based on the position of the alignment marks (Fig. 3B) are used to pattern the substrate with a second set of 50-nm parallel ODT SAM lines (contact force ~0.1 nN, scan speed = 4 Hz) (Fig. 3C). These lines are placed in interdigitated manner and with near perfect registry with respect to the first set of MHA SAM lines (Fig. 3C).

An additional capability of DPN, which we refer to as "overwriting," involves generating one soft structure out of one type of ink and then filling in with a second type of ink by raster scanning across the original nanostructure. Because water is the transport medium in the DPN experiment and the water solubilities of the inks used in these experiments are very low, there is no detectable exchange between the molecules used to generate the nanostructure and the ones used to overwrite on the exposed gold (Fig. 4). We used a MHA-coated tip to generate three geometric structures, a triangle (80-nm linewidth, 30-s writing time per side), square (60-nm linewidth, 20-s writing time per side), and pentagon (30-nm linewidth, 8-s writing time per side) (contact force ~0.1 nN, scan speed = 4 Hz, relative humidity ~35%). The tip was then changed, and a 3 μm by 3 μm area that comprised the original nanostructures was overwritten with an ODT-coated tip by raster scanning four times across the substrate (contact force ~0.1 nN, scan speed = 4 Hz). Increasing the scan size to 4.3 μm by 4.3 μm and imaging the patterned areas with an uncoated tip (contact force ~0.1 nN, scan speed = 5 Hz, relative humidity ~35%) shows the MHA-patterned areas (white, high friction), the ODT-overwritten areas (dark blue, low friction), and the surrounding unmodified Au (light blue, medium friction).

The multiple ink capabilities of DPN will offer opportunities to begin studying the interactions between highly sophisticated, multicomponent nanostructures and molecules in solution or the gas phase on a scale that was previously unattainable. Moreover, they will empower those in the field of molecule-based electronics to generate and evaluate customized multicomponent soft nanostructures that are interfaced with macroscopically addressable circuitry.

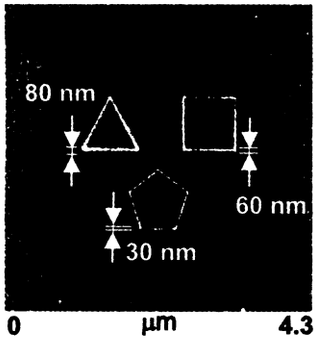

Fig. 4. SAMs in the shapes of polygons drawn by DPN with MHA on an amorphous Au surface. An ODT SAM has been overwritten around the polygons.

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β-Defensins: Linking Innate and Adaptive Immunity Through Dendritic and T Cell CCR6

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Defensins contribute to host defense by disrupting the cytoplasmic membrane of microorganisms. This report shows that human β-defensins are also chemotactic for immature dendritic cells and memory T cells. Human β-defensin was selectively chemotactic for cells stably transfected to express human CCR6, a chemokine receptor preferentially expressed by immature dendritic cells and memory T cells. The β-defensin–induced chemotaxis was sensitive to pertussis toxin and inhibited by antibodies to CCR6. The binding of iodinated LARC, the chemokine ligand for CCR6, to CCR6-transfected cells was competitively displaced by β-defensin. Thus, β-defensins may promote adaptive immune responses by recruiting dendritic and T cells to the site of microbial invasion through interaction with CCR6.

Defensins, a family of small (3.5 to 4.5 kD) cationic antimicrobial peptides with three to four intramolecular cysteine disulfide bonds, are found in mammals, insects, and plants (*1–4*). On the basis of the position and bonding of six conserved cysteine residues, defensins in vertebrates are divided into two categories, designated as α- and β-defensins (*1, 2*). Unlike α-defensins that are produced by neutrophils and intestinal Paneth cells, β-defensins are primarily expressed by epithelial cells of the skin, kidneys, and tracheabronchial lining of nearly all vertebrates, where they can be released upon microbial invasion or up-regulated by stimulation with lipopolysaccharide and tumor necrosis factor–α (TNF-α) (*2, 5, 6*). Two types of human β-defensins (HBDs), β-defensins 1 and 2

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(HBD1 and HBD2, respectively), have been identified (*6, 7*). Inactivation of the antimicrobial activity of HBDs is reported to contribute to the recurrent airway infections in patients with cystic fibrosis (*8, 9*).

