Diagnostic

Influence of macrophage activation on their capacity to bind bacterial antigens studied with atomic force microscopy

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Abstract

In this work we studied interactions between bacterial antigens and receptors on the surface of macrophages using atomic force microscopy (AFM). We used two bacterial cell wall components: lipopolysaccharide (LPS) derived from gram-negative Escherichia coli and exopolysaccharide (EPS) derived from gram-positive Lactobacillus rhamnosus. Interactions between these bacterial antigens and immune cell receptors were studied in peritoneal macrophages derived from two strains of mice, CBA and C3H/J, in which the Toll-like receptor 4 (TLR4) is genetically disabled. We collected 500 force-distance curves for LPS-activated cells using an EPS-covered AFM tip, and for EPS-activated cells using an LPS-covered AFM tip. Nonactivated cells were tested as reference cells. The results show that LPS-primed macrophages decrease their ability to bind EPS. Surprisingly, EPS-activated macrophages maintain or even increase their ability to bind LPS. This may suggest that in vivo commensal enteric bacteria, such as lactobacilli, will enhance the defense potential of local macrophages against pathogens expressing LPS.
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Macrophages are involved both in initiation of immunologic responses as antigen-presenting cells and the effector phase of immunity as inflammatory, tumoricidal and microbicidal cells. The activation of macrophages is a general feature of early stages of infection. Macrophages express specialized pattern recognition receptors for bacterial antigens on their surface, such as CD14, Toll-like receptors (TLRs), and mannose receptors. The interaction of these receptors with bacterial products, such as lipopolysaccharide (LPS) or peptidoglycan (PGN), leads to activation of macrophages and subsequent secretion of biologically active agents that are involved in the regulation of immune responses [1,2].

The two most commonly studied components of gram-negative and gram-positive bacterial cell walls are LPS and PGN, respectively. Proinflammatory effects of these bacterial cell wall components occur both in vitro after treatment of mononuclear phagocytes and in vivo after exposure of whole animals, with cells and animals being more sensitive to LPS than to PGN [3]. Moreover, a synergistic effect of LPS with PGN has been observed. Much less is known about the role of exopolysaccharide (EPS), one of the major products of lactobacilli, beneficial bacteria of the intestinal tract that are commonly used as probiotics. In this study we have focused on EPS and LPS interactions with receptors
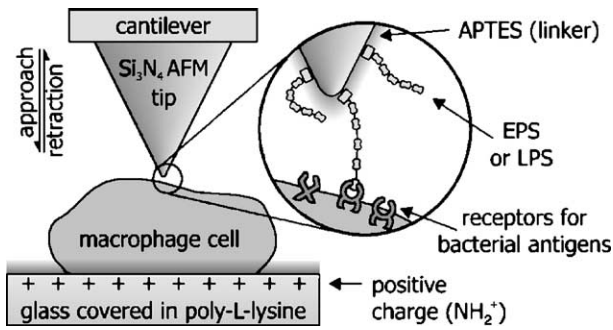
Fig 1. AFM setup used to gather force-distance curves. The EPS or LPS-covered tip (linked by APTES) approaches the macrophage cell surface. Antigen-receptor bonds that are formed rupture at a certain force upon retraction of the tip. The macrophages are firmly attached to the glass surface with poly-L-lysine because of their positive charges.

expressed on macrophages. However, both LPS and EPS antigens bind to pattern recognition receptors on the macrophage surface. Each of these receptors can link to different types of antigens, although some specialize in specific types of antigens; that is, their interaction is stronger. LPS binds mainly to CD14 and TLR4 receptors, whereas it is not known to which receptors EPS binds [4-7]. Both of these compounds have a polymer structure with long polysaccharide chains and additional lipid or peptide groups [8,9].

Atomic force microscopy (AFM) is a high-resolution microscopic method that is being used ever more frequently in biology, medicine, chemistry, and biomaterials investigations. AFM permits the performance of experiments in physiologic solutions with control of experimental parameters such as temperature or pH. AFM was originally designed as an imaging tool but was later modified to operate in force spectroscopy. It allows for direct measurements of single intermolecular interactions. By performing experiments in natural conditions, physiologic interactions can be characterized in terms of the strength of antigen-receptor or antigen-antibody bonds [10-14], the adhesion forces responsible for cell assembly [15], or the specific interaction between a cell and a molecule [16,17]. The tip of the atomic force microscope can be covered with antigens with the aid of a special chemical linker. Once the cantilever makes contact with the cell surface, an antigen-receptor bond is created (with a certain probability), as seen in Figure 1. This bond is ruptured when the tip is pulled away from the substrate surface. By collecting force-distance curves, it is possible to directly measure the strength necessary to destroy an antigen-receptor bond (rupture force), as well as the probability of forming a bond (adhesion probability).

