

RESEARCH REPORT

Suppression of cell-spreading and phagocytic activity on nano-pillared surface: *in vitro* experiment using hemocytes of the colonial ascidian *Botryllus schlosseri***L Ballarin¹, N Franchi¹, F Gasparini¹, F Caicci¹, A Miyauchi², E Hirose^{1,3}**¹*Department of Biology, University of Padua, Padua, Italy*²*Hitachi Research Laboratory, Hitachi Ltd., Omika, Hitachi, Ibaraki 319-1292, Japan*³*Faculty of Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan**Accepted February 23, 2015***Abstract**

Nano-scale nipple array on the body surface has been described from various invertebrates including endoparasitic and mesoparasitic copepods, but the functions of the nipple array is not well understood. Using the hydrophilized nanopillar sheets made of polystyrene as a mimetic material of the nipple arrays on the parasites' body surface, we assayed the cell spreading and phagocytosis of the hemocytes of the colonial ascidian *Botryllus schlosseri*. On the pillared surface, the number of spreading amoebocytes and the number of phagocytizing hemocytes per unit area were always smaller than those on the flat surface (Mann-Whitney test, $p < 0.05 - 0.001$), probably because the effective area for the cell attachment on the pillared surface is much smaller than the area on the flat sheet. The present results supports the idea that the nipple array on the parasites' body surface reduces the innate immune reaction from the host hemocytes.

Key Words: hemocyte; innate immunity; nanopillar sheet; nipple array; endoparasite**Introduction**

The surface structure of integumentary tissue influences its interaction with various factors in external environment. The moth-eye corneal nipple array is one of the most well-known examples, where the refractive index gradient reduces eye glare to decrease visibility to predators (e.g., Bernhard, 1967; Stavenga *et al.*, 2006). Similar surface structures have been described in aquatic or parasitic invertebrates such as annelids (Hausen, 2005), entoprocts (Iseto and Hirose, 2010; Nielsen and Jespersen, 1997), echinoderms (Holland, 1984), and tunicates (Hirose *et al.*, 1997, 1999). The size of nipples is almost uniform in each species but varies among species: 150 - 20 nm in height in ascidians (Hirose *et al.*, 1997). Although these nipple arrays may have various functions, it is often difficult to examine the functions using live materials. The development of nanoimprint technology has enabled the fabrication of polymer sheets with nanoscale structures such as nanopillars (Kuwabara and Miyauchi, 2008). Using nanopillar sheets as a mimetic model for the nipple arrays, we can examine the properties of the nipple arrays in various experimental conditions, and we recently

demonstrated the bubble repellency and anti-glare property of the nipple array in the water (Hirose *et al.*, 2013; Hirose *et al.*, in press). This biomimetic approach can contribute not only for better understanding the functions of biological structure but also for technological application of the functions.

Nipple array was also found on the cuticle surface of the body in some endoparasitic and mesoparasitic copepods infesting marine fish and invertebrates (Østergaard and Bresciani, 2000; Hirose and Uyeno, 2014). Depending on the species, the size of each nipple is quite various: from 1 μm to 20 nm in height. For these parasites, neither anti-glare nor bubble repellence would be important functions, since the parasite's body is enveloped in the host tissue. Nomura *et al.* (2005) showed that the HeLa cells do not spread on the synthetic nanopillar sheet, and thus, the pillared surface may have suppressive effects on cell attachment of the host organisms. Therefore, the nipple array on parasitic organisms may have protective function against the immunocytes of the host organisms. To test this hypothesis, hemocytes from a marine invertebrate was incubated on the nanopillar sheets as a mimetic model of the nipple array on the parasite integuments.

