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ELECTROPHYSIOLOGY OF PHAGOCYTIC MEMBRANES

II. MEMBRANE POTENTIAL AND INDUCTION OF SLOW HYPERPOLARIZATIONS IN ACTIVATED MACROPHAGES

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Summary

The potential differences measured on the cell surface and after penetration into the cytoplasm of activated macrophages are described. Linear regressions are made of the measured potential differences as functions of the tip potential of each microelectrode. The surface potential of the macrophage is not significantly different from zero.

Mouse macrophages have a transmembrane potential of −26 mV, whereas in guinea-pig cells this value is −18 mV. The input resistances of guinea-pig cells are higher than those of mouse macrophages. The cytoplasmic location of the electrode was characterized both by fluorescent dye injection and by electric criteria.

Slow membrane hyperpolarizations are directly elicited by mechanical stimulation. Electric responses evoked by current pulses were further characterized.

Our results lead to the establishment of objective criteria to validate intracellular recordings from macrophage.

Introduction

Little is known about the in vivo role of the macrophage in the control of immune responses. The macrophage acts as the interface of the immune system

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Abbreviation: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

with the external world and probably plays a major role in immune induction as well as in cell mediated immune reactions. Knowledge of certain processes at the level of the cell membrane appear to us a necessary step for the understanding of phenomena such as antigen presentation to interacting lymphocytes [1], phagocytosis, cell motility and pinocytosis [2,3].

Glass microelectrodes [4] are still the principal means of recording transmembrane potentials. Errors and artifacts are inherent to electrophysiological techniques. Although their significance is small when one deals with the electrical phenomena of large amplitude characteristic of excitable cells [5], they are of importance in the study of small cells with relatively low membrane potentials, such as macrophages.

The impalements require very fine electrode tips, which imply higher electrode-specific junction potentials. Although several laboratories have tried to probe the electrophysiology of the macrophage, few reports have appeared in the literature [6—8].

The studies reported here on activated macrophages deal with the problems of cell impalement with microelectrodes and characterize two distinct electric profiles in records from macrophages. The 'real' membrane potential is estimated by the use of electrode with a wide range of electrode tip potentials [9] and by extrapolating to zero the linear plot between the measured potential differences and the tip potentials. The cytoplasmic location of the electrode is controlled by dye injection that fills this compartment and is retained by the cell throughout the period of intracellular recording.

In the preceding paper of this series [7] we have shown that the slow hyperpolarization displayed by macrophage membrane [6] is due to increased potassium permeability. In this paper we establish criteria for acceptance of electric measurements from the motile membranes of phagocytic cells. We also show the first direct evidence of mechanical stimulation of the hyperpolarizing responses and discuss threshold situations for electrically evoked slow responses.

Although the functional significance of these potential changes has not been established, it is suggested that the underlying mechanism is a calcium-dependent potassium channel. This kind of channel has been described by Gardos [10] in the red blood cell, but probably represents a more general biological phenomenon since it is present in a variety of cells such as transformed fibroblasts [11] and several excitable cells (see Ref. 12 for a review).

Materials and Methods

Preparation

Macrophages were obtained from dextran or oil-induced peritoneal exudate of mice as previously described [7]. Briefly, the procedure involved washing the cells after collection and incubating them for 2—3 h in plastic Petri dishes with culture medium containing 10% fetal bovine serum. Non-adherent cells were washed away and the macrophage monolayer was incubated at 37° C in a 5% CO_2 humidified atmosphere for up to 72—96 h. Macrophages from oil-induced guinea-pig peritoneal exudates were cultivated on glass cover-slips or resin-coated plastic dishes (Sylgard 184-Dow Corning Midland, MI) using the same protocol as for mouse macrophages.

Before experiments fresh HEPES-buffered medium was added to the cells and changed at regular intervals (about 15 min).

Electrical arrangement

Intracellular recordings were performed in the complete culture medium by means of a standard electrophysiological arrangement [13]. Glass microelectrodes filled with 2.5 M KCl and with resistances of 40—80 MΩ were used. Under our conditions this is equivalent to a tip diameter below 200 nm [14]. After the measurements were made, the tips were broken and the tip potential was recorded. The same electrode provided simultaneous current injection and voltage recordings [13]. The reference electrode was a Ag | AgCl | KCl half cell, similar to that connected to the recording electrode, in contact with the culture dish by means of an agar salt bridge (resistance below $20 \cdot 10^3$ Ω).