The α-defensins may also have roles in protecting the host, based on their capacity to chemoattract T cells (*10*), to promote host immunity (*11*), and to activate the classical complement pathway (*12*). Because β-defensins are released upon microbial invasion and are located at the host-environment interface, such as mucosal surfaces and skin (*2, 5, 6*), they may also function to alert the adaptive immune system of vertebrates. We therefore investigated whether HBDs could chemotactically mobilize human dendritic cells (DCs), monocytes, and T cells (*13, 14*). Psoriatic skin-derived pure HBD2 (skin HBD2), synthetic HBD2 (sHBD2), and recombinant HBD2 (rHBD2), all induced substantial in vitro migration of $CD34^+$ progenitor–derived DCs in a dose-dependent manner, with optimal concentrations of HBD2 usually at 1000 ng/ml (Fig. 1A). Liver and activation-regulated chemokine (LARC, also known as MIP-3α), which selectively acts on immature DCs (*15*), was also chemotactic (Fig. 1A). Fluorescence-activated cell sorting (FACS) analyses (*16*) revealed that these DCs expressed very little CD83, low amounts of CD1a and CD86, and a moderate amount of human leukocyte antigen–DR (HLA-DR) (Table 1), which are phenotypic characteristics of immature DCs (*17*). Mature DCs (*14*) demonstrated high expression of CD1a, CD83, CD86, and HLA-DR (Table 1) and did not migrate in response to HBD2 (*18*). Examination of other mononuclear cell types revealed that sHBD2 chemoattracted only the memory subset of human peripheral blood T ($CD4^+/CD45RO^+$) cells (Fig. 1B); naïve T ($CD4^+/CD45RA^+$) cells or monocytes were not chemoattracted (*18*). Moreover, migration of DCs was inhibitable by treatment with pertussis toxin (PTX) (*18*), suggesting that HBDs might use one or more seven–transmembrane domain receptor or receptors coupled to Giα protein. Thus, HBDs might use one or more of the chemokine receptors (*19*).

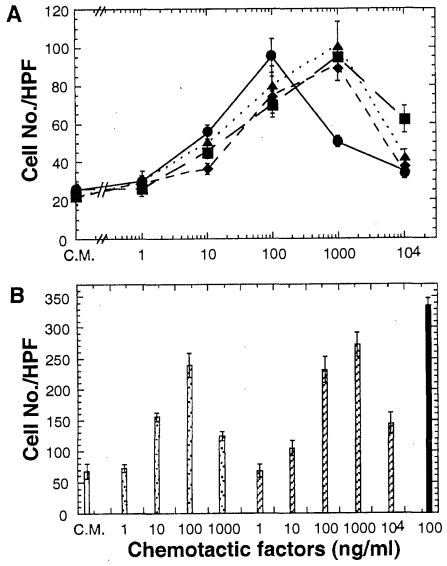

Fig. 1. Chemotaxis by HBDs of human immature DCs and memory T cells. (**A**) Migration of $CD34^+$ progenitor–derived DCs in response to skin HBD2 (■), rHBD2 (◆), sHBD2 (▲), and LARC (●). (**B**) Chemoattraction of human peripheral blood memory T cells by LARC (dotted bars), sHBD2 (hatched bars), and SDF-1α (black bar). The migration of immature DCs and memory ($CD4^+/CD45RO^+$) T cells (*14*) in response to HBDs and chemokines was examined by chemotaxis assay (*13*). Filter membranes of 5-μm pore size, uncoated or coated with fibronectin, were used to assay the migration of immature DCs and memory T cells, respectively. A representative of two to three experiments is shown. The results are presented as the number of cells per high power field (HPF). Error bars, SD of triplicated wells.