Ascidians are sessile, marine invertebrates and they have various endoparasites, including notodelphyid copepods (e.g., Monniot, 1990). Although integumentary ultrastructures were rarely

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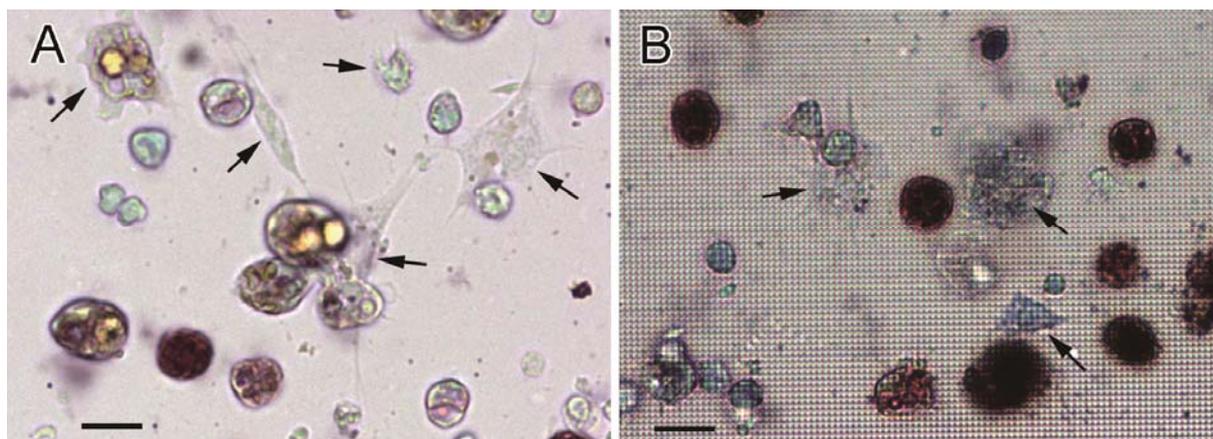


Fig. 1. Giemsa-stained hemocytes of *B. schlosseri* on hydrophilized polystyrene flat (A) and pillared (B) sheets. Arrows indicate spreading hemocytes. Scale bars = 10 µm.

investigated, tiny nipple arrays were found in an endoparasitic copepod (Uyeno, unpublished). As a primitive chordate, the colonial ascidian *Botryllus schlosseri* is one of the model animals among marine invertebrates for studies on innate immunity and colonial allo-recognition (Ballarin, 2008). A common vascular system is anastomosed within the colony, and several types of hemocytes circulate throughout the colony. To date, seven types of four categories of hemocytes were described from *B. schlosseri* and characterized morphologically and cytochemically for the functional characterization (Ballarin and Cima, 2005), and many of these hemocytes are involved in innate immunity and colonial allo-recognition (Sabbadin *et al.*, 1992; Ballarin, 2008, 2012). Moreover, immunocytotoxicity to the *Botryllus* hemocytes was utilized for the assessment of the toxic effects of marine pollutants (*e.g.*, Ballarin and Cima, 2001, Cima *et al.*, 2008). In *B. schlosseri*, there are two types of amebocytes, *i.e.*, hyaline amebocytes and granular amebocytes, that are respectively involved in phagocytosis and cytotoxicity (Ballarin and Cima, 2005). The granular amebocytes are precursors of morula cells that are the major effector cells releasing phenoloxidase against foreign cells, such as

bacteria and yeasts, and in the allogeneic rejection reactions (Ballarin *et al.*, 1995, 2005). In another colonial ascidian *Aplidium yamazii*, phagocytic amebocytes are involved in the formation of cell aggregation to reject allogeneic colonies (Ishii *et al.*, 2008). To date, it is uncertain whether the botryllid ascidians have endoparasites with nipple arrays or not, due to the lack of studies. Nevertheless, *B. schlosseri* is convenient marine invertebrates to study the potential function of the nipple arrays reducing the immune responses of the host organisms, since the hemocytes involved in innate immunity has been well documented as mentioned above. In this study, we compare the cell spreading and phagocytic activity of the *Botryllus* hemocytes on flat and nanopillared surfaces to elucidate the potential function of the nipple array on parasite integument to immune responses from the host animals.