In previous experiments the interaction between bacterial antigens (LPS, EPS, PGN) and a native (inactive) form of macrophages was measured. In this work an additional step was taken to study the interactions between bacterial antigens and pattern recognition receptors on the macrophage surface after activation of the macrophage.

Methods

Bacterial components

EPSs were extracted from a lactic acid bacterium (*Lactobacillus rhamnosus* strain KL37C) isolated from human intestinal flora [8]. LPSs were taken from *Escherichia coli* serotype 0111:B4 (Sigma, St. Louis, MO).

Tip functionalized with bacterial antigens

Standard V-shaped silicon nitride (Si_3N_4) cantilevers (Microlevers; Veeco Instruments SAS, Dourdan, Cedex, France) with a nominal spring constant of 0.01 N/m were used. The tips were functionalized with bacterial antigens: LPS and EPS. Before each experiment the tips were thoroughly cleaned by a 5-minute immersion in acetone, then washed in deionized water and irradiated with ultraviolet light for 20 minutes. They were then immersed in a 3-aminopropyltriethoxysilane (APTES) 10% (v/v) water solution (Sigma, St. Louis, MO) for 3 hours. This compound strongly binds to Si_3N_4 and contains many positively charged amino groups. The tips were rinsed three times in deionized water and then functionalized by immersing them into a 100-µg/mL bacterial antigen/phosphate-buffered saline (D′ PBS) solution for 2 hours. The antigen-covered tips were immediately used for measurements.

Macrophage cell preparation

Mice

CBA and C3H/J mice (8-12 weeks of age, 18-22 g) were maintained in the Animal Breeding Unit, Department of Immunology, Jagiellonian University Medical College, Cracow. All mice were housed four to five per cage in the laboratory room with water and standard diet ad libitum. The appropriate permission for using mice in this project was obtained from the Local Ethical Committee.

Cells

Peritoneal mouse macrophages were elicited by intraperitoneal injection of 2 mL Thioglycolate (Sigma, St. Louis, MO). Cells were collected 48 hours later by washing the peritoneal cavity with 5 mL of D′ PBS. Cells were centrifuged twice at 2000 rpm and suspended in a 5-mL D′ PBS solution containing 100 µL of 2% bovine serum albumin. Then, 200 µL of a bacterial antigen solution (100 µg/mL) were added. Next, these were dropped onto a glass coverslip and incubated at 37°C in 5% CO_2 for 4 hours. Afterward, the glass coverslip with macrophages was rinsed in D′ PBS and immersed in 1.5% (v/v) glutaraldehyde D′ PBS solution for 1 minute. Glutaraldehyde causes stabilization of receptors in the cell membrane. After 1 minute the coverslip was rinsed three times in D′ PBS and immediately used for measurements. To determine whether the incubation time has an influence on macrophage activation, four experiments where performed with incubation times of 1, 2, 4, and 12 hours.
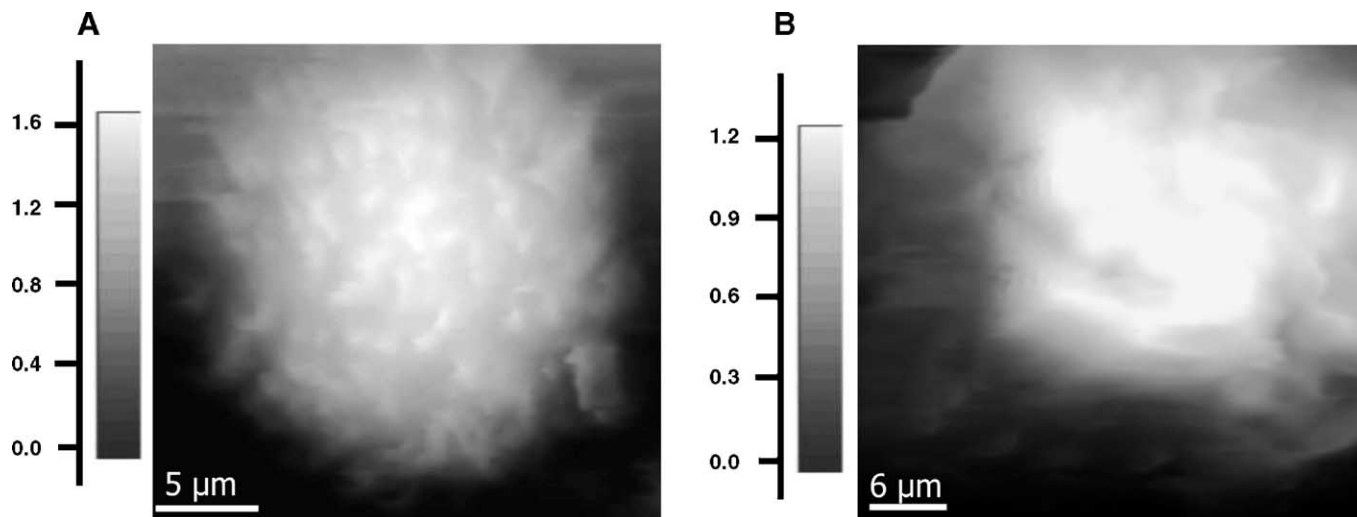
A

