that a population change due to chloroquine pressure is not responsible for this temporal fluctuation. A possible explanation for this fluctuation is that the area B 1994 samples and 1995–1996 samples were from different villages. However, for both the 1994 samples and 1995–1996 samples, we found no difference in distribution of *msp1* haplotypes among villages. Also, within a single village (Tadhimboko in area B), the distribution differed significantly between 1994 samples and 1995–1996 samples. Thus, there was a temporal change in distribution within area B. A previous study has shown considerable temporal variation in distribution of *msp1* 5′ recombinant types in hypoendemic areas in Brazil.[38] The temporal variation in Brazil may be due to migration of laborers. In addition to such migration, strain-specific immunity to certain *msp1* haplotypes may be involved in the temporal variation presently observed in the distribution in the Solomon Islands. Studies of *P. falciparum* populations from Irian Jaya suggest that strain-specific immunity is a factor in temporal variation in frequency of *msp2* alleles.[39]

In the present study, Guadalcanal *P. falciparum* populations did not exhibit seasonal change in the distribution of *msp1* 5′ recombinant types, the rate of multiple infections, or multiplicity of infections. A clear seasonal variation in the diversity of antigen genes and multiplicity has been observed in low-transmission areas such as Sudan,[40] where transmission ceases nearly completely during the dry season. In the Solomon Islands, transmission of malaria is perennial, although it decreases during the dry season. Therefore, a direct comparison of seasonal changes in transmission cannot be made between the Solomon Islands and Sudan. In a perennial transmission area in Benin, reduced transmission had no substantial influence on the diversity of *msp2* alleles or multiplicity of infections.[41] Thus, seasonal changes in malaria transmission do not always affect the diversity of *msp1* alleles and multiplicity of infections in area with relatively high transmission.

The present study is the first to document the prevalence of particular *pfcrt* alleles in the Solomon Islands. *Plasmodium falciparum* populations in northern Guadalcanal exhibited monomorphic prevalence of a Papua New Guinea type of chloroquine-resistant *pfcrt* in both 1994 samples and 1995–1996 samples. Chloroquine is the mainstay for treatment of malaria and is effective against *P. vivax* malaria in the Solomon Islands[24] and Vanuatu.[42] Thus, the persistence of chloroquine pressure may be the cause of the monomorphic prevalence of chloroquine-resistant *pfcrt* in the southwestern Pacific.

In conclusion, the present study presents evidence that allelic diversity of *P. falciparum msp1* as measured by *msp1* haplotypes is not entirely dependent on the intensity of transmission. Populations from the Solomon Islands had significantly lower diversity compared with that from Thailand with a lower level of transmission. Linkage disequilibrium in *msp1* was also significantly higher in the Solomon Islands compared with Thailand. These findings indicate that frequency of recombination events in *msp1* is determined not only by transmission intensity but also by the number of *msp1* alleles prevalent in an area and multiplicity of infections.

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Review

# MALARIA ENDEMIC PATTERNS ON LOMBOK AND SUMBAWA ISLANDS, INDONESIA

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**ABSTRACT:** Nusa Tengara Barat (NTB) province consists of two main islands, Lombok and Sumbawa, to the east of Bali Island, Indonesia. Most of the area is known to be moderately malaria endemic, but the exact malaria epidemiology has not been elucidated. At least 30 deaths per year are thought to be caused by falciparum malaria in Lombok alone, judging from the hospital data. According to the Gebrak Malaria Team in West Lombok, the annual incidence in the district of West Lombok from 1996 to 1999 was consistently over 40‰.

In the present report, we describe the small malaria endemic foci in the West Lombok and Sumbawa districts. Falciparum malaria is predominant over vivax malaria and other types of malaria. There are 11 species of *Anopheles* vector, but three of these species, *An. subpictus*, *An. maculates* and *An. barbirostris*, are of primary importance in malaria transmission and *An. sundaicus* and *An. aconitus* are of secondary importance. Our data from Sekotong, West Lombok, and Sumbawa supported the importance of *An. subpictus* in coastal areas but suggested the existence of different transmission peaks according to environmental conditions. The usual transmission peak comes in the dry season but is affected by climatic and geographical conditions. Although there were many malaria endemic foci along the coast, the width and grade of the foci varied widely. The presence of malaria endemic foci inland, although likely, has not been definitively reported to date.

## INTRODUCTION

Indonesia is known as a country where tourists are at a high risk for malaria infection. But the incidence of malaria varies widely among different islands and even among different areas of the same island. It is important to obtain exact information on the epidemiological conditions of malaria on each island. In this report, we describe the epidemiology of malaria in parts of Lombok and Sumbawa islands on the basis of our experience and local data (Fig.1).

### 1) Malaria situation in Indonesia;

Malaria is still a major public health problem in Indonesia. In 1995, the National Health Household Survey estimated that around 32,000 deaths were caused by malaria. [1]. Indonesia is a large archipelago consisting of 12,508 islands of various sizes and shapes located along the equator and had a total population of 209 million in 1999. About 70% of the population live in Java and Bali, where malaria has been mostly eradicated, although even today small outbreaks are reported every year. In the outer islands, however, a much higher incidence of malaria is seen in general. But the incidence of malaria varies from hypo-to hyperendemic depending on the environmental and socioeconomic conditions of an area. The natural and social environment of the Indonesian islands varies widely, resulting in different malaria conditions. Furthermore, even on a single island, malaria endemic situations vary in degree and

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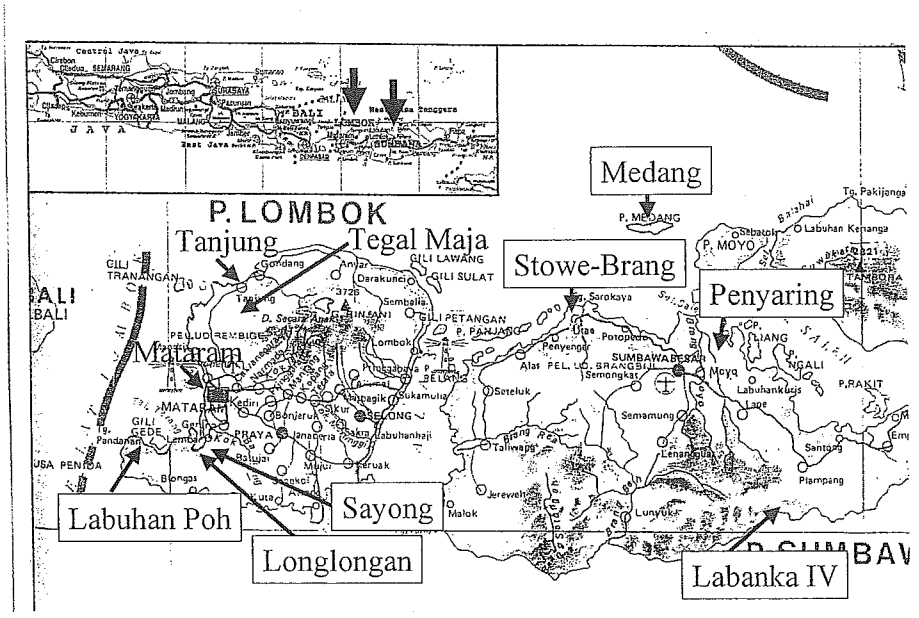


Fig. 1 Location of places described in the text on Lombok and Sumbawa islands, Indonesia

size according to geographical conditions. No exact information on malaria epidemiology in each area has been published, especially in English. In this review, we introduce the conditions of malaria in Lombok and Sumbawa islands based on our epidemiological studies conducted under Japan Society for the Promotion of Science (JSPS) sponsorship, and on local data and reports offered by concerned health organizations.

**2) The collaborative survey of Indonesian and Japanese researchers;** In 1991 we had an opportunity to conduct an epidemiological malaria survey on Lombok island as one of the collaborative works in a large scale cooperative study between Kobe University School of Medicine, Japan and the Tropical Disease Center (TDC), Airlangga University, Surabaya, Indonesia under the sponsorship of JSPS. The malaria epidemiological study was carried out continuously in Lombok and later in Sumbawa for ten years until the JSPS project reached completion in 2000.

**3) Malaria situation in West Lombok;** The West Lombok district is located in the western part of Lombok island, NTB province. The province is composed of two major islands, Lombok and Sumbawa, each of them containing three districts. The capital of the province is the municipality Mataram, which is located in the center of West Lombok district. West Lombok consists of nine subdistricts, in all of which malaria is endemic. According to the report by Gerback Malaria Team in West Lombok [2], the annual incidence in the district was over 40 per 1,000 population every year from 1996 to 1999. Falcipaum malaria is more common than vivax and other types of malaria. The transmission peak is usually observed between July and September. There are 11 species of *Anopheles* vector but, of these, three, *An. subpictus*, *An. maculates* and *An. barbirostris*, are of primary importance in malaria transmission and *An. sundaicus* and *An. aconitus* are of secondary importance.