Materials and Methods

Animals

Colonies *Botryllus schlosseri* from the Venetian Lagoon were attached on glass plates, reared in aerated aquaria in the laboratory and fed with Liquifry

Table 1 The number of spreading amebocytes per optical field*

Trials	Cell density ($\times 10^6$ cells/ml)	Flat sheet Average \pm SD	Pillared sheet Average \pm SD	Statistic difference [#]
1	1.5	1.77 \pm 1.12	0.90 \pm 0.83	$p < 0.01$
2	1.0	1.90 \pm 1.51	1.10 \pm 1.25	$p < 0.05$
3	2.1	2.40 \pm 1.56	1.40 \pm 1.17	$p < 0.05$
4	1.4	1.83 \pm 1.13	1.00 \pm 0.93	$p < 0.01$
5	1.5	2.13 \pm 1.20	1.10 \pm 0.94	$p < 0.001$

*Averages are based on the cell counts in 30 optical fields for each.

[#]Mann-Whitney test.

Marine (Liquify Co., Dorking, England). The blastogenetic cycle of each colony was checked under a stereomicroscope, because the composition of hemocyte types changes depending on the phase of the cycle (Ballarin *et al.*, 2008).

Hemocyte collection

For hemocyte collection, we used the colonies in which the combination of the developmental stages of zoid and buds was 9/8/2, *i.e.*, phase B (see Watanabe, 1953; Sabbadin, 1955; Manni *et al.*, 2007). One to three systems containing 10 - 30 zooids were immersed in filtered seawater (FSW) containing 0.38 % Na-citrate (pH 7.5) to prevent hemocyte clustering. The tunic vessels and zooids in the colonies were punctured with fine tungsten needles and hemocytes leaked from the colonies were collected in a micro tube on ice and incubated for 5 min to allow debris sedimentation. The supernatant was then transferred to another micro tube and centrifuged at 780 *g* for 10 min. Pellets were re-suspended in FSW at $1 - 2.2 \times 10^6$ cells/ml. Cell concentration was determined using a Bürker hemocytometer. Two hundred μL of hemocyte suspension were spread over a polystyrene sheet (20x20 mm) that was hydrophilized by oxygen plasma etching (EXAM, Shinko Seiki) to imitate an organic surface. Each sheet had a flat surface or arrays of 1- μm -high pillars of 0.5 μm diameter at 1 μm interval between the centers of the neighbor pillars (Kuwabara and Miyauchi, 2008). The hemocytes on the sheet were left to adhere to the polystyrene sheets for 30 min at room temperature.

Number of spreading amebocytes

Hemocytes on the sheets were fixed in FSW containing 1 % glutaraldehyde and 1 % sucrose for 20 min at room temperature, washed in distilled water and stained with 10 % Giemsa's solution for 10 min. The polystyrene sheets were mounted with distilled water on glass slides and the Giemsa-stained hemocytes were observed under an Olympus CX31 light microscope equipped with a CCD camera (Infinity 2, Lumenera Corp.). The number of the spreading hemocytes was counted in 30 optical fields, at the magnification of 400x, for each sheet. One optical field was about 120x160 μm . The hemocytes having pseudopodia (*e.g.*, filopodia and lamellipodia) were regarded as the spreading amebocytes. We made five replicates using different clones of colonies. These spreading amebocytes potentially include two cell types: hyaline amebocytes and granular amebocytes that are precursors of macrophage-like cells and morula cells, respectively (Ballarin and Cima, 2005). Here, we did not distinguish the two types, because they were often difficult to be distinguished particularly on the pillared surface. The detachment of the amebocytes was also tested preliminarily. A sheet with hemocytes was gently dipped in FSW for six times before fixation, and the number of spreading hemocytes on the sheet was determined as reported above so to compare the cell number on the same type of sheet without dipping. The number of all types of hemocytes was also counted in 30 optical fields.

Phagocytosis activity

Ordinary baker's yeast (*Saccharomyces*

cerevisiae) was suspended in FSW at a concentration around 1.5×10^7 cells/ml and 200 μl of the yeast suspension were added on the flat and pillared sheet on which hemocytes were attached. After 1-h incubation at room temperature, the sheets were dipped in FSW to remove free yeast cells, and the hemocytes were fixed as described above. Following Giemsa's staining, the number of hemocytes containing yeast cells was counted in 20 optical fields at the magnification of 400x for each sheet. We made four replicates: a pair of duplicate tests using the same colony and two independent tests using different clones of colonies.