B

Fig 2. AFM images of (**A**) nonactivated and (**B**) EPS-activated macrophages. Once activated, the cell grows in size and deforms irregularly.
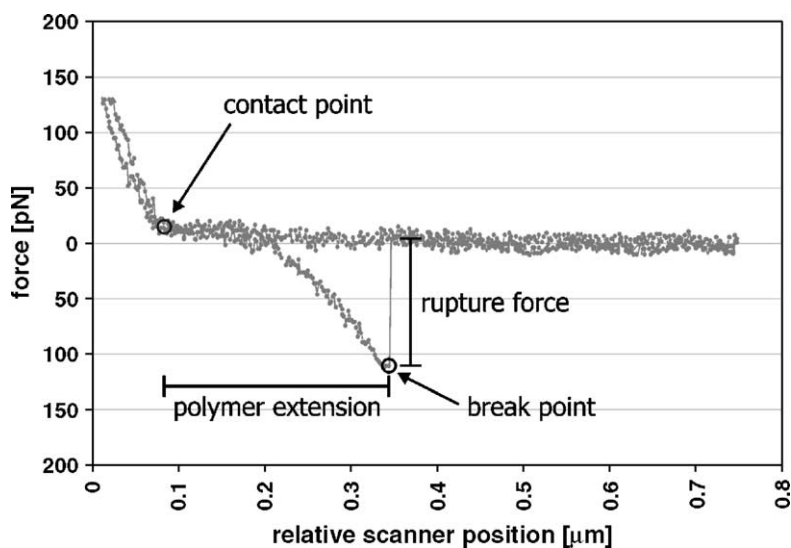

Fig 3. Typical force-distance curve for a specific antigen-receptor interaction and its characteristic parameters. Notice the shift in the force response to the polymer extension.

Two types of macrophages were used in this experiment: macrophages taken from C3H/J genetically modified mice with defective TLR4 receptors and control macrophages taken from CBA mice.

AFM measurements

A commercial instrument, the Thermomicroscopes CP (Veeco Instruments SAS, Dourdan, Cedex, France) equipped with a multimode head and a 100-μm scanner, was used for measurements. The deflection of the cantilever was detected by a position-sensing photodiode detector. All experiments were performed in D′ PBS solution (pH 7.4) by using a commercial liquid cell (Thermomicroscopes CP).

Raw force-distance curves were obtained as the cantilever deflection versus the z-displacement of the scanner. The cantilever deflection, initially in millivolts (mV), was converted to a distance in nanometers (nm) by calibrating

the cantilever on a rigid surface. Then, the cantilever spring constant, measured experimentally, was used to obtain the corresponding force by using Hooke's equation (methods described by E. Wojcikiewicz et al [15]).