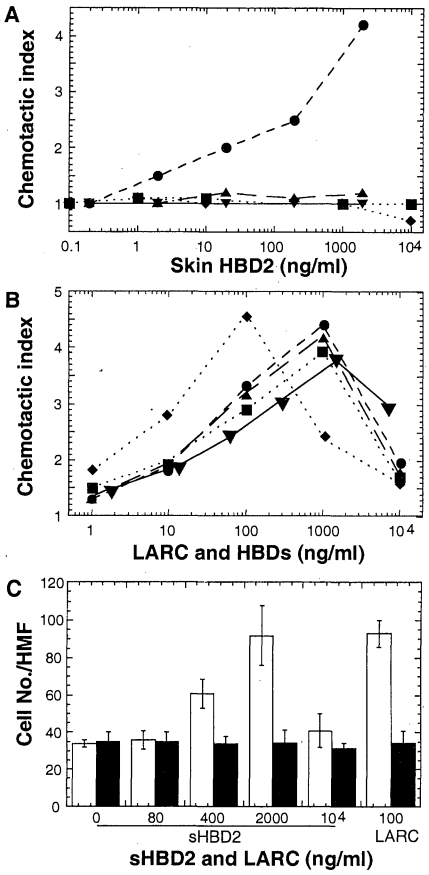

Fig. 2. Selective induction of CCR6/293 cell migration by HBDs. (**A**) Migration of HEK293 (▼), CXCR4/293 (■), CCR1/293 (▲), CCR5/293 (◆), or CCR6/293 (●) cells to skin HBD2. (**B**) Migration of CCR6/HEK293 cells in response to LARC (◆), skin HBD2 (▲), rHBD2 (●), sHBD2 (■), and sHBD1 (▼). Cell migration was investigated by chemotaxis assay (*13*), with a 10-μm filter membrane precoated with rat-tail collagen (Sigma). Spontaneous cell migration varied, but it was typically in the range of 30 to 50 cells per HPF. Error bars are omitted for clarity. (**C**) Sensitivity of sHBD2-induced CCR6/293 cell migration to PTX. The cells were incubated at 37°C for 30 min without (white bar) or with (black bar) PTX (100 ng/ml) (Sigma) before chemotaxis assays. Similar results were obtained from at least two separate experiments. Error bars, SD of triplicated wells.

Only four of the chemokine receptors (including CXCR4, CCR1, CCR5, and CCR6) are expressed on $CD34^+$ progenitor–derived immature DCs (*15, 20–22*). We therefore tested whether HBDs could attract human embryonic kidney 293 (HEK293) cell lines stably transfected to express these receptors (*23–26*). Skin HBD2 induced the migration of CCR6-expressing HEK293 (CCR6/293) cells in a dose-dependent fashion but did not induce directional migration of CXCR4/293, CCR1/293, CCR5/293, or parental HEK293 cells (Fig. 2A). Skin HBD2, rHBD2, sHBD2, sHBD1, and LARC were chemotactic for CCR6/293 cells in a typical bimodal manner (Fig. 2B). LARC was more potent than HBDs in chemoattracting immature DCs, memory T cells (Fig. 1), and CCR6/293 cells (Fig. 2B), although HBDs and LARC showed similar efficacy. This may be attributable to the smaller size of HBD peptides. Because sHBD2 is indistinguishable from natural HBD2 with respect to its antimicrobial activity and structure (*27*) and because it showed chemotactic potency and efficacy similar to that shown by other HBDs (Figs. 1 and 2B), we used sHBD2 for most of our subsequent experiments. As expected, CCR6/293 cell migration induced by sHBD2 and LARC was blocked by 30 min before treatment with PTX (100 ng/ml) at 37°C (Fig. 2C).