Statistics

The Student *t*-test was used to compare the mean cell number in pillared and flat sheets or in the sheets with and without dipping, when the normality of the data distribution could be assumed, passing the Kolmogorov-Smirnov test. The Mann-Whitney test was used when the normal distribution was not supported. We performed the analyses using InStat software (v. 3.1a, Graphpad Software, 2004).

Scanning electron microscopy

The sheets attaching the hemocytes were fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer containing 1.7 % sodium chloride (pH 7.4) on ice. The specimens were dehydrated through an ethanol series and dried at critical point. The sheets were cut into small pieces and sputter coated with gold-palladium. The hemocytes on the sheet were examined with a JEOL JSM-6060LV scanning electron microscope at 15 kV.

Results

Number of spreading amebocytes

Hemocytes of *B. schlosseri* adhered to the hydrophilized polystyrene sheet regardless of the presence of nanopillars. Among the various types of the hemocytes on the sheets, amebocytes spread their cytoplasm and extended the pseudopodia on the flat sheet (Fig. 1A) and the pillared sheet (Fig. 1B). The number of these spreading amebocytes per optical field (ca. 120x160 μm) was always larger on the flat sheets than on the pillared sheets (Table 1). Significant differences were shown in the all five replicates using different clones of colonies (Mann-Whitney test, $p < 0.05 - 0.001$).

Dipping the sheets in FSW had no significant effect on the detachment of the spreading amebocytes on the pillared sheet (Mann-Whitney test, $p > 0.05$): cell numbers per optical field were 1.2 ± 1.34 (average \pm SD) on the dipped sheet and 1.1 ± 0.96 on the control (no dipping). Similarly, there was no significance on the flat sheet (Mann-Whitney test, $p > 0.05$): cell numbers per optical field were 1.57 ± 1.2 on the dipped sheet and 2.13 ± 1.22 on the control. However, the number of all cell types on the pillared surface was significantly decreased by dipping (Mann-Whitney test, $p < 0.01$): 7.63 ± 2.10 on the dipped sheet and 10.8 ± 2.83 on the control. Significant difference was not found for the number of all cell types on the flat sheets (Student *t*-test, $p > 0.05$): 8.9 ± 3.33 on the dipped sheet and 9.73 ± 2.61 on the control.

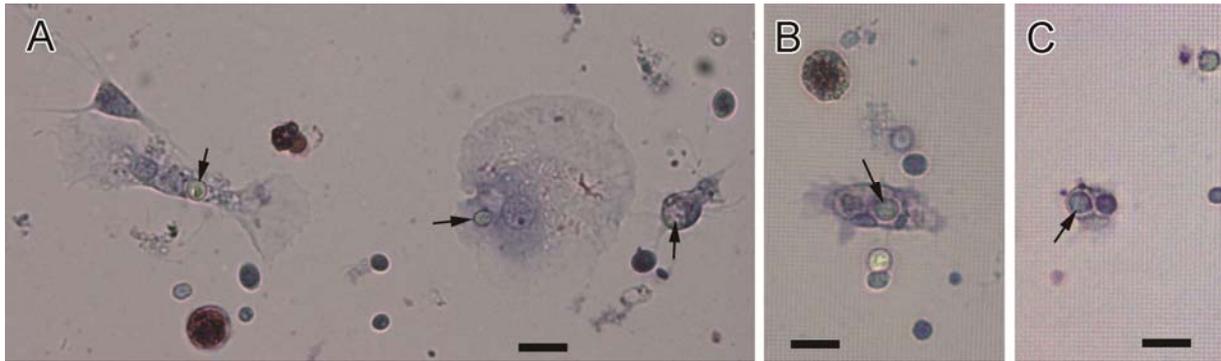


Fig. 2. Hemocytes of *B. schlosseri* on the flat (A) and the pillared (B, C) sheets, incubated with yeast cells. Arrows indicate yeast cells contained in the hemocyte. Scale bars = 10 μm .