Results and discussion

The goal of this research was to study the influence of macrophage activation on the interaction between pattern recognition receptors and bacterial antigens. Two bacterial antigens were used: EPS extracted from *L. rhamnosus* and LPS extracted from *E. coli*. Each antigen was used on two types of macrophages isolated from CBA and C3H/J mice. The macrophages were activated by the addition of EPS or LPS to the macrophage suspension (as described under Methods) and an incubation of a predetermined duration. Force-distance curves were collected in the treated cells
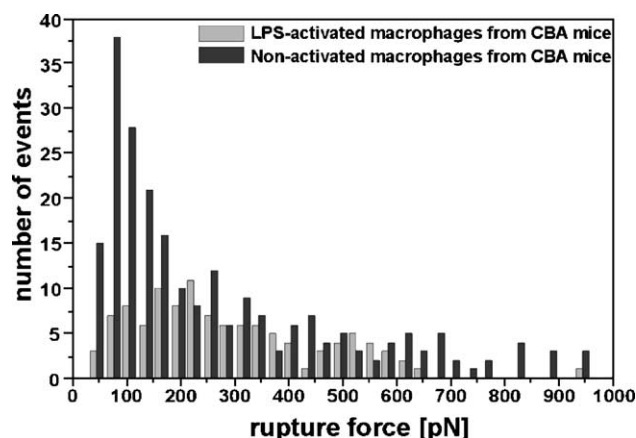
Fig 4. Histogram presenting the distribution of rupture forces measured from the 500 force-distance curves performed with an EPS-covered AFM tip, for each set, on macrophages from CBA mice. These cells have all receptors active. Only the specific interactions were binned.

with an AFM tip covered with bacterial antigens. For EPS-activated macrophages, the tip was covered with LPS, whereas for LPS-activated macrophages the tip was covered with EPS. Nonactivated macrophages from CBA and C3H/J mice were used as controls.

Cell images

Before collecting force-distance curves in a cell, it was imaged via AFM. Figure 2, A and B illustrate nonactivated and activated cells, respectively. A significant difference can be seen in the size and topography of both cells. Nonactivated macrophages are characteristically round, with an average diameter of 15 to 18 im and small protrusions on the surface. Once activated, the cells grow and deform irregularly, assuming an amoeboid shape.

Force-distance curves

To determine the mean unbinding force characteristic of a particular antigen-receptor complex, force-distance curves were collected using an AFM tip coated with a particular kind of bacterial antigen. The deflection is measured both while the cantilever approaches the surface of a sample and when it retracts to its starting point. The rupture force is defined as the force necessary to break the bond. Its value can be determined on the basis of the characteristic jump (breakpoint) visible on the force-distance curves. Examples of the force-distance curves showing bond breaking are presented in Figure 3.

The shape of the force-distance curve indicates whether a bond was created between antigens on the tip and receptors on the cell. Because EPSs and LPSs have long polymer structures, an antigen-receptor bond breaking produces a characteristic shift in its rupture point (Figure 3) [18,19]. Such a rupture is called specific to distinguish it from nonspecific ruptures caused by direct adhesion of the cell membrane to the tip. The average unbinding force value is determined from a histogram corresponding to a family of

Table 1
Summary of the relevant values extracted from Figures 4 and 5*

Type of cell	LPS activated	EPS-covered tip	
		Peak force (pN)	Probability of adhesion
CBA	Yes	–	0.14
CBA	No	90 ± 20	0.51
C3H/J	Yes	–	0.03
C3H/J	No	100 ± 20	0.37

For activated cells no peaks were visible, but there is a definite decrease in the probability of adhesion for both cell types.
* The peak values represent the mean and width of the Gaussian distribution used to model the peaks.

force-distance curves; this results from the statistical character of the bond breaking process. The applied bin size was governed by the size of the noise present in the AFM system [20], which corresponds to the cantilever thermal fluctuations and its electronic noise (converted into force units). The amplitude of these fluctuations sets a lower limit to the resolvable adhesion force [16], which in this case was 35 ± 5 pN. For each complex, 500 force-distance curves were acquired. Additionally, for each interacting pair, a probability of adhesion events was measured.