Fluorescein injection

Microelectrodes were filled with distilled water which was replaced shortly before use by 20 mM sodium fluoresceinate in 12.5 mM KCl solution. The fluorescent anion was injected into the cytoplasm with rectangular current pulses (amplitude below $1 \cdot 10^{-8}$ A, 300 ms duration at a rate of about one per 3 s) for a short period (about 1 min). Extreme care must be taken to avoid current damage to the macrophage, an event detected by the prompt leakage of the dye into the medium. Fluorescence was excited using a blue filter (Leitz BG12) and observed in a dark field microscope through a yellow filter (Leitz OG1).

Results and Discussion

The surface potential of macrophage

When an electrode is forced against a cell a small dimple is observed in the membrane's surface. At the same time a positive potential is recorded, as shown in the initial portion of Fig. 1A. Mechanical forces at the electrode's tip are not responsible for this potential change, since it is not reproduced when an electrode touches an inert surface such as plastic or agar.

Plotting the surface potential of mouse macrophage against the value of tip potential (Fig. 1B, open symbols) gives a value of −3 mV for the y intercept, with a 95% confidence interval of ±4.6 mV. This implies that the surface potential of the macrophage is not significantly different from zero, assuming an ideal situation where no electrode tip potential is recorded and junction potentials are constant.

Low impedance unbalancing of the bridge circuit (below 10 MΩ) was detected along with the positive prepotential. It was not possible to determine the exact location of the electrode tip during these measurements. Both potential and resistance changes were promptly reversed when the contact with the cell surface was broken.

A positive surface potential has been described as a characteristic of cell coating in lymphocytes, malignant cells and trophoblastic cells, but it is not found in several normal cell types tested specifically for this purpose [15]. This

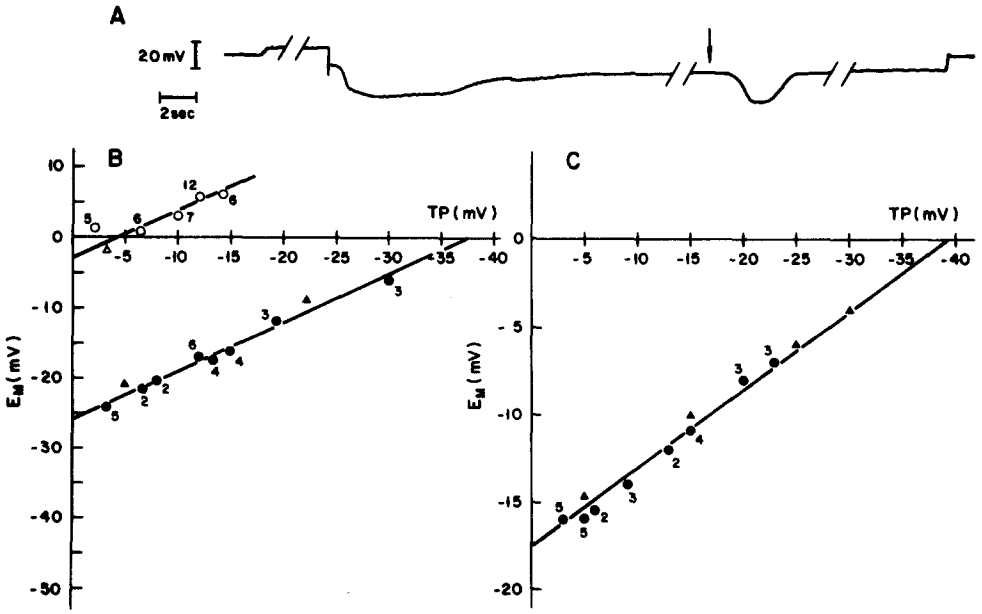

Fig. 1. Membrane potential of macrophages. A. Typical recording of the electrical profile in a guinea-pig macrophage. Note the positive potential before impalement and the slower hyperpolarization that follows. Interruptions correspond to 4 s, 8 s and 2 min, respectively. Arrow indicates the moment that mechanical stimulation (tap on the manipulator's base) triggered an electrical response. B. Potential differences (E_M) as functions of tip potentials (TP) in mouse macrophages. Open symbols correspond to the 'surface potential' and solid symbols to the cytoplasmic potential. Single cell measurements indicated by triangles. Circles are mean values (number of cells indicated) of different impalements with the same electrode. C. Regression line for tip potentials and the cytoplasmic potential measured for guinea-pig cells. Symbols are the same as in B.

potential varies with the hydrogen ion concentration of the bathing medium, as expected from a fixed charge matrix.