Phagocytic activity

Phagocytosis of yeast cells was observed on both of the flat sheets (Fig. 2A) and the pillared sheets (Figs 2B, C). The number of the cell engulfing the yeast cell per optical field (ca. $120 \times 160 \mu\text{m}$) was always larger on the flat sheets than that on the pillared sheets (Table 2). Significant differences were shown in the all trials (Mann-Whitney test, $p < 0.05 - 0.0001$).

Cell morphology on the sheets

Both spherical and spreading hemocytes were found on the sheets, but many of the well-spreading, fattened cells were partially damaged in the specimens for SEM observation (Figs 3A, E). The hemocytes attached on the substrate with various forms of pseudopodia (e.g., lamellipodia and filopodia). On the flat surface, the hemocytes appeared to adhere to the substrate with the almost entire substratum-side of the pseudopodia (Figs 3B - D). On the pillared surface, the hemocyte adhered only to the tips of the pillars, and the pseudopodia did not adhere to the lateral surface of the pillars (Figs 3F - I). The pillars at the periphery of the lamellipodia were broken at their bases (arrowheads in Fig. 3G) probably due to the shrinkage of the cells during the critical-point drying, indicating that the pseudopodia adhered to the pillars. The filopodia also adhere to the pillar tips, and they strode over the intervals between the pillars (Fig. 3I).

Discussion

When foreign materials are introduced in the ascidian body, hemocytes endocytize the small materials (phagocytosis) and adhered to the large materials (encapsulation) (e.g., Anderson, 1971; Wright and Cooper, 1975; Parrinello *et al.*, 1984). These are primary innate immune reactions to protect the body from pathogens and parasites. The colonial ascidian *B. schlosseri* contains various types of hemocytes and some of the hemocyte types have phagocytic activity and some are involved in inflammatory responses (Ballarin and Cima, 2005; Ballarin *et al.*, 1993; Ballarin, 2008). Here, using the hydrophilized nanopillar sheets made of polystyrene as a mimetic material of the nipple arrays on the body surface of endoparasitic/mesoparasitic organisms, we assayed the cell spreading and phagocytosis of the hemocytes of the colonial ascidian *B. schlosseri* to reveal the influence of the nipple array on immunocyte functionality. The ascidian hemocytes well adhered to the hydrophilized polystyrene sheets regardless of the presence or absence of nanopillars. However, the number of spreading amebocytes was significantly larger on the flat surface than the pillared surface, indicating that the spreading of amebocytes was reduced on the pillared surface. Moreover, when the hemocytes were incubated with yeast cells, the number of hemocytes phagocytizing yeast cells was

Table 2 The number of yeast-containing cells per optical field*

Trials	Cell density ($\times 10^6$ cells/ml)	Flat sheet Average \pm SD	Pillared sheet Average \pm SD	Statistic difference [#]
1 [†]	2.2	2.30 \pm 1.53	0.40 \pm 0.68	$p < 0.0001$
2 [†]	2.2	1.75 \pm 0.97	0.75 \pm 0.85	$p < 0.001$
3	1.3	2.20 \pm 0.95	0.95 \pm 0.89	$p < 0.001$
4	1.5	1.45 \pm 0.94	0.80 \pm 0.68	$p < 0.05$

*Averages are based on the cell counts in 20 optical fields for each.

[#]Mann-Whitney test.

[†]These two trials were performed as duplicate tests using the same colony.