Figure 2 shows the monthly slide-positive cases observed in 2000 and 2001 in Tanjung and Tegal Maja villages by the health center in Tanjung, which is located in the northernmost part of West Lombok. The village of Tanjung lies on the northern coast while the village of Tegal Maja is located south of it in the inland. At the former, the clear peak of malaria cases was seen in October, which was two months later than the usual transmission peak between July and

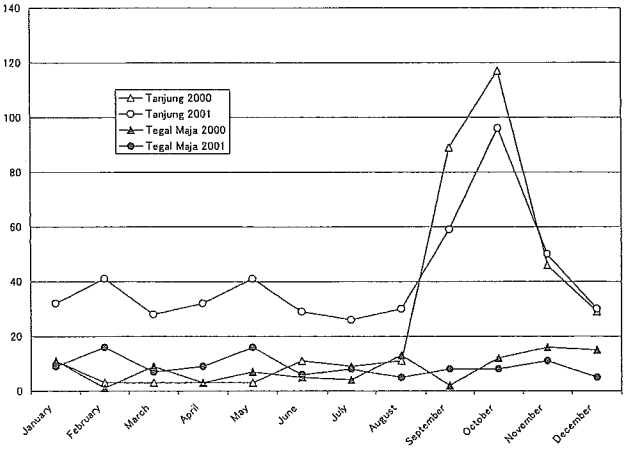


Fig. 2 Number of slide positive cases in two villages of Tanjung health center

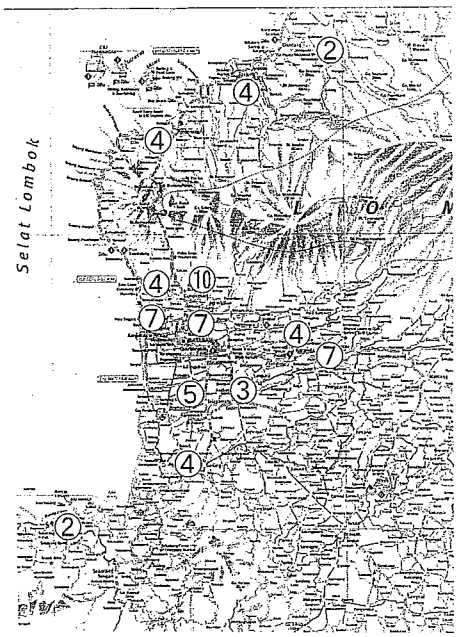


Fig. 3 Number of cerebral malaria cases at Mataram hospital shown by the place of residence

September mentioned in the local report [2]. This type of transmission is thought to be caused by *An. sundaicus* rather than *An. subpictus* according to a previous study by one of the present authors [3]. We obtained the data on malaria patients hospitalized in the Mataram hospital in 2001 and the first half of 2002. In total, 809 malaria patients were hospitalized. These were composed of 580 falciparum malaria patients including 71 with cerebral malaria, 54 vivax malaria patients and 175 clinical malaria patients. A total of 39 died of falciparum malaria, and 29 of these were cerebral malaria patients. We marked the number of cerebral malaria patients on a map according to the place of residence recorded in their patient-reports (Fig. 3). The cases were distributed equally in coastal and inland areas. This indicates that more careful attention should be given to the inland areas to identify malaria endemic foci.

## STUDIES IN WEST LOMBOK

### 1) Survey areas in West Lombok

Malaria epidemiological study target areas were selected on the basis of discussions between TDC and the Nusa Tenggara Barat (NTB) provincial health office. The subdistricts (kecamatan) Batulayar and Sekotong were selected for the preliminary survey. Subjects for blood and spleen examination were randomly selected from different subvillages (dusun) in the two subdistricts. A total of 36 subvillages, or 10 from Batulayar and 26 from Sekotong, were subjected to the survey. Although the number of persons examined in each subvillage was too small for evaluation, we selected three subvillages in Sekotong for the longitudinal survey, namely, dusun Labuhan Poh from desa (village) Sekotong Barat, and dusun Longlongan and Sayong from desa Sekotong Tengah. (Fig. 1, Fig. 4 and Table 1).

### 2) Geographical differences among subvillages (Table 1)

At subvillage Labuhan Poh, the land gradually rises away from the coast toward the inland. The largest river in this village passes through the subvillage and creates a wide lagoon during the dry season (Photo. 1). Many branches of

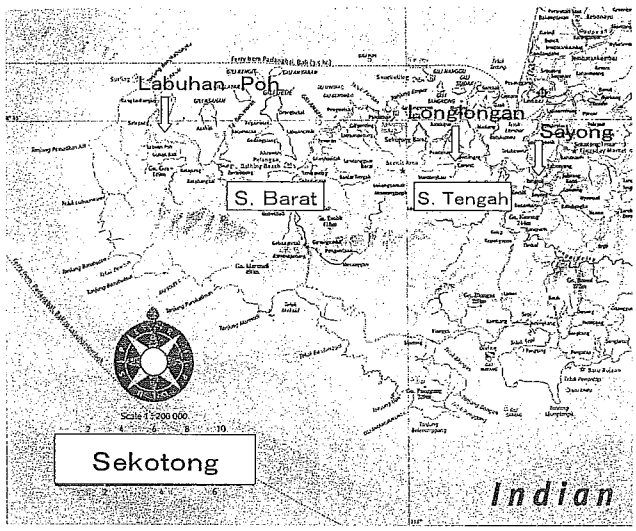


Fig. 4 Geographical distribution of the three subvillages selected for malaria survey at Sekotong

Table 1 Geographical features and population of three subvillages of Sekotong Barat and Tengah in 1992

| Subvillage | hill | wet rice field | beach | dry field | population |
|---|---|---|---|---|---|
| Labuhan Poh | 16.5% | 0 | 21.3% | 62.2% | 855 |
| Sayong | 30.6% | 67.7% | 1.7% | 0 | 1,247 |
| Longlongan | 71.5% | 4.3% | 16.1% | 8.1% | 805 |

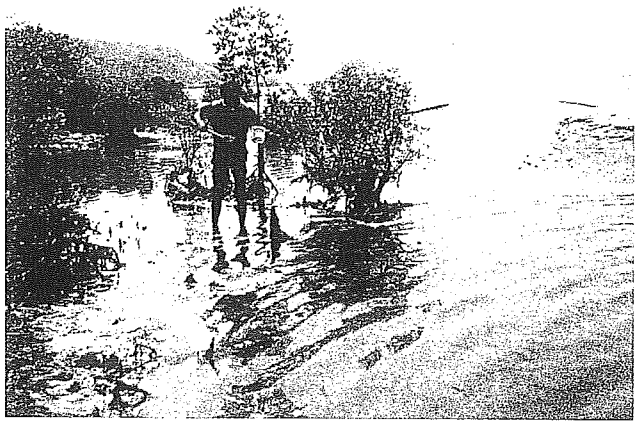

Photo 1. A huge lagoon formed after closing the river's exit to the sea at Labuhan Poh, Sekotong, West Lombok

the river extend into mangrove areas where large mangrove trees have been cut for fuel, leaving many water pools exposed to sunshine and resulting in breeding places for brackish species of *Anopheles* mosquitoes such as *An. sundaicus* and *An. subpictus*. Subvillage Sayong lies on flat ground. Most of the area is occupied by wet rice fields, and in the coastal part of the flat land once covered by mangrove forest, fish ponds were made after the removal of mangrove trees. Subvillage Longlongan has a complex topography; the narrow flat land along the coast, originally mangrove forest, was developed for fish ponds, and the following sharp sloping land leads to a rather flat hilly area where rice fields were developed between stands of grass or bush in the rainy season.

### 3) Survey methods and subjects

The longitudinal survey was started in August 1992 and carried out five times until June 1993 [4]. At the initial step, in order to determine the seasonal changes in malaria transmission, we intended to collect blood samples from the same subjects randomly selected from all age groups through all the surveys in a year. After the third survey, however, we had to replace the subjects with a new group composed of almost the same proportion of age groups, because of difficulties encountered in obtaining informed consent and cooperation from the former subjects. In the survey, the subjects were usually gathered in one place such as a school or a village health office (pustu) on an appointed date, and a 1–2 ml venous blood sample was obtained from each person along with in a syringe, a drop of blood for thin smear and another drop for thick smear on separate slide-glasses. The blood in a syringe was transferred into a small tube for serum collection. All the samples were carried to TDC for parasitological and serological examination. Medical examination was administered to each person after blood collection, and if necessary, medicines were given. On the same day the entomological survey was conducted, consisting of the examination of breeding sites and larva collection in the daytime, and adult mosquito collection at night.

### 4) Malaria prevalence in the survey areas

Table 2 and Fig. 5 present the results of the blood examinations. We cannot accurately compare the results of the first three surveys with those of the last two surveys because of the replacement of subjects. In total, the malaria positive rate gradually declined after the first survey in August 1992. However, the malaria transmission trend in each subvillage differed from that in others (Fig. 5). A relatively stable slide positive rate was found in dusun Labuhan Poh in August, October and December 1992, while in other subvillages it varied by month, especially in dusun Longlongan.

Table 2 Results of blood examinations in a longitudinal malaria survey conducted in three subvillages of Sekotong, Lombok from August 1992 to June 1993

| | August 1992 | | | | | October 1992 | | | | | December 1992 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subvillage | PR | Number of positive cases | | | | PR | Number of positive cases | | | | PR | Number of positive cases | | | |
| | % | Pf | Pv | Pm | Mix | % | Pf | Pv | Pm | Mix | % | Pf | Pv | Pm | Mix |
| Labuhan Poh | 11.5 (14/122) | 10 | 4 | 0 | 0 | 9.8 (12/122) | 9 | 2 | 0 | 1 | 12.3 (15/122) | 11 | 3 | 0 | 1 |
| Sayong | 11.9 (18/151) | 6 | 11 | 0 | 1 | 6.0 (9/150) | 4 | 5 | 0 | 0 | 6.0 (9/150) | 4 | 5 | 0 | 0 |
| Longlongan | 21.1 (20/95) | 12 | 8 | 0 | 0 | 6.4 (6/95) | 5 | 1 | 0 | 0 | 2.1 (2/95) | 1 | 1 | 0 | 0 |
| Total | 14.1 (52/368) | 28 | 23 | 0 | 1 | 7.4 (27/367) | 18 | 8 | 0 | 1 | 7.1 (26/367) | 16 | 9 | 0 | 1 |

| | April 1993 | | | | | June 1993 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Subvillage | PR | Number of positive cases | | | | PR | Number of positice cases | | | |
| | % | Pf | Pv | Pm | Mix | % | Pf | Pv | Pm | Mix |
| Labuhan Poh | 3.7 (4/107) | 3 | 1 | 0 | 0 | 1.1 (1/89) | 0 | 0 | 1 | 0 |
| Sayong | 0.72 (1/139) | 1 | 0 | 0 | 0 | 0 (0/129) | 0 | 0 | 0 | 0 |
| Longlongan | 1.0 (1/98) | 1 | 0 | 0 | 0 | 9.0 (8/89) | 5 | 2 | 0 | 1 |
| Total | 1.7 (6/344) | 5 | 1 | 0 | 0 | 2.9 (9/307) | 5 | 2 | 1 | 1 |

PR, positive rate; Pf. *Plasmodium falciparum*; Pv, *Plasmodium vivax*; Pm, *Plasmodium malariae*; Mix, mix infection; ( ), actual number

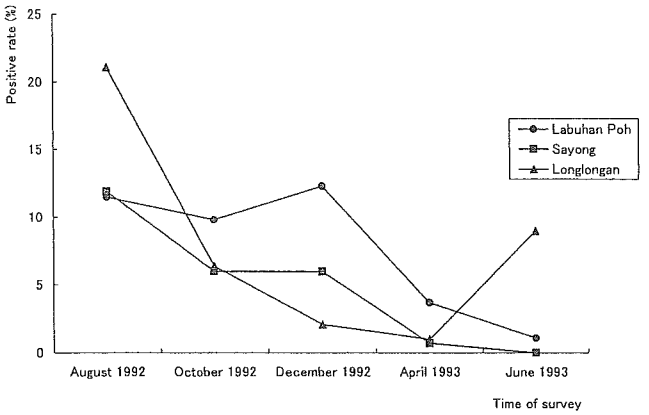


Fig. 5 Slide positive rates in the three subvillages of Sekotong, Lombok from August 1992 to June 1993

This difference may be attributable to the different environmental and geographical conditions of each subvillage (Table1). Especially in dusun Longlongan, the malaria in the hilly area may have a different transmission mode.