In the case of macrophages our data indicate that an ideal electrode, with zero tip potential, would not record a prepotential in the cell's surface.

We have no explanation for the correlation coefficient of 0.91 between tip potential (T) and the measured potential difference (PD) observed when the electrode touches the surface of mice macrophages. The linear relationship obeys the equation $PD = 0.66 \cdot T - 2.987$. Fine-tipped pipettes have provided much information pertaining to electrical events at different levels of cell and tissue organization. The exact nature of the mechanisms by which the microelectrode senses electrical changes in its microdomain remains unclear. Two possibilities have been suggested to explain the tip potential: (a) interfacial potentials between glass and the electrolyte solutions [16]; or (b) hydration and swelling of the glass wall near the tip resulting in a system similar to a complete glass membrane [17].

The relationship between measured surface and tip potentials may result from the interaction of the fine tip with the surface charge or to a reaction of the phagocytic membrane to the electrode penetration. At the present stage it is not possible to distinguish between these mechanisms, which may occur in conjunction or separately.

Membrane potential of the macrophage

When the electrode is forced against the cell's surface two different patterns may be recorded. In some cases a typical penetration with sharp voltage drop is observed (Fig. 1A). A simultaneous unbalance of the bridge circuit indicates an increase in resistance between electrode and earth. This abrupt impalement usually occurs after a gentle tap on the table or the passage of a large current pulse ($1 \cdot 10^{-8}$ A). It is generally followed by a longer slow hyperpolarization as shown in Fig. 1A. If the electrode is withdrawn, the bridge circuit returns to the balanced situation and a sharp return to baseline is observed. In other cases, after the surface positive shift there is slow and progressive establishment of the transmembrane potential. In both cases the equilibrium is attained at about the same value of transmembrane potential.

Mice activated macrophages yield a potential difference that is a linear function of the tip potential, according to the equation.

$$PD = 0.64 \cdot T - 25.72 \quad (1)$$

The computed regression line (Fig. 1B, solid symbols) has a correlation coefficient of 0.986 and the 95% confidence interval for the Y intercept is estimated at ±2.8 mV. It is thus concluded that the extrapolation to about −26 mV is the 'real' transmembrane potential of these phagocytes.

Guinea-pig activated macrophages are easier to impale. More often the penetration is accompanied by a sharp voltage drop. Our impression is that they seem more resistant than mouse cells to membrane damage during impalement. However, if the same careful two-step procedure used to impale mouse cells is employed, a similar electrical profile is observed for guinea-pig macrophages with the appearance of both components of the electrical profile. When the correlation with tip potential (T) is made the regression line corresponds to the equation

$$PD = 0.68 \cdot T - 17.56 \quad (2)$$

with a correlation coefficient of 0.988. In this case again the extrapolation to zero tip potential gives a value that can be taken as the membrane potential (about −18 mV) since the 95% confidence interval for the Y intercept is ±1.7 mV (Fig. 1C).

It was not possible to determine which ion or ions dominate the transmembrane potential of the macrophages. Increasing the extracellular K^+ concentration results in a smaller membrane depolarization than that expected from a K electrode (unpublished observations).

The many compartments of the macrophage preclude an easy estimation of the ionic contents of its cytoplasm. Since the total potassium content and ionic conductance of the cytoplasm are unknown, we have not attempted a study of ionic influences in the membrane potential of the macrophage. During the slow hyperpolarization it is quite evident that the potassium permeability dominates the transmembrane potential [6,7] but its contribution to the electrically quiescent cell remains to be established.

Membrane resistance

The total input resistance for guinea-pig macrophages ranges from 50 to 190 MΩ, with an apparently normal distribution as shown in Fig. 2A (52 cells measured). The mean value is in the class interval of 110—130 MΩ. In the case of mouse macrophages the input resistance for 85 cells ranged from 10 to 150 MΩ, with an average in the class interval of 70 to 90 MΩ (Fig. 2B). This value is in accordance with measurements in human macrophages, where a mean value of 84.4 MΩ jas been reported [4,8].