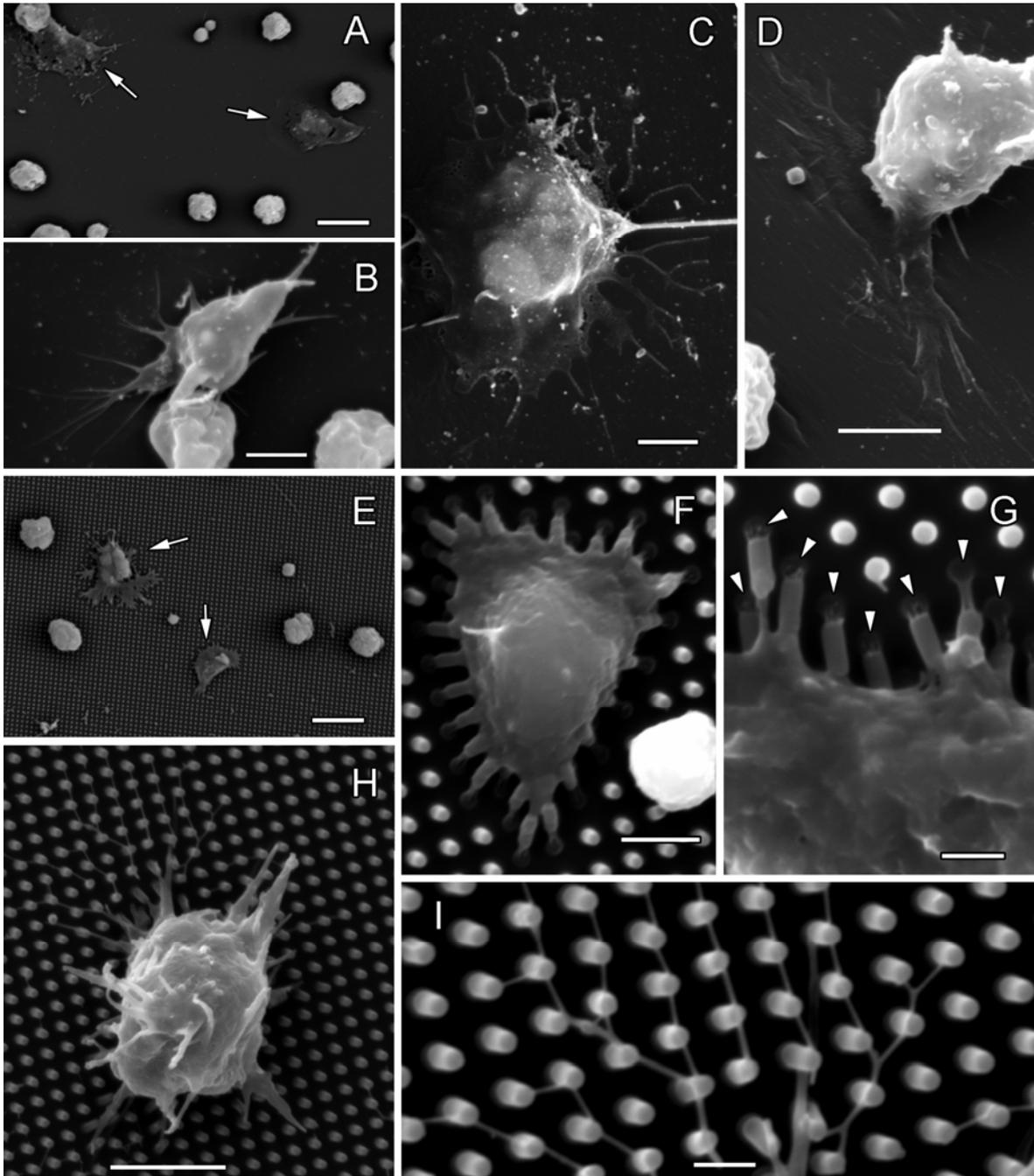


Fig. 3. SEM micrograph of round and spreading hemocytes of *B. schlosseri* on the flat (A - D) and on the pillared (E - I) sheets. Spreading hemocytes were partially damaged during the sample preparation (arrows in A and E). They extended pseudopodia on the flat substrate (B - D) and lamellipodia on the pillared substrate (F). Enlargement of the attachment between the cell and the pillar tips is shown in G. Pillars at the cell periphery were broken at their bases (arrowhead in G). Spreading hemocytes extended branching filopodia on the pillared substrate (H; enlarged in I). Filopodia adhered to the pillar tips and strode over the intervals between the pillars (I). Scale bars = 10 μm (A, E), 5 μm (B, D, H), 2 μm (C, F), 1 μm (G, I).