**5) Entomological observation in the survey area**

The results of the entomological examination also showed a wide variety (Table 3 and 4). As expected, a relatively stable number of adult *Anopheles subpictus* mosquitoes were captured at all three subvillages, especially at dusun Labuhan Poh (Table 3), but *An. sundaicus*, *An. barbirostris* and *An. aconitus*, which have been recognized as malaria vectors in Indonesia, were captured sporadically once or twice in five surveys conducted one year except *An. sundaicus* at dusun Labuhan Poh [4, 5]. The fluctuation in the number of captured mosquitoes suspected to be malaria vectors did not correspond to the parasitological data (Table 2, 3 and Fig. 5). The most stable larva collection of the brackish *Anopheles* species was obtained at lagoon and mangrove areas in dusun Labuhan Poh but at fish-ponds in dusun Sayong and Longlongan (Table 4). These results, taken with the parasitological data, indicate that *An. subpictus* (and additionally *An. sundaicus*) play a major role in malaria transmission in these subvillages. The previous intensive study on mosquito fauna in Lombok island by Lee et al.

Table 3 Adult collection of *Anopheles* species known as malaria vector in three subvillages of Sekotong (1992-1993)

| SUBVILLAGE | COLLECTION METHOD | Aug 92 | Oct 92 | Dec 92 | Apr 93 | Jun 93 |
|---|---|---|---|---|---|---|
| | | (species / no. mosq. collected per night*) | | | | |
| LABUHAN POH | Outdoor Human Bait (OHB) | sub/14<br>sun/4 | sub/9<br>sun/8 | sub/6<br>sun/2 | bar/14<br>sub/4 | sub/6<br>sun/3 |
| | Indoor Human Bait (IHB) | sub/2 | sub/11<br>sun/22 | sub/6<br>sun/5 | 0<br>0 | sub/4 |
| | Indoor Resting (IR) | 0 | sun/5 | 0 | 0 | 0 |
| | Bednet Trap (BT) | 0 | sub/7<br>sun/16 | 0 | 0 | 0 |
| | Cattle Bait (CB) | sub/11<br>sun/1<br>bar/1 | sub/12 | sub/1 | sub/2<br>acon/1 | sub/5<br>sun/1 |
| SAYONG | OHB | sub/7 | sub/14<br>sun/3 | sub/10<br>acon/5 | sub/8<br>acon/1 | sub/1 |
| | IHB | 0 | sub/4 | sub/9<br>acon/2 | acon/2 | 0 |
| | IR | 0 | 0 | 0 | 0 | 0 |
| | BT | bar/1 | 0 | 0 | 0 | 0 |
| | CB | sub/5 | sub/16 | sub/6 | acon/4<br>sub/9 | sub/9 |
| LONGLONGAN | OHB | sun/3 | sub/15<br>sun/3 | sub/4 | sub/19 | 0 |
| | IHB | 0 | sub/3<br>sun/1 | sub/17<br>sun/1 | sub/3 | sub/1 |
| | IR | 0 | 0 | 0 | 0 | sub/1 |
| | BT | sun/1 | 0 | 0 | 0 | — |
| | CB | sub/7<br>bar/1 | sub/17 | sub/8 | sub/10<br>acon/7 | sub/9<br>acon/1 |

*40 min per hour from 6 pm–12 pm.

sub, *Anopheles subpictus*; sun, *An. sundaicus*; acon, *An. aconitus*; bar, *An. barbirostris*

Table 4 Type of breeding place and density of *Anopheles* larvae (per dip) in three subvillages of Sekotong

| Type Br. Pl. | *Anoph.* Species | Aug. 92 | Oct. 92 | Dec. 92 | Apr. 93 | Jun. 93 |
|---|---|---|---|---|---|---|
| Labuhan Poh | | | | | | |
| I. Lagoon | 1. *An. subpictus* | n. d. | 1.87 | 1.30 | 1.40 | 0.95 |
| | 2. *An. sundaicus* | n. d. | 0.70 | 0.40 | 0 | 0.30 |
| II. River | 1. *An. flavirostris* | 1.00 | 0 | 0 | 0 | 0 |
| | 2. *An. minimus* | 0.50 | 0 | 0 | 0.70 | 0 |
| | 3. *An. vagus* | 0 | 0 | 0 | 0.11 | 0.10 |
| | 4. *An. subpictus* | 1.56 | 0 | 1.50 | 0 | 0 |
| | 5. *An. sundaicus* | 0.12 | 0 | 0 | 0 | 0 |
| III. Mangrove | 1. *An. subpictus* | 1.00 | 0 | 1.60 | 0.67 | (−) |
| | 2. *An. sundaicus* | 0 | 0 | 0.40 | 0 | (−) |
| IV. Rice field | 1. *An. vagus* | (−) | (−) | 1.40 | (−) | 0.10 |
| V. Fishpond | 1. *An. subpictus* | (−) | (−) | (−) | 0.10 | 0 |
| Sayong | | | | | | |
| I. Fishpond | 1. *An. subpictus* | 0.80 | 0.80 | 1.00 | 0.05 | 0.90 |
| | 2. *An. sundaicus* | 0 | 0 | 0 | 0.05 | 0 |
| | 3. *An. annularis* | 0 | 0 | 0 | 0.05 | 0 |
| II. Rice field | 1. *An. aconitus* | 0 | 0 | 0.40 | 0.60 | 0.25 |
| | 2. *An. barbirostris* | 0.70 | 0 | 0 | 0 | 0 |
| | 3. *An. vagus* | 0 | 1.50 | 2.20 | 0 | 0.28 |
| III. Fresh water | 1. *An. barbirostris* | 0.60 | 0 | 0 | 0 | 0 |
| | 2. *An. annularis* | 0 | 0 | 3.40 | 0.60 | 0 |
| Longlongan | | | | | | |
| I. Well | 1. *An. barbirostris* | 0.40 | 0 | 0 | 0 | 0.35 |
| | 2. *An. annularis* | 0 | 0 | 0 | 0.10 | 0 |
| | 3. *An. vagus* | 0 | 0 | 1.20 | 3.00 | 0.70 |
| II. River | 1. *An. aconitus* | 0.02 | 0 | 0 | 0 | 0 |
| | 2. *An. barbirostris* | 0.77 | 0 | 0 | 0.40 | 0 |
| | 3. *An. vagus* | 0.85 | 0.67 | 1.80 | 0 | 0.23 |
| III. Ricefield | 1. *An. barbirostris* | 0 | 0.05 | 0 | 0.25 | 0.01 |
| | 2. *An. annularis* | 0 | 0 | 0 | 0.01 | 0.01 |
| | 3. *An. vagus* | 1.00 | 0.80 | 4.20 | 2.07 | 0.55 |
| IV. Fishpond | 1. *An. subpictus* | 0.08 | 1.38 | 1.20 | 1.80 | 1.03 |

(−), no water at the time examined; Br. Pl., Breeding Place; n. d., not done

identified three *Anopheles* species, *An. annularis*, *An. barbirostris* and *An. subpictus*, as potential vectors [6]. Recently, Miyagi et al also found *An. subpictus* and *An. sundaicus* in coastal areas and *An. barbirostris*, *An. leucosphyrus* group and *An. minimus* in fresh water and cited them as potential vectors [7]. In 2001, Sukowati, S. et al., Health Ecology Research Center, NIHR&D found *Plasmodium falciparum* (*P. f.*) sporozoite-positive *An. subpictus* in this area (report to the Indonesian health ministry). In the subvillage Longlongan, in addition to the coastal area, malaria was found in the hilly area where more than half of the population of this subvillage live, but we could not determine the vector mosquitoes there. Because of windy conditions and the collection confined to one night during the survey, the entomological staff were able to capture only a few adult mosquitoes. From the larvae examination we inferred two probable transmission vectors. One is *An. barbirostris*, the larvae of which were found in rice-fields, stagnant water along small rivers and wells, and the other is *An. subpictus*, which was consistently found in fish ponds along the coast and is thought to be able to move back and forth between the coast and the hills with the wind.

**6) Endemic situation of malaria**

We selected subjects equally from all the age groups to determine the degree of endemicity. Our results showed no difference in malaria prevalence among age groups [4] (data, not shown), indicating a hypo-or meso-endemic pattern in the area. The additional serological examination of antibodies to *P. f.* crude antigens using ELISA also demonstrated a meso-endemic pattern at dusun Labuhan Poh (Fig. 6), that is, the positive rate was low (about 20%) at the age of 0 but rose to nearly 100% at the age of 6 or over. In this area, three *Plasmodium* species were detected, that is, about

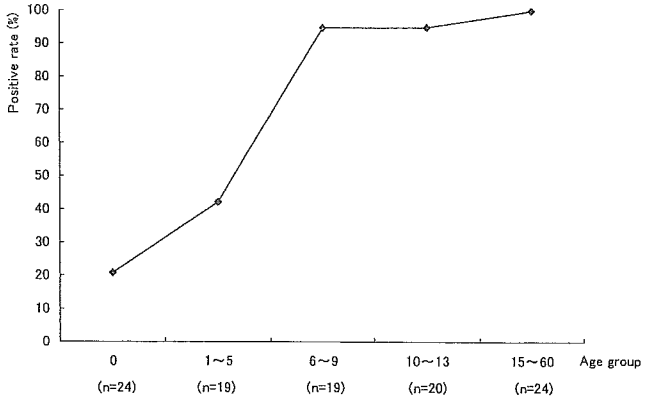


Fig. 6 Sero-positive rate to P. f. crude antigens among persons examined in subvillage Labuhan Poh in October 1992

60% *P. falciparum*, 40% *P. vivax* and only one *P. malariae* (Table 2). The *P. malaria* case was confirmed by PCR using the ribosomal DNA sequence [8]. Our results did not confirm the peak of transmission between July and September as described in the local report [2].