LPS-activated cells

Figure 4 compares the LPS-activated and nonactivated macrophages from CBA mice tested with an EPS-covered tip. The histogram for the nonactivated cell has a distinct maximum for low rupture forces that is clearly not present on its activated counterpart. However, both cases share a similar distribution of rupture events once the large peak is disregarded. These data suggest that LPSs blocked many of the receptors frequently useful for EPS binding at low rupture forces but left other receptors unhindered. Table 1 presents the changes in the probability of adhesion for both situations: the cells show a definite decrease in the probability of adhesion with EPS once activated by LPS. Table 1 also presents the parameters of the peak used to model the data for the nonactivated cells. This model consists of a Gaussian distribution floating on an exponential one, which approximates the interactions with other receptors and noise, as plotted in Figure 4.

Figure 5 presents two histograms: one for LPS-activated and one for nonactivated macrophages from C3H/J mice interacting with an EPS-covered tip. The large probability recorded for the nonactivated cells, as shown in Figure 5 and summarized in Table 1, suggests that LPS causes a considerable decrease in EPS binding on the LPS-activated macrophage. Similar to the previous system, this result suggests that LPS blocks many receptors on the C3H/J cell membrane responsible for EPS binding.

It is also important to note the resemblance between the results in Figure 5 and those in Figure 4. This similarity exists because EPS does not bind to TLR4 and thus recognizes no difference between macrophages from CBA and C3H/J mice. The difference in the probabilities of
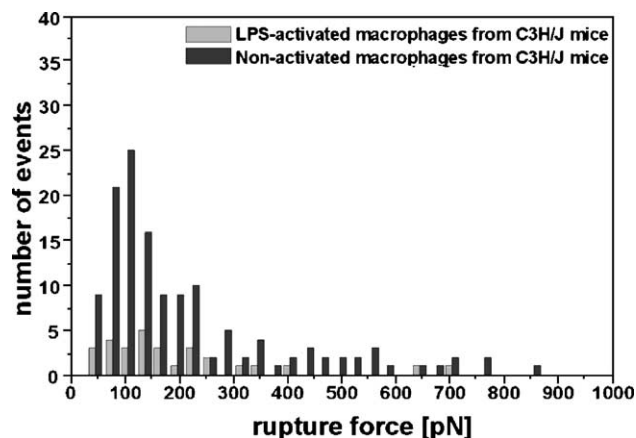
Fig 5. Histogram presenting the distribution of rupture forces measured from the 500 force-distance curves performed with an EPS-covered AFM tip, for each set, on macrophages from C3H/J mice. These cells have TLR4 receptors genetically disabled. Only the specific interactions were binned.
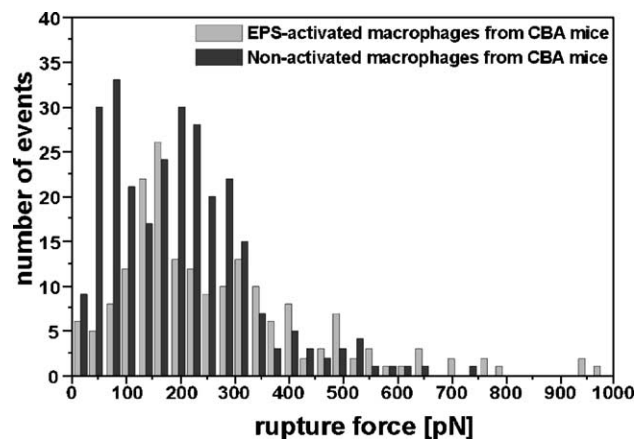

Fig 6. Histogram presenting the distribution of rupture forces measured from the 500 force-distance curves performed with an LPS-covered AFM tip, for each set, on macrophages from CBA mice. These cells have all receptors active. Only the specific interactions were binned.

Table 2
Summary of the relevant values extracted from Figures 6 and 7*

Type of cell	EPS activated	LPS - covered tip		
		First peak (pN)	Second peak (pN)	Probability of adhesion
CBA	Yes	150 ± 30	300 ± 60	0.40
CBA	No	60 ± 20	220 ± 70	0.58
C3H/J	Yes	120 ± 50	310 ± 50	0.31
C3H/J	No	110 ± 40	230 ± 70	0.12