also significantly larger on the flat surface than the pillared surface. The smaller number of spreading amebocytes on the pillared sheet possibly causes the smaller number of phagocytizing cells than that on the flat sheet. However, the number of spreading amebocytes does not simply indicates the number of

phagocytic cells on the sheet, because some phagocytic hemocytes, e.g., hyaline amebocytes and granular amebocytes, would not spread on the pillared sheet. In any cases, the phagocytic activity in the same amount of blood is significantly reduced on the pillared surface.

The primary cause suppressing the cell spreading is supposed to be the reduction of the area of the cell adherence to the substratum. On the pillared sheets, the hemocytes exclusively adhered to the tip of the pillars (0.5 μm diameter-circle), whereas the hemocytes appeared to adhere to the flat sheets with almost the entire area of the cells. According to the specifications of the nanopillar sheet, the effective area for the cell attachment on the pillared surface is one fifth of the area on the flat sheet. Less stable cell-attachment on the pillared surface may also explain the suppression of the phagocytic activity of the hemocytes. Actually, cell adhesion to any materials involves several molecular steps, and opsonization by various molecules enhances phagocytosis of foreign materials. Therefore, the present results suggest the potential function of nano-surface structures on endoparasites only from the viewpoint of cell spreading and phagocytosis but the real interactions between hosts and parasites should be more complex and remain uncertain. It should be also considered that microorganisms and/or debris might partly (or entirely) cover the surface and change the properties of the surface in natural condition. However, the nipple arrays of the body surfaces were often free from epibionts and debris in electron micrographs (e.g., Østergaard and Bresciani, 2000; Hirose and Uyeno, 2014).

Nomura *et al.* (2005) reported that the HeLa cells on the pillared sheet were easily detached by pipetting, probably because the area of the cell adherence to the substratum is small on the pillared surface. In the present study, dipping of the sheet significantly reduced the number of total hemocytes on the pillared sheets but not on the flat sheet. This is consistent with the results on HeLa cells. In contrast, dipping of the sheet did not reduce the number of the spreading amoebocytes on the pillared and flat sheet, indicating that the spreading cells firmly adhered even on the pillared surface enough to remain on the sheet through dipping. In SEM observation, the pillars at the periphery of the lamellipodia were broken at their bases; the cell shrinkage during the critical-point drying probably pull the pillars down. This may indicate that the pseudopodia attached to the pillar tips with considerable adhesive strength. It should be noted that the glutaraldehyde-fixation harden the cell and each pillar (0.5 μm diameter) was soften at the condition of critical-point drying ($> 35^\circ\text{C}$, $> 7.4 \text{ MPa}$) in the preparation of SEM specimens.

Nano-scale nipple array on the body surface has been described from various invertebrates including endoparasitic and mesoparasitic copepods (Østergaard and Bresciani, 2000; Hirose and Uyeno, 2014). In the host tissue, these parasites should evade or resist host immune responses, such as encapsulation by hemocytes. The present results showed the nanopillar structure significantly reduces cell spreading and phagocytic activity of ascidian hemocytes. The pillared surface would be difficult to adhere and easy to separate for the hemocytes, because of the small area for the cell attachment. Accordingly, the nipple array on the parasite body surface may reduce the innate immune reaction from the host hemocytes. Recently, synthetic micro-dimple arrays, 3 - 7 μm in depth, were shown to decrease friction of the surface (Hirai *et al.*, 2013), and nipple

arrays may also make low friction surfaces. For instance, the parasites may be able to evade the encapsulation or have an extension of time until the completion of the encapsulation. The affinity of the pillared surface to the hemocytes would vary depending on the diameter of the pillars and the interval among the pillars. The nanopillar sheets used in this study is not exactly the same as those of the parasites' body surface, and, optimizing the parameters, the pillared surface may have lower affinity to the cells.

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