## STUDIES IN SUMBAWA

### 1) Survey areas in Sumbawa

In Sumbawa, four subvillages in different subdistricts were examined for prevalence of malaria from 1996 to 1999 (Fig. 1). One subvillage, dusun Medang, is a small island accessible in one hour from Sumbawa Besar by small motorboat. In this subvillage, the preliminary spleen examination was

Table 5 Parasite positive rate and spleen rate in the longitudinal survey conducted in three subvillages of Sumbawa

| | December 1996 | | | | | July 1997 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Subvillage | Positive Rate (%) | Number of positive cases Pf | Pv | Mix | Spleen Rate (%) | Positive Rate (%) | Number of positive cases Pf | Pv | Mix | Spleen Rate (%) |
| Penyaring | 7.1 (8/112) | 7 | 1 | 0 | 0 | — | — | — | — | — |
| Labangka IV | 14.3 (16/112) | 10 | 6 | 0 | 8.0 | 7.1 (8/112) | 4 | 4 | 0 | 14.3 |
| Stowe Brang | 33.9 (38/112) | 8 | 25 | 5 | 25.9 | 15.3 (17/111) | 13 | 3 | 1 | 34.2 |
| Total | 18.5 (62/336) | 25 | 32 | 5 | 11.3 | 11.2 (25/223) | 17 | 7 | 1 | 16.0* |
| | March 1998 | | | | | October 1998 | | | | |
| Subvillage | Positive Rate (%) | Number of positive cases Pf | Pv | Mix | Spleen Rate (%) | Positive Rate (%) | Number of positive cases Pf | Pv | Mix | Spleen Rate (%) |
| Penyaring | 1.8 (2/112) | 2 | 0 | 0 | 0 | 0.9 (1/112) | 1 | 0 | 0 | 0 |
| Labangka IV | 0.9 (1/112) | 1 | 0 | 0 | 0 | 13.0 (9/69(74))** | 4 | 3 | 2 | 23.0 |
| Stowe Brang | 8.0 (9/112) | 7 | 2 | 0 | 12.5 | 1.8 (2/112) | 1 | 1 | 0 | 4.5 |
| Total | 3.6 (12/336) | 10 | 2 | 0 | 4.2 | 14.1 (12/293) | 6 | 4 | 2 | 7.4 |
| | December 1998 | | | | | February 1999 | | | | |
| Subvillage | Positive Rate (%) | Number of positive cases Pf | Pv | Mix | Spleen Rate (%) | Positive Rate (%) | Number of positive cases Pf | Pv | Mix | Spleen Rate (%) |
| Penyaring | 0.9 (1/112) | 1 | 0 | 0 | 0 | 3.6 (4/111) | 3 | 1 | 0 | 0 |
| Labangka IV | 5.3 (5/95) | 1 | 4 | 0 | 1.1 | 5.4 (6/111) | 2 | 3 | 1 | 0 |
| Stowe Brang | 0 (0/112) | 0 | 0 | 0 | 0 | ND (−/108) | ND | ND | ND | 0.9 |
| Total | 1.9 (6/319) | 2 | 4 | 0 | 0.3 | 4.5 (10/222) | 5 | 4 | 1 | 0.3 |

P. f., *Plasmodium falciparum*; P. v., *Pl. vivax*; Mix, mix infection
−,data lost
* The number of subjects in Penyaring was assumed to be 112.
** 74 persons were subjected to spleen examination, but only69of these underwent blood examinations.
ND, not done

conducted on 161 1st and 2nd grade school-children and showed a 42.9% spleen rate (meso-endemic), but afterwards neither blood nor mosquito examinations was conducted because of the risk of the available boat capsizing. Therefore, three subvillages were selected for the longitudinal survey. The methods were the same as those used in Lombok.

**2) Malaria prevalence in the Sumbawa survey areas**

The slide positive rates and spleen rates at three subvillages are shown in Table 5. All three subvillages are located along the coast. Subvillage Penyaring and Stowe Berang face the ocean to the north and subvillage Labangka IV to the south. The former two subvillages are geographically similar. They have mangrove beaches and flat lands. The mangrove beaches were developed for fish ponds in both subvillages. Despite the environmental similarities, subvillage Penyaring showed a very low slide-positive rate and 0% spleen rate, while dusun Stowe Berang showed rather high positive rates for both examinations. Subvillage Labangka IV showed a medium endemic pattern with seasonal epidemics.

**3) Entomological observation and epidemiological analysis**

The entomological examination clearly demonstrated a high density of adults and larvae of *An. subpictus* at subvillage Stowe Berang but a very low density at subvillage Penyaring (data, not shown). This was due to the difference in breeding sites between the two subvillages, namely, many abandoned fish-ponds with algae and weeds were found at the former (Photo. 2) while most of the fish ponds were well maintained at the latter. The sharp decline in positive rates for spleen and blood examinations at dusun Stowe Berang from October 1998 was due to two malaria control projects conducted from January 1998 for a year, that is, the distribution of insecticide impregnated mosquito-nets and the cleaning of abandoned fish-ponds (Fig. 7). Our reports in 1996 and 1997 note that these control projects were conducted by the Sumbawa district health office, and suggest that the control methods worked effectively. In subvillage Labangka IV, an outbreak of malaria was observed just before our survey in October 1998. This subvillage has a very narrow sandy beach with a steep cliff rising behind. A rather flat hilly area spreads away from the cliff. The entomological survey found that the captured *Anopheles* mosquitoes were exclusively *An. subpictus* and that there were several lagoons on a small beach where *An. subpictus* larvae bred. According to staff in the Sumbawa district health office, the outbreak may be related to the custom of villagers to gather around the cliff (cape) at night to catch a species of bird during this season.

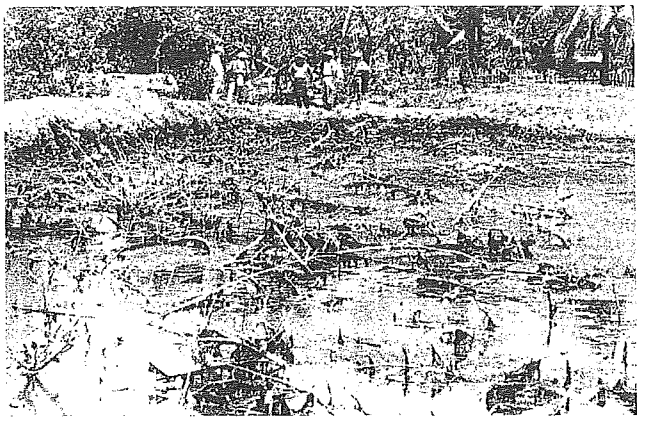

Photo 2. An abandoned fish pond at Stowe Brang, Utan, Sumbawa

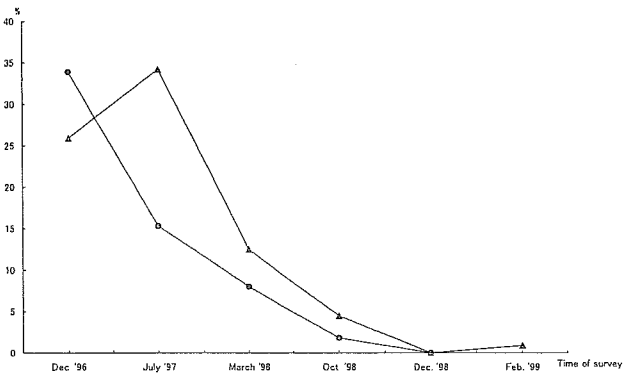


Fig. 7 Changes in slide-positive rate (●) and spleen rate (▲) in Stowe Brang, Sumbawa during the three-year period after malaria control activities in January 1998

## SUMMARY

Although our data are still insufficient to determine the full range of epidemiological features, we can draw the following conclusions about malaria in Lombok and Sumbawa.

1) Malaria endemic areas are located mainly along the seacoast and less frequently inland.

2) The degree of endemicity is hypo-endemic to meso-endemic.

3) The main transmission vectors are *Anopheles subpictus* and *An. sundaicus*, which breed in brackish water.

4) Although similar species of vector play a role in transmission in coastal endemic foci, the mode and the season of transmission vary with the ecological characteristics of the vector and social and environmental conditions.

5) Small endemic foci are found in hilly areas inland, but the responsible vector species have not been determined.

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# IL-18 with IL-2 protects against *Strongyloides venezuelensis* infection by activating mucosal mast cell–dependent type 2 innate immunity

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**C57BL/6 (B6) and B6 background STAT6$^{-/-}$ mice pretreated with IL-18 plus IL-2 showed prominent intestinal mastocytosis and rapidly expelled implanted adult worms of the gastrointestinal nematode *Strongyloides venezuelensis*. In contrast, identically pretreated mast cell–deficient W/W$^{v}$ mice failed to do so. Thus, activated mucosal mast cells (MMC) are crucial for parasite expulsion. B6 mice infected with *S. venezuelensis* third-stage larvae (L3) completed parasite expulsion by day 12 after infection, whereas IL-18$^{-/-}$ or IL-18R$\alpha^{-/-}$ B6 mice exhibited marked impairment in parasite expulsion, suggesting a substantial contribution of IL-18–dependent MMC activation to parasite expulsion. Compared with IL-18$^{-/-}$ or IL-18R$\alpha^{-/-}$ mice, *S. venezuelensis* L3–infected STAT6$^{-/-}$ mice have poorly activated MMC and sustained infection; although their IL-18 production is normal. Neutralization of IL-18 and IL-2 further reduces expulsion in infected STAT6$^{-/-}$ mice. These results suggest that collaboration between IL-18–dependent and Th2 cell–dependent mastocytosis is important for prompt parasite expulsion.**

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Abbreviations used: MMC, mucosal mast cell; mMCP-1, mouse mast cell protease-1; L3, third-stage larvae; VCU, villous crypt units.