To test for chemokinesis, we performed a simplified checkerboard analysis by comparing sHBD2 and LARC, the sole chemokine ligand identified so far for CCR6 (*15, 18, 19, 22*). Synthetic HBD2 and LARC were chemotactic for CCR6/293 cells because equal concentrations of these agents in the upper and lower wells of the chemotaxis chamber did not increase cell migration over medium control (Fig. 3A, compare bars 3 and 5 with bar 1). The presence of LARC in the upper wells largely attenuated sHBD2-induced CCR6/293 migration and vice versa. Consequently, sHBD2 and LARC appeared to compete with each other for use of CCR6. This possibility was further tested by examining whether sHBD2 could competitively inhibit the binding of LARC to CCR6/293 cells. Although less potent than unlabeled LARC, sHBD2 displaced the binding of iodinated LARC (^{125}I-LARC) to CCR6/293 cells in a dose-dependent manner with a median effective concentration of ~700 ng/ml (Fig. 3B). This difference in affinity correlates with the data showing that HBDs were less potent than LARC in attracting immature DCs, memory T

cells, and CCR6/293 cells (Figs. 1 and 2B).

Next, the role of CCR6 in DC migration induced by HBDs (Fig. 1A) was investigated. About 50% of immature DCs generated (*14*) were shown by FACS analysis to express CCR6 on their surface (*28*). Moreover, DC migration induced by a suboptimal dose (100 ng/ml) of sHBD2 was inhibited by 50 μg/ml of antibodies to CCR6 [immunoglobulin G2b (IgG2b) (R&D Systems, Minneapolis, MN)], but it was not inhibited by 50 μg/ml of isotype-matched antibodies to CCR5 (IgG2b) (Fig. 3C, dotted bars). This inhibition was antibody dose-dependent (*28*). Additionally, antibody to CCR6 alone had no influence on background DC migration, but it did inhibit DC migration induced by suboptimal dose (10 ng/ml) of LARC (Fig. 3C, hatched bars). Therefore, CCR6 expressed by DCs was involved in the migration of DCs in response to HBDs, but the possibility that β-defensins also use other receptors cannot be ruled out.

Collectively, the results indicate that β-defensins use CCR6, at least, as a receptor. Although β-defensins show no sequence homology with LARC, they presumably have similar tertiary CCR6 binding sites and act as "microchemokines." This is analogous to data showing that a number of viral genome-encoded proteins, some of which, although apparently sharing no sequence homologies, can nevertheless interact with chemokine receptors (*29*). The affinity of HBDs for CCR6, which is lower than that of LARC, may be compensated for by the availability of HBDs in higher concentrations at the sites of microbial invasion. Because DCs and T cells are important in adaptive immune responses (*17*), HBDs in vivo may, through their interaction with CCR6, function to recruit immature DCs and memory T cells to cutaneous or mucosal sites of microbial invasion. Therefore, we propose that β-defensins have developed the capacity to play important roles in both innate and adaptive immune responses against microbial invasion.

Table 1. Surface marker expression of DCs. Immature and mature DCs were generated from $CD34^+$ progenitor cells as described (*14*) and analyzed by FACScan (*15*). At least 2500 events were collected for each sample. The mean ± SD of two separate experiments is given. MFI, median fluorescence intensity.

Marker	Immature DCs		Mature DCs	
	Positive (%)	MFI	Positive (%)	MFI
CD1a	24.9 ± 3.7	2.6 ± 0.3	83.1 ± 11.0	11.9 ± 5.6
CD83	2.2 ± 1.2	1.5 ± 0.1	68.8 ± 0.1	3.2 ± 0.8
CD86	26.2 ± 4.4	9.0 ± 1.5	77.4 ± 2.0	46.3 ± 3.2
HLA-DR	94.3 ± 0.4	47.7 ± 4.0	97.0 ± 1.2	317.0 ± 10.0