The light-gray values represent a peak that is not considered significant.
  * The peak values represent the mean and width of the Gaussian distribution used to model each peak.
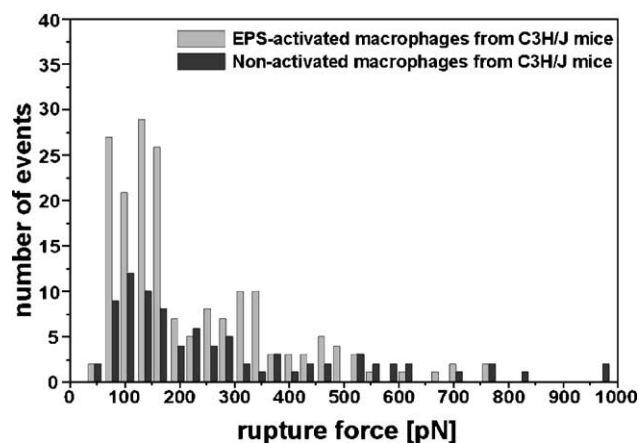

Fig 7. Histogram presenting the distribution of rupture forces measured from the 500 force-distance curves performed with an EPS-covered AFM tip, for each set, on macrophages from C3H/J mice. These macrophages have TLR4 receptors genetically disabled. Only the specific interactions were binned.

adhesion for both pairs of histograms is explained by the deactivation of TLR4 on macrophages from C3H/J mice.

EPS-activated cells

Figure 6 presents two combined histograms for the EPS-activated and nonactivated macrophages from CBA mice interacting with an LPS-covered tip.

For the EPS-activated macrophages from CBA mice the histogram consists of two peaks. These peaks are attributed to interactions between LPS and receptors that still remained active for bonding after EPS activation. As previous studies showed, the EPS interactions with both types of macrophages (with and without TLR4) are comparable; therefore, one can conclude that TLR4 does not (or does very weakly) contribute to the EPS binding. On the basis of this conclusion we suggest that the greatest contribution to

interactions between the LPS-covered tip and EPS-activated macrophages comes from interactions with TLR4. However, the first and second peaks of the histogram for the EPS-activated macrophages from CBA mice are mainly attributed to single and double interactions with TLR4. As expected, the second peak has a force approximately double that of the first. A model with three Gaussian distributions on an exponential noise floor was used to extract the parameters of the two first peaks. The third was not taken into consideration because its error is too large, but necessary to prevent the first two from artificially skewing toward the right. The result is summarized in Table 2.

For the nonactivated macrophages from CBA mice (Figure 6), the first large peak represents the interaction with low affinity between LPS and the group of receptors that have been previously inhibited by EPS. The second peak attributed to a strong specific interaction between LPS with TLR4 receptors is similar to the first peak from the system of LPS on the tip approaching EPS-activated macrophages from CBA mice. Once the first peak is taken into account, the probability of adhesion (of the remaining TLR4) changes insignificantly from one histogram to the next.
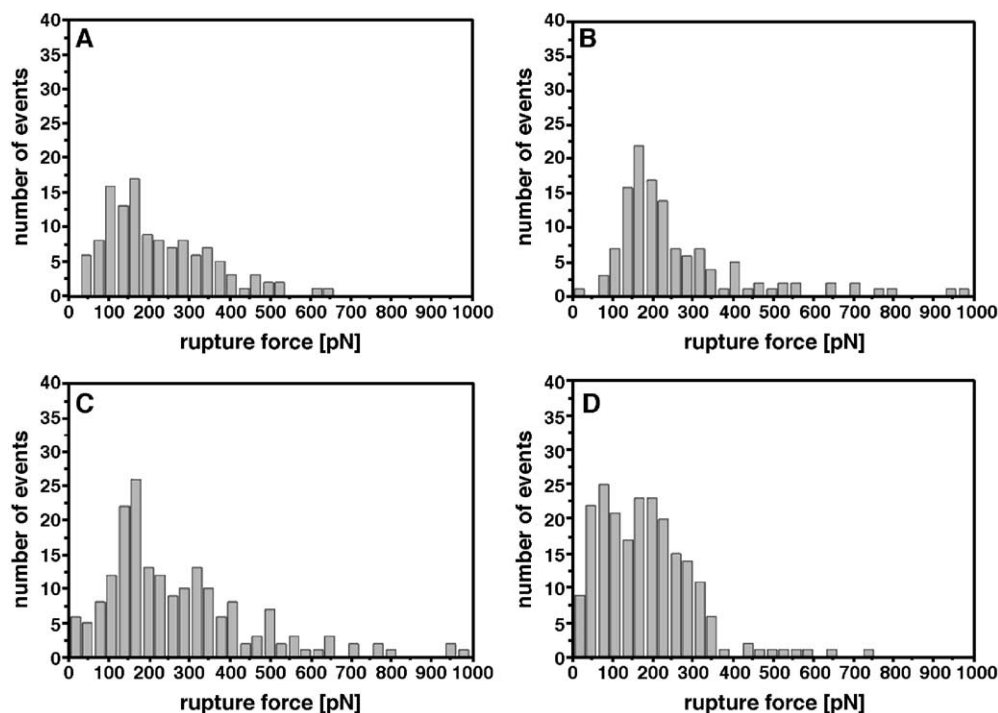
Fig 8. Results from force-distance curves performed on macrophages from CBA mice, incubated in EPS for 1 (**A**), 2 (**B**), 4 (**C**), and 12 hours (**D**). The first three are very similar, suggesting that activation occurs within an hour. The last one differs and resembles the nonactivated macrophages histogram in Figure 6, suggesting that the macrophages returns to its native form between 4 and 12 hours.