Microbes can be classified into intracellular and extracellular types. In general, intracellular microbes are expelled by cell-mediated immunity (Th1 responses), whereas extracellular microbe eradication often is mediated by the function of humoral immunity (Th2 responses; references 1–6). Upon infection with intracellular microbes, macrophages or DCs produce various types of proinflammatory cytokines in response to Toll-like-receptor/MyD88-mediated signaling (7, 8). Among the proinflammatory cytokines produced, IL-12 and IL-18 are most important up-stream cytokines of IFN-γ and synergistically induce T cells, B cells, NK cells, macrophages, and DCs to produce IFN-γ (9–16). Resultant IFN-γ then activates macrophages to produce nitric oxide, leading to eradication of intracellular pathogens (3–5). Indeed, IL-12– and/or IL-18–deficient mice show markedly reduced host resistance against *Cryptococcus neoformans* or *Leishmania major* (17–19). Thus, both IL-12 and IL-18 are important for host defense against intracellular microbes. However, our recent studies clarified that IL-18 without help from IL-12 induces Th2 cytokines in T cells, basophils, and mast cells (4, 20–23). Most surprisingly, administration of IL-18 or IL-18 plus IL-2 into naive mice induces IgE in a CD4$^{+}$ T cell–, IL-4–, and STAT6–dependent manner (20–22). Moreover, transgenic mice overexpressing IL-18 in their keratinocytes spontaneously produce IgE (21, 24) and develop atopic dermatitis (25). Thus, IL-18 regulates both Th1 and Th2 responses depending on its cytokine milieu (4).

It is well known that expulsion of some types of helminthes depends on the action of activated mast cells (2). Here we demonstrate that treatment of mice with daily injection of IL-2 plus IL-18 induces significant increases in the number of intestinal mucosal mast cells (MMCs) and in their release of mouse mast cell protease-1 (mMCP-1), which are hallmarks of infection with gastrointestinal nematode (2, 26–29). Furthermore, this pretreatment prepares them to expel implanted adult worms of *Strongyloides venezuelensis* rapidly. In contrast, identically pretreated W/W$^{v}$ mice that lack mast cells (30) failed to expel implanted worms. These results suggest that IL-18– and

IL-2–dependent MMC activation is indispensable for rapid parasite expulsion. WT mice inoculated with *S. venezuelensis* third-stage larvae (L3) showed significant increases both in serum IL-18 and mMCP-1 levels and completed worm expulsion by 12 d. In contrast, IL-18–deficient (IL-18$^{-/-}$) or IL-18R$\alpha^{-/-}$ mice required longer period to complete worm expulsion. STAT6$^{-/-}$ mice infected with *S. venezuelensis* L3 showed more severe impairment in parasite expulsion, and neutralization of IL-18 and IL-2 further reduced their capacity to expel the parasite. Here, we demonstrate that both IL-18–dependent and Th2 cytokine–dependent MMC activation pathways are critically involved in induction of rapid parasite expulsion.

## RESULTS

### Intestinal MMC accumulation in WT mice injected with IL-18 plus IL-2

We first tested whether daily i.p. injection of IL-2 and/or IL-18 induces accumulation of MMC in intestines of the mice. Stained jejunal sections revealed that administration of IL-18 (2 μg/d) and IL-2 (2,000 U/d) induced MMC, although treatment with each component alone did not induce or induced it weakly (Fig. 1 A). Titration study indicated that IL-18 stimulated a dose-dependent increase in MMC when combined with IL-2 (2,000 U/d) (Fig. 1 B). We injected 2,000 U/d of IL-2. Much higher doses of IL-2 (e.g., $10^4$ U/d) failed to enhance this response (unpublished data). We compared the degree of accumulation of MMC in the mice treated with IL-2 and IL-18 with that in mice inoculated with *S. venezuelensis* L3 2 wk earlier and found that infection with *S. venezuelensis* L3 showed more potent MMC-inducing activity (Fig. 1 A, d and f, and B). The effect of IL-2 and IL-18 was prominent in intestines, and no mast cell accumulation was observed in other organs, such as lungs, spleens, livers, and kidneys.

Because IL-3, IL-4, and IL-9 are well-known potent mast cell growth factors (30–32), we simultaneously measured serum levels of these cytokines at various time points after treatment of mice with IL-2 plus IL-18. As shown in Fig. 1 C, administration of IL-18 (2 μg/d) and IL-2 (2,000 U/d) induced increases in serum levels of IL-3, IL-4, IL-9, and IL-13. In general, these cytokines are below the detection level in normal mice. However, serum levels of IL-3, IL-4, and IL-9 increased in mice injected with IL-2 and IL-18 and peaked at d 7; IL-13 was persistently elevated even beyond d 7. IL-18 treatment alone induced IL-4 very modestly (30 pg/ml). Neither IL-10 nor IFN-γ was detected in the sera of mice injected with IL-18 plus IL-2 (unpublished data).

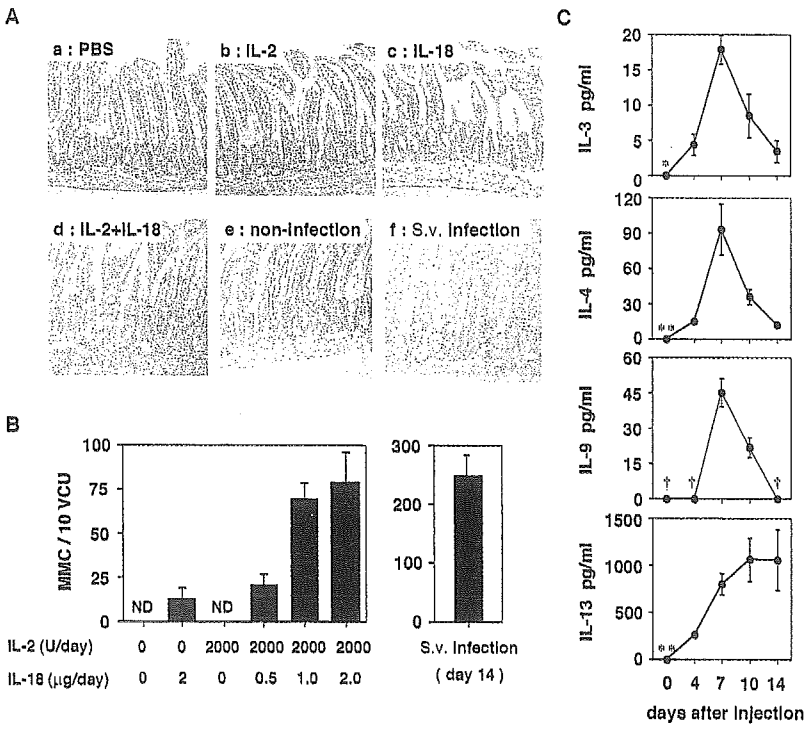


**Figure 1. IL-18– plus IL-2–induced intestinal MMC accumulation in vivo.** (A) C57BL/6 mice (six to eight mice per group) were injected daily i.p. with PBS alone or with IL-18 (0.5–2 μg/d) and/or IL-2 (2,000 U/d) for 13 d or were inoculated with 5,000 *S. venezuelensis* L3. 14 d after cytokine treatments or inoculation with *S. venezuelensis* L3, intestine tissue samples (8–10 cm from the pyloric ring) were removed and fixed in Carnoy's fluid and stained with Alcian blue, pH 0.3, and Safranin-O. Original magnification, 200. (B) The number of mast cells in the epithelium and lamina propria mucosa was counted in 10 VCU. Results are geometric means ± SD. ND; not detected. (C) C57BL/6 mice (six to eight mice per group) were injected daily with IL-18 (2 μg/d) plus IL-2 (2,000 U/d) for 13 d. They were bled 0, 4, 7, 10, and 14 d later, and serum IL-3, IL-4, IL-9, and IL-13 levels were measured by ELISA. Results are geometric means ± SEM. *, <3 pg/ml; **, <10 pg/ml; †, <20 pg/ml. Results are representative of three independent experiments.

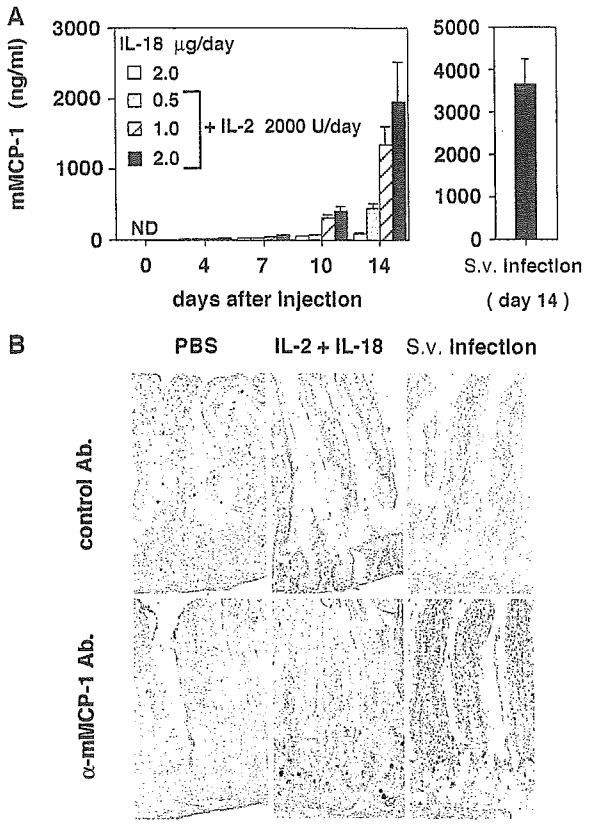


Figure 2. IL-18– plus IL-2–induced mMCP-1 production in vivo. (A) C57BL/6 mice (six to eight mice per group) were injected i.p. daily with IL-18 (2 μg/d) or with various doses of IL-18 (0.5–2 μg/d) and IL-2 (2,000 U/d) for 13 d or were inoculated with 5,000 *S. venezuelensis* L3. They were bled 0, 4, 7, 10, and 14 d later, and mMCP-1 in sera was measured by ELISA. Results are geometric means ± SEM. ND; not detected. (B) C57BL/6 mice (six to eight mice per group) were injected daily with PBS or with IL-18 (2 μg/d) and IL-2 (2,000 U/d) for 13 d or were inoculated with 5,000 *S. venezuelensis* L3. 14 d after treatments, upper intestines were removed and fixed with 4% paraformaldehyde. Immunohistochemical staining for mMCP-1 was performed as described in Materials and methods. Results are representative of three independent experiments.