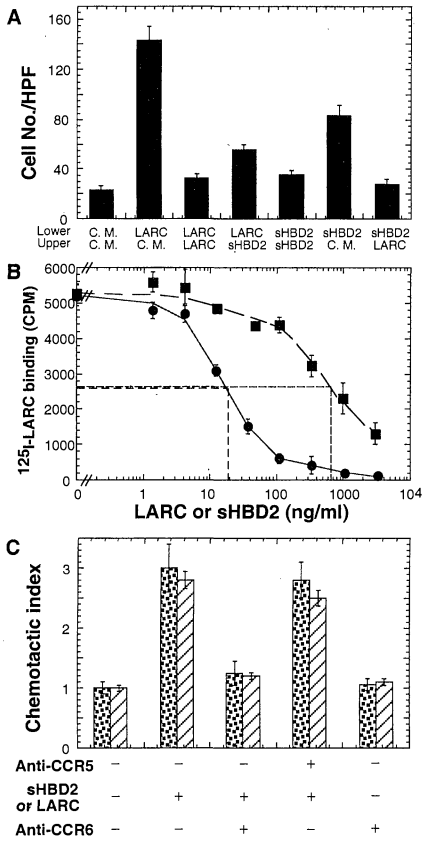

Fig. 3. Mediation by CCR6 of cell migration induced by HBDs. (**A**) Cross-desensitization of CCR6/293 cell chemotaxis by sHBD2 and LARC. LARC (100 ng/ml) and sHBD2 (1000 ng/ml) were added into the lower wells, and CCR6/293 cells in CM or in CM containing LARC (100 ng/ml) or sHBD2 (1000 ng/ml) were added into the upper wells of a chamber. The cell migration was evaluated as described (*13*). Error bars, SD of triplicated wells. (**B**) Binding inhibition. Competitive binding was performed in triplicate by adding a constant amount of ^{125}I-LARC and increasing amounts of sHBD2 (■) or LARC (●) to individual 1.5-ml microfuge tubes, each containing 2×10^6 CCR6/293 cells suspended in CM. After incubation at 24°C with constant mixing for 1 hour, the mixture was centrifuged through a 10% sucrose/PBS cushion, and the cell-associated radioactivity was measured with a 1227 Wallac gamma counter. CPM, counts per minute; error bars, SD of triplicated tubes. (**C**) Dependence on CCR6 of sHBD2-induced immature DC migration. Chemotaxis of DCs in response to sHBD2 (100 ng/ml) (dotted bars) or LARC (10 ng/ml) (hatched bars) in the absence (–) or presence (+) of antibodies (50 μg/ml) as specified was examined by chemotaxis assay (*13*). Antibodies were added at identical concentrations into the upper and lower wells of a chamber. DCs were pretreated with antibodies at 24°C for 30 min before they were added into the upper wells of a chamber. Error bars, SD of triplicated wells.