By incubating the macrophages in EPSs, a certain group of receptors was blocked while TLR4 remained unchanged. This explains why the EPS-activated macrophages still interact as strongly with LPS as the nonactivated cells. Therefore, EPSs and LPSs compete for macrophage surface receptors, but not in the case of TLR4, for which LPS has a definite affinity.

Figure 7 presents a histogram for EPS-activated macrophages from C3H/J mice interacting with LPS on the AFM tip, as well as an equivalent histogram for nonactivated cells. Three peaks, on an exponential noise floor, were fitted to obtain the mean and standard deviation of the rupture forces, although only the first two peaks were considered significant for the EPS-activated cells and only the first peak for the nonactivated cells. Comparing both histograms, a significant increase in LPS interaction with the EPS-activated C3H/J cells can be observed: the results presented in Table 2 show a definite increase in the probability of adhesion. Also, the second peak for the EPS-activated cell can be attributed to double bonds. On one hand, EPS blocks receptors that also bind to LPS, reducing the amount available for the latter. On the other hand, EPS activation can stimulate the macrophages and increases their efficiency for further binding, for example with LPS. The EPS binding with one type of receptor induces a signal for the other receptors to increase their affinity toward antigens. This is important in the case of C3H/J cells, wherein TLR4 is incapable of binding with LPS. After EPS activation, the macrophage undergoes an increase in LPS binding susceptibility, because the receptors in this direction (except for TLR4) are activated, although the rupture force remains unchanged.

Time dependence of EPS activation

In the experiments described above the incubation time for EPS activation was 4 hours. To observe the influence of the incubation time on macrophage activation, four experiments were performed with incubation times of 1, 2, 4, and 12 hours, as shown in Figure 8. The differences among the first three histograms are not statistically significant, suggesting that macrophage activation occurs quickly (within an hour) and lasts as long as 4 hours or slightly longer.

However, the rupture force average for the 12-hour activation experiment is comparable to the value from nonactivated cells, in Figure 8, suggesting that the cells return to their native form after a long period of time.

Conclusions

LPS is embedded in the cell membrane of potentially pathogenic bacteria. The addition of LPS to the suspension of macrophages activates them and stimulates phagocytosis. This experiment demonstrates that, after activation by LPS, the macrophages reduce their binding efficiency to EPS. We can conclude that EPS and LPS compete for the same receptors on macrophage membranes.

Contrary to the results obtained for LPS-activated macrophages, EPS activation does not reduce the probability of adhesion of LPS, because pathogenic bacteria are a primary target for macrophages. Although EPS and LPS compete for certain receptors on the cell surface, the EPS-activated cells increase their affinity toward additional antigen binding (with LPS, for example), compensating for the initial reduction in probability of adhesion because of the presence of EPS. This latter effect was much stronger for C3H/J cells, wherein TLR4, the main receptor for LPS binding, is deliberately disabled.

By using AFM we were able to directly determine the changes in interactions between bacterial antigen and pattern recognition receptors after activation of macrophages. These results are promising for improving the understanding of the role of commensal bacteria in organisms and their interaction with immune cells such as macrophages.

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