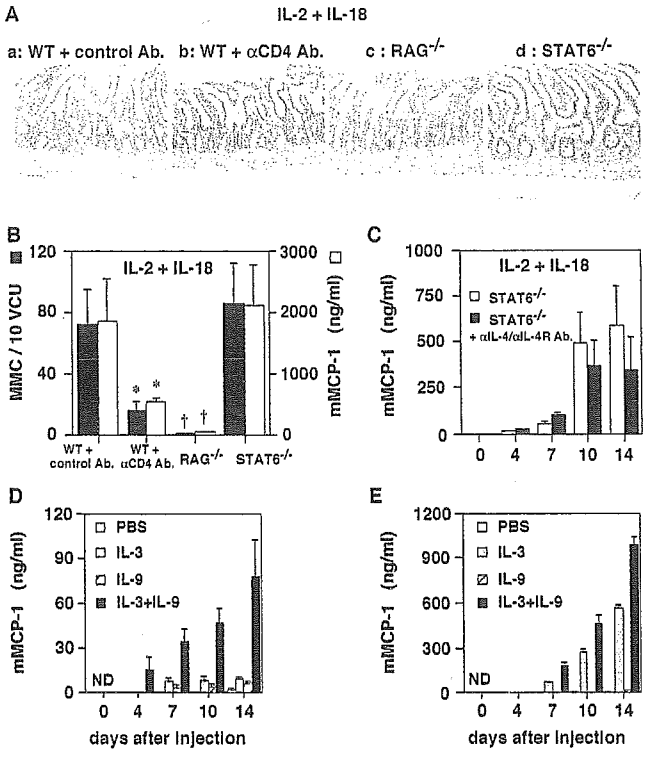


Figure 3. IL-18– plus IL-2–induced CD4+ T cell–dependent but STAT6-independent intestinal mastocytosis. C57BL/6 WT and C57BL/6-background RAG-2$^{-/-}$ (RAG$^{-/-}$) or STAT6$^{-/-}$ mice (six mice per group) were injected i.p. daily with IL-18 (2 μg/d) and/or IL-2 (2,000 U/d) for 13 d. To deplete CD4+ T cells, C57BL/6 mice received anti-CD4 (GK1.5; 0.5 mg/d) or control antibody (rat IgG2b; 0.5 mg/d) 7 and 4 d before IL-2 and IL-18 treatment and 0, 3, and 7 d after treatment. (A) 14 d after IL-2 and IL-18 treatment, tissue samples (8–10 cm) from the pyloric ring were removed and fixed in Carnoy's fluid and stained with Alcian blue, pH 0.3, and Safranin-O. Original magnification, 200. (B) The number of mast cells in the epithelium and lamina propria mucosa was counted in 10 VCU. Results are geometric means ± SD. Mice were bled 0, 7, 10, and 14 d later, and serum mMCP-1 was measured by ELISA. Results are geometric means ± SEM. *, $P < 0.01$; †, $P < 0.001$ by Student's *t* test as compared with mice treated with control antibody. Results are representative of three independent experiments. (C) STAT6$^{-/-}$ mice (eight mice per group) were injected i.p.daily with IL-18 (2 μg/d) and/or IL-2 (2,000 U/d) for 13 d. Four mice from each group received a mixture of anti-IL-4 antibody (10 mg/d) and anti-IL-4R antibody (10 mg/d) 1 d before IL-2 and IL-18 treatment and 4 and 8 d after treatment. They were bled 0, 4, 7, 10, and 14 d later, and serum mMCP-1 was measured by ELISA. Results are geometric means ± SEM. Results are representative of two independent experiments. (D and E) C57BL/6 normal mice (five mice per group) were injected daily for 13 d (D) with PBS alone or with IL-3 (0.013 μg/d) and/or IL-9 (0.5 μg/d) or (E) with PBS alone or with IL-3 (0.1 μg/d) and/or IL-9 (0.5 μg/d). They were bled 0, 4, 7, 10, and 14 d later, and serum mMCP-1 levels were measured by ELISA. Results are geometric means ± SEM. ND; not detected. Results are representative of three independent experiments.

### mMCP-1 production by activated intestinal MMC in mice injected with IL-18 plus IL-2

It is well known that the serum level of mMCP-1 correlates very well with the degree of intestinal MMC activation that is associated with mucosal mast cell degranulation (26–29). Thus, we examined whether accumulated MMCs were activated to produce mMCP-1 in mice treated with IL-2 plus IL-18. As shown in Fig. 2 A, the serum level of mMCP-1 was below the detection level under normal conditions. However, when mice received a daily injection of IL-18 (2 μg/d), the mMCP-1 level in their sera increased (day 7, 19.5 ng/ml; day 10, 39.58 ng/ml; day 14, 55.6 ng/ml). Coinjection of IL-2 markedly increased these serum mMCP-1 levels. IL-18 induced a dose-dependent increase in mMCP-1 levels in mice treated with IL-2 (2,000 U/d; Fig. 2 A). We found that serum level of mMCP-1 in the mice injected with IL-18 (2 μg/d) and IL-2 (2,000 U/d) was almost comparable with that in the mice inoculated with *S. venezuelensis* L3 (Fig. 2 A).

In addition to the effect of IL-18 and IL-2 on serum mMCP-1 level, stained jejunal sections indicated that this

treatment markedly increased the number of mMCP-1–positive cells (Fig. 2 B). Their distribution is very similar to that of MMC (Fig. 1 A and Fig. 2 B). Furthermore, like MMC, mMCP-1–positive cells were prominent in intestines and were not observed in the lungs, spleens, livers, and kidneys of the mice injected with IL-18 and IL-2 (unpublished data). Taken together, these results clearly indicated that treatment with IL-18 and IL-2 induces accumulation, maturation, and activation of intestinal MMC, namely intestinal mastocytosis.

### CD4$^+$ T cell–dependent but STAT6-independent mastocytosis in mice injected with IL-18 plus IL-2

To determine whether this intestinal mastocytosis induced by IL-18 plus IL-2 is dependent on the function of CD4$^+$ T cells, we injected IL-2 and IL-18 into C57BL/6 WT mice depleted of CD4$^+$ T cells by pretreatment with anti-CD4 antibody or into C57BL/6 RAG-2–deficient (RAG-2$^{-/-}$) mice lacking both T cells and B cells. Anti-CD4 treatment significantly impaired IL-2– and IL-18–induced intestinal mastocytosis (Fig. 3, A and B) and mMCP-1 production ($P < 0.01$; Fig. 3 B). Furthermore, RAG-2$^{-/-}$ mice injected with IL-18 plus IL-2 showed very poor accumulation of MMC and very low serum levels of mMCP-1 (Fig. 3, A and B). These results clearly indicated that IL-18– plus IL-2–induced accumulation of intestinal MMC is dependent on the function of CD4$^+$ T cells.

Injection of IL-2 and IL-18 induced increases in the serum levels of IL-3, IL-4, IL-9, and IL-13 (Fig. 1 C). We previously reported that IL-2 and IL-18 stimulate CD4$^+$ T cells from C57BL/6 or C57BL/6 background STAT6$^{-/-}$ mice to produce IL-3, IL-4, IL-9, and IL-13 (21, 22). Because IL-4 was shown to induce intestinal mastocytosis in a STAT6-independent manner (33, 34), we injected IL-2 and IL-18 into STAT6$^{-/-}$ mice and examined the accumulation of MMC. STAT6$^{-/-}$ mice displayed intestinal mastocytosis and high serum levels of mMCP-1 (Fig. 3, A and B) when injected with IL-2 and IL-18, suggesting STAT6-independent IL-4 induction of mastocytosis. However, blockage of IL-4 only partly inhibited the IL-2–plus IL-18–induced mMCP-1 response (Fig. 3 C). Thus, the effect of IL-4 was modest, and induction of mMCP-1 requires the participation of other factors. Because IL-3 and IL-9 can induce MMC in vivo (30–32), we daily injected IL-3 (0.013 μg/d) and IL-9 (0.5 μg/d) into C57BL/6 WT mice. This treatment only partially replaced the effect of IL-2 and IL-18 (Fig. 3 D). However, a higher dose of IL-3 (0.1 μg/d) even without IL-9 strongly increased the serum level of mMCP-1, and additional IL-9 stimulation enhanced this effect modestly (Fig. 3 E). Thus, IL-2 plus IL-18 treatment seems to induce mMCP-1 by virtue of IL-3 and IL-9 from CD4$^+$ T cells, and IL-3 seems to be most critical for mMCP-1 induction.

### Rapid expulsion of implanted adult *S. venezuelensis* by mice treated with IL-18 plus IL-2

Because the degree of parasite expulsion is closely associated with the degree of intestinal mastocytosis in the mice in-

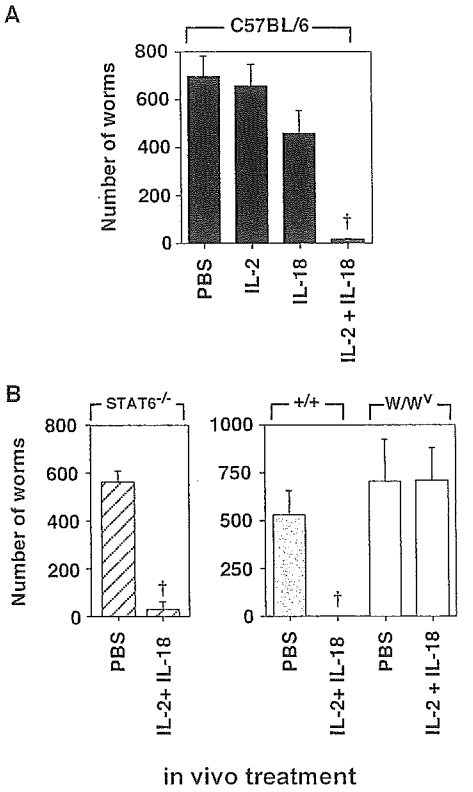


Figure 4. IL-18– plus IL-2–induced MMC-dependent expulsion of adult *S. venezuelensis*. Adult *S. venezuelensis* (1,500 worms/mouse) were implanted in the duodenum of recipient (A) C57BL/6 WT mice and (B) C57BL/6-background STAT6$^{-/-}$, WBB6F1$^{+/+}$, and WBB6F1-W/W$^v$ mice that had been injected i.p. daily with PBS alone or with IL-18 (2 μg/d) and/or IL-2 (2,000 U/d) for 13 d. 16 h after implantation, the mouse intestines ware removed, cut open longitudinally, and washed lightly to remove fecal matter and worms that were still in the lumen. Then the intestines were incubated in PBS at 37°C for 3 h. The number of worms that emerged from the intestines was counted. Results are geometric means ± SD of eight animals per group and are representative of more than three independent experiments. †, $P < 0.0001$ by Student's *t* test as compared with PBS control groups.