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13. Chemotaxis assay was performed and chemotactic factors were determined as follows. The migration of DCs, monocytes, T cells, and various transfectant cells was assessed with a 48-well microchemotaxis chamber technique as previously described [W. Falk, R. H. Goodwin Jr., E. J. Leonard, *J. Immunol. Methods* **33**, 239 (1980)]. The incubation time was 1.5 hours for DCs and monocytes, 4 hours for T cells, and 5 hours for parental and transfectant HEK293 cells. The cells were suspended in, and all chemotactic factors were diluted with, chemotactic medium (CM) (RPMI 1640 containing 1% bovine serum albumin). The results are presented as either number of cells per high power field or chemotactic index defined as the fold increase of cell migration in the presence of test factors over cell migration in the presence of CM alone. Chemotactic factors used in this study were from the following sources: Recombinant HBD2, stromal-derived factor–1α (SDF-1α), and LARC were from Pepro Tech (Rocky Hill, NJ); sHBD1 was from Magainin Research Institute (Plymouth Meeting, PA); sHBD2 was purchased from Peptide Institute (Minoh-shi, Osaka, Japan); skin HBD2 was purified from the shed psoriatic skin as described (*6*); and ^{125}I-LARC with a specific radioactivity of 2000 Ci/mmol was purchased from Amersham Life Science (Cleveland, OH).
14. Mononuclear cells were isolated from human peripheral blood or bone marrow of normal donors by routine Ficoll-Paque density gradient centrifugation. $CD34^+$ cells were then purified from the resulting mononuclear cells with a magnetic cell sorter CD34 progenitor isolation kit (Miltenyi Biotech, Auburn, CA), and peripheral blood T cell subsets were purified with human T cell subset enrichment columns (R&D Systems). To generate DCs, we cultured purified $CD34^+$ cells at a density of 5×10^5/ml in RPMI 1640 [RPMI 1640 (Biowhittaker, Walkersville, MD), 20% fetal bovine serum (FBS) (Hyclone, Logan, UT), 2 mM glutamine, 25 mM Hepes (Quality Biologicals, Gaithersburg, MD), penicillin (100 U/ml), and streptomycin (100 μg/ml)] in the presence of recombinant human granulocyte-macrophage colony-stimulating factor (100 ng/ml, specific activity of $\geq 10^7$ U/mg) (Pepro Tech) and recombinant human interleukin-4 (100 ng/ml, specific activity of $\geq 2 \times 10^6$ U/mg) (Pepro Tech) for 2 weeks with medium exchange every 3 days. Thereafter, the cells were cultured for another 7 days in the same medium in the presence of recombinant human TNF-α (100 ng/ml, specific activity of $\geq 10^7$ U/mg) (Pepro Tech) to induce maturation if required.
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16. FACS analysis was performed as follows. Human DCs (5×10^5/sample) were washed three times with

FACS buffer [phosphate-buffered saline (PBS), 1% FBS, and 0.02% NaN_3, at pH 7.4)]. Subsequently, the cells were directly stained with fluorescein isothiocyanate–or phycoerythrin-conjugated mouse monoclonal antibodies to CD83 (Immunotech, Marseille, France), CD1a, CD86, HLA-DR (PharMingen, San Diego, CA), or CCR6 (R&D Systems) at 24°C for 1 hour at a final concentration of 5 μg/ml in FACS buffer. As a negative control, DCs were stained with isotype-matched irrelevant antibodies. Thereafter, the cells were washed twice with FACS buffer and twice with PBS, resuspended in PBS containing 1% paraformaldehyde, and analyzed with a flow cytometer (Coulter or FACScan Epics Analyzer).

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26. Parental HEK293 epithelial cells were transfected with linearized CCR6 mammalian expression constructs by electroporation. After selection in Dulbecco's minimum essential medium (Biowhittaker), 10% FBS, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) containing G418 (400 μg/ml) (Life Technologies, Gaithersburg, MD) for 2 weeks, single-cell cloning was performed. The expression of functional CCR6 was confirmed by saturation binding, FACS analysis of surface CCR6, and chemotaxis of the transfectant cells in response to LARC.
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30. We thank P. W. Gray and M. Tsang for human CCR6 expression constructs and antibody to CCR5, respectively; N. Dunlop for technical assistance; and C. Fogle and C. Nolan for secretarial assistance. Supported in part by a fellowship from the Office of International Affairs of the National Cancer Institute (NCI) of NIH (to D.Y.) and by contract N01-CO-56000 from NCI of NIH (to O.C. and O.M.Z.H.). The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

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A New Primate from the Middle Eocene of Myanmar and the Asian Early Origin of Anthropoids

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A new genus and species of anthropoid primate, *Bahinia pondaungensis* gen. et sp. nov., is described from the Yashe Kyitchaung locality in the Late Middle Eocene Pondaung Formation (Myanmar). It is related to *Eosimias*, but it is represented by more complete remains, including upper dentition with associated lower jaw fragment. It is interpreted as a new representative of the family Eosimiidae, which corresponds to the sister group of the Amphipithecidae and of all other anthropoids. Eosimiidae are now recorded from three distinct Middle Eocene localities in Asia, giving support to the hypothesis of an Asian origin of anthropoids.