fected with *S. venezuelensis* (35, 36), we examined the protective role of IL-2– and IL-18–induced MMC against *S. venezuelensis* infection. Thus, we implanted adult worms in the duodenum of mice pretreated with IL-2 and/or IL-18 for 13 d. We recovered invading parasites at 16 h after implantation (Fig. 4 A; reference 36). A considerable number of the parasites were shown to invade intestines of the mice that received PBS, IL-2, or IL-18 alone. Furthermore, these mice remained heavily infected up to 7 d after treatment (unpublished data). In contrast, mice pretreated with IL-18 plus IL-2 almost completely rejected them ($P < 0.0001$; Fig. 4 A), revealing this expulsion was a rapid response. In these experiments, we injected 2μg/d of IL-18 together with IL-2 into naive mice. However, we found that a much lower dose of IL-18 (0.5 μg/d) equally prepared mice to expel implanted parasites rapidly (unpublished data). It is important to start injection of a mixture of IL-2 and IL-18 before implan-

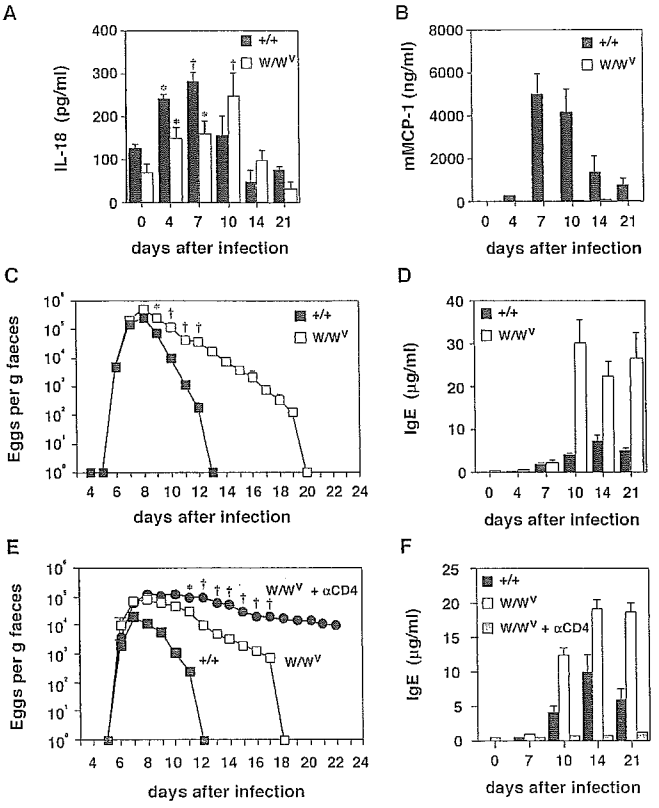


**Figure 5. Role of MMC against *S. venezuelensis* L3.** (A and B) Serum levels of (A) IL-18 and (B) mMCP-1 from WBB6F1$^{+/+}$ and WBB6F1-W/W$^{v}$ mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means ± SEM of five animals per group and are representative of three independent experiments. *, $P < 0.01$; †, $P < 0.001$ by Student's *t* test as compared with day 0. (C) Kinetics of the number of eggs per g feces and (D) serum levels of IgE from WBB6F1$^{+/+}$ and WBB6F1-W/W$^{v}$ mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means ± SEM of five animals per group. *, $P < 0.01$; †, $P < 0.005$ versus corresponding value for WBB6F1$^{+/+}$ mice. (E) Kinetics of the number of eggs per g feces and (F) serum levels of IgE from WBB6F1$^{+/+}$ mice, WBB6F1-W/W$^{v}$ mice, and CD4$^{+}$ T cell–depleted WBB6F1-W/W$^{v}$ mice inoculated with 5,000 *S. venezuelensis* L3. To deplete CD4$^{+}$ T cells, WBB6F1-W/W$^{v}$ mice were injected with anti-CD4 antibody (0.5 mg/d) on days 7 and 4 before infection and two times per wk after infection. Results are geometric means ± SEM of five animals per group. *, $P < 0.01$; †, $P < 0.005$ versus corresponding value for WBB6F1-W/W$^{v}$ mice without anti-CD4 antibody treatment.

tation (unpublished data), because appreciable induction of MMC required >10 d (Fig. 2 A). These results clearly indicated that only mice pretreated with IL-2 plus IL-18 gained the capacity to expel implanted adult parasites rapidly. As expected from the results shown in Fig. 3, STAT6$^{-/-}$ mice pretreated with IL-2 and IL-18 also gained the capacity to reject implanted parasites rapidly (Fig. 4 B).

Next, we examined whether mastocytosis induced by IL-2 plus IL-18 is critically involved in the expulsion of implanted parasites. Thus, we treated mast cell–deficient W/W$^{v}$ mice with IL-2 and IL-18 and examined their capacity to expel parasites. We found these mice failed to respond to this treatment by intestinal MMC accumulation and mMCP-1 production (unpublished data). Furthermore, they could not reject implanted parasites (Fig. 4 B). These results clearly indicated that rapid expulsion of implanted adult worms is mediated by the function of activated MMC.

### Partial contribution of activated MMC to expulsion of S. venezuelensis L3

In the preceding section, we demonstrated that MMC activation is indispensable for rapid expulsion of implanted adult worms. Next, it was important to determine whether MMCs are also critically involved in host defense against *S. venezuelensis* L3 infection. Thus, we inoculated control mice and mast cell–deficient W/W$^{v}$ mice (37) with 5,000 *S. venezuelensis* L3. We daily counted fecal egg number (eggs/g of feces). We simultaneously tested whether this *S. venezuelensis* L3 infection induced IL-18 production and found that both types of mice clearly produced IL-18 (Fig. 5 A). However, only control mice, but not W/W$^{v}$ mice, produced mMCP-1 (Fig. 5 B). Thus, MMCs are the only cell source of mMCP-1 during *S. venezuelensis* L3 infection. Inoculated control mice completed parasite expulsion by day 13, whereas production of the parasites' eggs was significantly prolonged in W/W$^{v}$ mice (Fig. 5 C). These results suggest that activated MMC markedly shortened the time required for parasite expulsion. However, we should be cautious in concluding that delayed worm expulsion by W/W$^{v}$ mice reflects only a mast cell defect. These mice have defects in peristalsis (38) and intestinal T cell populations (39), as well as a mast cell defect. Despite these defects, W/W$^{v}$ mice eventually expelled parasites by day 20 (Fig. 5 C), suggesting contribution of MMC-independent parasite expulsion mechanism. Indeed, W/W$^{v}$ mice infected by *S. venezuelensis* showed a markedly augmented IgE response (Fig. 5 D), and depletion of CD4$^{+}$ T cells abrogated their capacity to expel parasite and to produce IgE (Fig. 5, E and F). Thus, Th2 cells also play a protective role for parasite expulsion.

### Impaired expulsion of *S. venezuelensis* L3 without help from endogenous IL-18

To address the role of endogenous IL-18 in the host defense against *S. venezuelensis* L3 infection, the capacity of IL-18$^{-/-}$ mice and IL-18Rα$^{-/-}$ mice to expel *S. venezuelensis* was examined. C57BL/6 WT, C57BL/6 background IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice were inoculated with 5,000 *S. venezuelensis* L3. As shown in Fig. 6 A, IL-18$^{-/-}$ and IL-18Rα$^{-/-}$ mice, compared with infected WT mice, exhibited significantly prolonged production of the parasites' eggs, but they could expel worms by day 16. We found these deficient mice also exhibited significantly reduced levels of mMCP-1 at day 4, 7, and 10 after infection (Fig. 6 B), suggesting the importance of endogenous IL-18 for early induction of mMCP-1. However, these deficient mice and WT mice

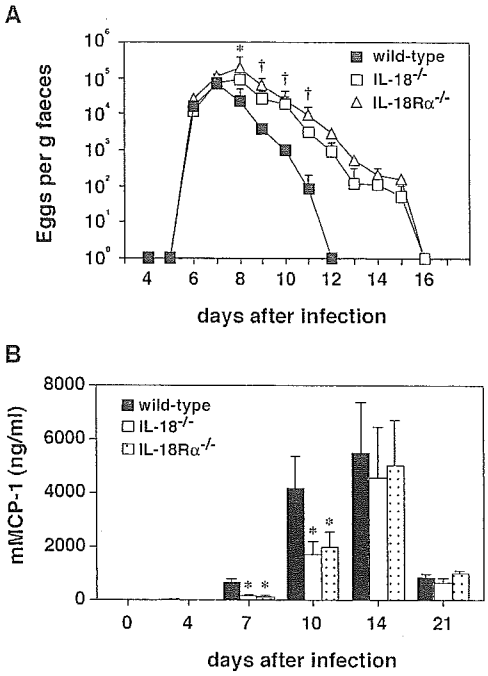


Figure 6. Role of endogenous IL-18 against *S. venezuelensis* L3. Kinetics of (A) the number of eggs per g feces and (B) serum levels of mMCP-1 from WT, IL-18$^{-/-}$, and IL-18R$\alpha^{-/-}$ mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means ± SEM of eight animals per group and are representative of three independent experiments. *, $P < 0.01$; †, $P < 0.005$ versus corresponding value for WT mice.

showed comparable levels of mMCP-1 in their sera at day 14 (Fig. 6 B), suggesting that this late mMCP-1 induction is independent of endogenous IL-18 and possibly is dependent on Th2 cells generated thereafter.