Anthropoid primates are represented during the Eocene in Southeast Asia by three derived genera, which belong to a monophyletic group, the Amphipithecidae (*1*). Two of them, *Amphipithecus* and *Pondaungia*, are recorded from the Late Middle Eocene Pondaung Formation in Myanmar, and *Siamopithecus* has been described from the Late Eocene Krabi Formation in Thailand (*2*). However, these forms give little information concerning their origins. *Eosimias* is a much more primitive primate known from the Middle Eocene of China (*3–5*), and its systematic position is debated; some authors recognize it as a basal anthropoid, therefore supporting an Asian origin of the group (*3, 4*), while others interpret it as having possible affinities with tarsiiformes (*6*). Some have even doubted its anthropoid nature (*7*).

During the November 1998 fieldwork organized in the frame of the Myanmar-French Pondaung Expedition Project, we recovered the remains of a new small primitive anthropoid, *Bahinia pondaungensis* gen. et sp. nov., at the Yashe Kyitchaung locality in Central Myanmar. The fossils (two fragmentary maxillaries and a broken lower jaw) were collected from a carbonate nodule in a reddish clay level which also yielded a complete lower jaw of *Amphipithecus mogaungensis* in the same stratigraphical level (*1*). The stratigraphic sequence shows normal magnetic polarity (*8*).

Systematics: Suborder Anthropoidea Mivart, 1864; Family Eosimiidae Beard *et al.*, 1994; *Bahinia pondaungensis* gen. et sp. nov.

Holotype: Left and right maxilla and associated jaw fragment. The right maxilla [National Museum of Myanmar Primate (NMMP) 15], shows I^{1-2}, C, P^{2-3}, broken P^4, M^{1-2} (Fig. 2A), and the premaxillary-maxillary suture (Fig. 1). The left maxilla (NMMP 14) (Fig. 2B) displays I^{1-2}, C, P^{2-4}, M^{1-2}, and broken M^3. The two maxillaries were separated in the nodule by carbonate matrix; thus, they belong to a single individual. The fragmentary right lower jaw (NMMP 16) (Fig. 2C) preserves the posterior part of the socket for canine and P_2-M_1.

Horizon and locality: Late Middle Eocene of Pondaung Formation, Yashe Kyitchaung locality (Primate Resort), Bahin village, Myaing Township.

Diagnosis: Very small anthropoid primate with dental formula 2.1.3.3. Two upper incisors vertically implanted with I^1 slightly larger than I^2 (judging from the remaining roots), strong vertical upper and lower canine with rounded crown sections, reduced and single-rooted P_2, P_3 as large as P_4, simple with their crown obliquely orientated and high protoconid, M_1 with low paraconid, metaconid widely separated from protoconid, and buccodistally projecting hypoconulid. Very reduced P^2, broad upper molars with well-developed cingulum surrounding the crowns, very slight hypocone swelling on M^1 and M^2 and strongly reduced paraconule and metaconule, lingually bent paracone and metacone on M^2. Mesiodistally reduced M^3. Differs from *Eosimias* species by its larger size, less reduced P_2, more simple P_4 and P^3, M_1 with less oblique cristid obliqua and trigonid only slightly higher than talonid, and by its upper molars with very small conules. Differs from *Catopithecus* and *Oligopithecus* by the retention of P^2, its single-cusped P_4, its P_{3-4} of about the same size, its hypoconid non-twinned with entoconid on lower molars, its upper premolars without inner cusps, and by its broader upper molars with only very slight hypocone swelling. Differs from *Proteopithecus* by its deeper lower jaw, its P_2 smaller than P_3, its more simple P_4, its M_1 paraconid in mesiolingual position and non-twinned hypoconulid and entoconid, its upper premolars lacking an inner cusp, and by its upper molars without well-developed hypocone (dimensions in Table 1).

Etymology: Genus name after Bahin village, and species name after the stratigraphic

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