### Remarkable prolongation of *S. venezuelensis* L3 expulsion without help from Th2 cytokines and endogenous IL-18

To determine the relative contribution of Th2 cells and endogenous IL-18 to parasite expulsion, we compared the capacity of STAT6$^{-/-}$ mice receiving control antibody or anti-IL-18 and/or anti-IL-2 antibodies to expel *S. venezuelensis*. STAT 6$^{-/-}$ mice inoculated with *S. venezuelensis* L3 increased their serum IL-18 levels during infection (Fig. 7 A). Inoculated STAT 6$^{-/-}$ mice required 23 d to expel parasites (Fig. 7 B), perhaps because of their inability to produce mMCP-1 promptly (Fig. 7 C). However, they started to produce mMCP-1 at day 14 and sustained substantial levels of mMCP-1 up to day 21 after infection (Fig. 7 C). Anti-IL-18 antibody treatment only modestly prolonged the time required for parasite expulsion (Fig. 7 B) and reduced serum mMCP-1 level (Fig. 7 C). However, STAT6$^{-/-}$ mice injected with anti-IL-2/anti-IL-18 exhibited more profound defects in their ability to expel *S. venezuelensis* and to produce mMCP-1 than did STAT6$^{-/-}$ mice (Fig. 7, B and C), although neutralization of IL-2 alone did not affect parasite expulsion and serum levels of

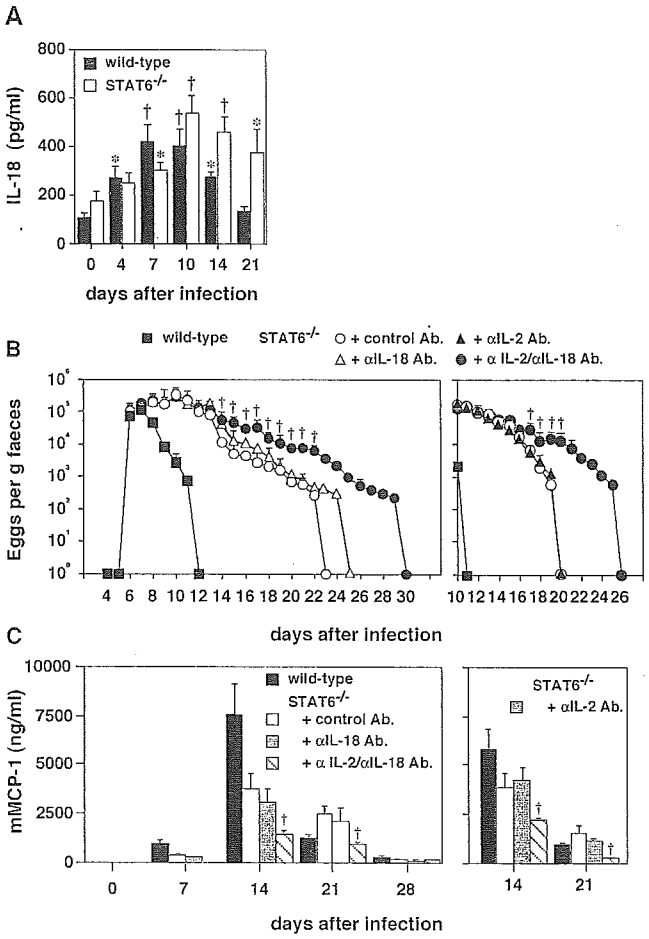


Figure 7. Role of Th2 cytokines and endogenous IL-18 against *S. venezuelensis* L3. (A) Serum levels of IL-18 from WT and STAT6$^{-/-}$ mice inoculated with 5,000 *S. venezuelensis* L3. Results are geometric means ± SEM of five animals per group and are representative of three independent experiments. Ab, antibody; *, $P < 0.01$; †, $P < 0.001$ by Student's *t* test as compared with day 0. (B) Kinetics of the number of eggs per g feces and (C) serum levels of mMCP-1 from WT and STAT6$^{-/-}$ mice inoculated with 5,000 *S. venezuelensis* L3. STAT6$^{-/-}$ mice inoculated with *S. venezuelensis* L3 were injected intravenously with anti-IL-18 (800 μg/d) and/or anti-IL-2 (100 μg/d) or control antibody (rabbit IgG and rat IgG2b) two times per wk for 2 wk after infection. Results are geometric means ± SEM of five animals per group and are representative of three independent experiments. Ab, antibody; †, $P < 0.005$ versus corresponding value for STAT6$^{-/-}$ mice injected with control antibody.

mMCP-1 (Fig. 7, B and C). These results taken together indicated involvement of two types of intestinal MMC activation, IL-18– plus IL-2–dependent and Th2 cell–dependent activation, for *S. venezuelensis* expulsion.

## DISCUSSION

Here we show that pretreatment with IL-18 and IL-2 prepares mice to expel implanted adult worms promptly by induction of intestinal mastocytosis. We also show the relevant role of endogenous IL-18 for expulsion of *S. venezuelensis* L3

by using IL-18$^{-/-}$ or IL-18R$\alpha^{-/-}$ mice. Finally, we show the collaborative action of IL-18–dependently activated MMC and Th2 cell–dependently activated MMC for rapid parasite expulsion.

The role of intestinal MMC in worm expulsion has been studied extensively in various experimental host–parasite systems. In the case of infection with *S. venezuelensis* L3, host mice complete parasite expulsion within 2 wk, and the expulsion is tightly associated with level of intestinal mastocytosis (35, 36). In contrast, mast cell–deficient W/W$^v$ mice infected with *S. venezuelensis* L3 show a significant delay in parasite expulsion (35). Furthermore, parasite expulsion is more severely impaired in W/W$^v$ mice that are deficient for IL-3 gene expression. In these mice, MMC responses are almost completely absent, and *S. venezuelensis* continue to parasitize in the intestine for >50 d (35). In this study, we have demonstrated that depletion of CD4$^+$ T cells in W/W$^v$ mice further abrogated their capacity of to expel the parasite (Fig. 5 E). Thus, Th2 cells display their protective role against parasite even in the absence of activated MMC.

It is well established that mMCP-1, selectively expressed by intestinal MMC, participates in the effecter-phase response to expulsion of intestinal nematodes (2, 26–29). Miller et al.. reported that mMCP-1 is not detectable in the culture of BM-derived mast cells stimulated with IL-3 and IL-9 (40). However, mast cells begin to produce mMCP-1 when additionally stimulated with SCF and TGF-β. Here, we have demonstrated that daily injection of a mixture of IL-18 and IL-2 induces intestinal mastocytosis (Fig. 1 A) and an increase in the serum level of mMCP-1 (Fig. 2 A). This serum level of mMCP-1 is somewhat lower than that seen in WT mice infected with *S. venezuelensis* L3 for 14 d (Fig. 2 A). We have also demonstrated that mast cell–deficient W/W$^v$ mice failed to produce mMCP-1 in response to the treatment with IL-18 and IL-2 or to the inoculation with *S. venezuelensis* L3 (Fig. 5 B). Based on these observations, we could conclude that MMC is the only producer of mMCP-1 in these circumstances.

Treatment of normal mice with IL-18 and IL-2 induced IL-3, IL-4, IL-9, and IL-13 (Fig. 1 C). Like WT mice treated with Il-18 plus IL-2, STAT6$^{-/-}$ mice displayed intestinal mastocytosis and increased serum levels of mMCP-1 after IL-18 plus IL-2 treatment (Fig. 3, A and B). Finkelman et al. have previously reported that IL-4 treatment significantly increased the number of MMC in both WT and STAT6$^{-/-}$ mice and that a much larger increase was observed in the latter, indicating that signaling through STAT6 seems to suppress IL-4 induction of MMC (33, 34). However, the IL-18–plus IL-2–induced mMCP-1 response in STAT6$^{-/-}$ mice was inhibited only partly by blocking IL-4 (Fig. 3 C), suggesting contributions of other cytokines to mMCP-1 induction. Because CD4$^+$ T cells stimulated by IL-18 plus IL-2 produce IL-3 and IL-9 as well as IL-4 and IL-13, we examined whether daily treatment with IL-3 (0.013 μg/d) and IL-9 (0.5 μg/d) for 2 wk could replace IL-18 and IL-2-treatment. We found this treatment only partly replaced the effect of IL-2 plus IL-18 treatment on serum mMCP-1 level. However, a much higher dose of IL-3 (0.1 μg/d), even without IL-9, replaced the effect of IL-2 and IL-18 (Fig. 3 E). Thus, we considered the possibility that a set of Th2 cytokines, including IL-3, IL-4, and IL-9, have an orchestrated action on mMCP-1 induction, and that neutralization of single factor could not effectively inhibit this orchestration.

In this report, we first showed that induction of intestinal mastocytosis by treatment with IL-18 plus IL-2 is sufficient to expel implanted adult worms. However, as we reported in the subsequent section, host defense against *S. venezuelensis* L3 infection is more complicated. We examined the contribution of endogenous IL-18 to parasite expulsion by using IL-18$^{-/-}$ or IL-18R$\alpha^{-/-}$ mice. WT mice infected with *S. venezuelensis* L3 showed a significant increase in serum levels of IL-18 (days 4–14) and mMCP-1 (days 7–21) and completed worm expulsion within 12 d (Fig. 6 A). In contrast, IL-18$^{-/-}$ or IL-18R$\alpha^{-/-}$ mice infected with *S. venezuelensis* L3 exhibited significantly reduced serum levels of mMCP-1 at days 4, 7, and 10 after infection, and worm expulsion was significantly delayed as compared with WT mice (Fig. 6 A). A reason for this delay might be that, like WT mice, both IL-18$^{-/-}$ and IL-18R$\alpha^{-/-}$ mice inoculated with *S. venezuelensis* L3 generated a Th2 response (unpublished data; reference 22). Thus, we assumed the possible contribution of Th2 cells to this late-phase worm expulsion.

IL-18 also is definitively involved in the host defense of STAT6$^{-/-}$ mice. Because of their defective Th2 cell development, they required a much longer period to expel parasites. Anti-IL-2 or anti-IL-18 treatment alone showed little effect on parasite expulsion, whereas anti-IL-18 plus anti-IL-2 treatment markedly impaired the parasite expulsion and significantly reduced mMCP-1 production ($P < 0.005$; Fig. 7, B and C). Nevertheless, STAT6$^{-/-}$ mice treated with anti-IL-2/anti-IL-18 still displayed mMCP-1 response after parasite infection. These results suggest participation of other factors in induction of mMCP-1. Recent reports have clearly demonstrated that nematode infection induces Th2 cytokines from basophils in the lung, liver, and spleen (41, 42), suggesting their contribution to mMCP-1 induction in STAT6$^{-/-}$ mice.

As noted previously, neutralization of IL-18 and IL-2 showed profound effects on parasite expulsion, whereas neutralization of IL-2 or IL-18 alone showed very modest effects. At present, we have no explanation that accounts for this discrepancy. We considered the possibility that antibody neutralization of IL-18 might be incomplete, and residual IL-18 in combination with endogenous IL-2 might induce mMCP-1. Alternatively, an unidentified factor might partly replace the effect of IL-2 or IL-18. Thus, regulation of the mMCP-1 response is complicated, and we need to study further how STAT6$^{-/-}$ mice expel infected parasites.

We suspected that IL-18 might be produced by various types of cells that are activated either directly or indirectly by