However our results do not agree with a previous report in electrophysiology of murine macrophages transformed by SV40 [18]. Calculating from the recordings of Levy et al. a value of about 1 MΩ is obtained for the input resistance. Even considering the possibility that those cells were making intercellular communication, as stated, we cannot explain the marked differences observed. The exceedingly large current pulses used by those authors could account for significant underestimation of cellular input resistances. Macrophages can be easily damaged by current injection, as we have observed in fluorescein injected cells. Damage can be observed by bleb formation and a marked fall in the input resistance when pulses of the order of $1 \cdot 10^{-7}$ A are used continuously.

The input resistance of an adherent cell depends on: (a) specific resistance of the membrane; (b) specific resistance of the cytoplasm; (c) total area of the membrane in contact with bathing medium. In the present case we have no reason to believe in differences in a or b. It is concluded that the range of our measurements is in consequence both of experimental error and differences in c.

Determination of the area of the cell membrane in the phagocyte cultures may give rise to large errors because of the irregular cell shape and the extensive membrane folding. It is then impossible to estimate the resistance for a unit membrane area with any degree of certainty.

Fluorescent dye injection

The difficulties in impaling the phagocytes and the controversy that may

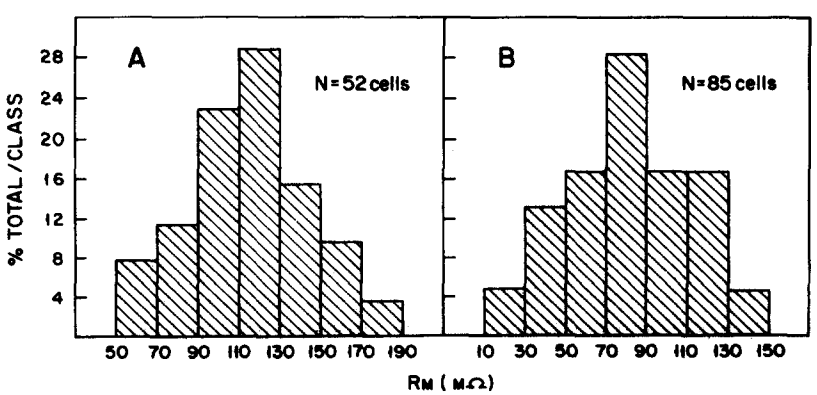

Fig. 2. Input resistances of macrophages. A. Class distribution of total cell resistance in guinea-pig cells (*N* = number of cells measured). B. Histogram for equivalent cell resistance in mouse macrophages. Note differences both in range and mean class intervals.

arise from the above discussion have led us to make some experiments in order to probe the exact location of our electrode tips.

When the microelectrode was positioned by the same procedure as used to record membrane potentials, fluoresceinate was injected into the cell. The dye filled the cell completely and was retained for 10—15 min (five cases). Fig. 3 shows an example of those experiments in murine macrophages. Sometimes a small vacuole around the tip of the micropipette was filled with fluorescein, but it did not retain the dye for more than a few seconds.

These experiments confirm the intracellular location of our electrodes and reveal no leakage to the bathing medium. This also validates our measurements of input resistances.

Triggering of slow hyperpolarizations

By the ability to display slow hyperpolarizations it was possible to subdivide the cells into three groups: (a) quiescent cells, where the hyperpolarizations can be elicited by mechanical or electrical stimulation; (b) cells that exhibit spontaneous slow membrane hyperpolarizations; and (c) electrically quiescent cells, which have a stable membrane potential and do not respond to stimulation.

Fig. 4A illustrates an experiment where a second empty electrode was driven to touch the phagocyte's surface and the mechanical stimulation of the electrical response is observed. Electrical stimulation was obtained both with inward and with outward current (Figs. 4B and 5B).

In some cases it was possible by a gentle tap at the base of the micromanipulator to induce for several periods slow hyperpolarizations (Fig. 5A). In one case, two electrodes were driven into the same guinea-pig cell and when mechanical stimulation elicited electrical response it was detected both in the current injecting electrode and in the second recording electrode. Electrical stimulation varies from cell to cell and the threshold current needed to elicit the response is extremely variable. An increase in pulse duration could trigger the response in the same cell (Fig. 5B).

Both the transformed fibroblasts [11] and guinea-pig macrophages [6]

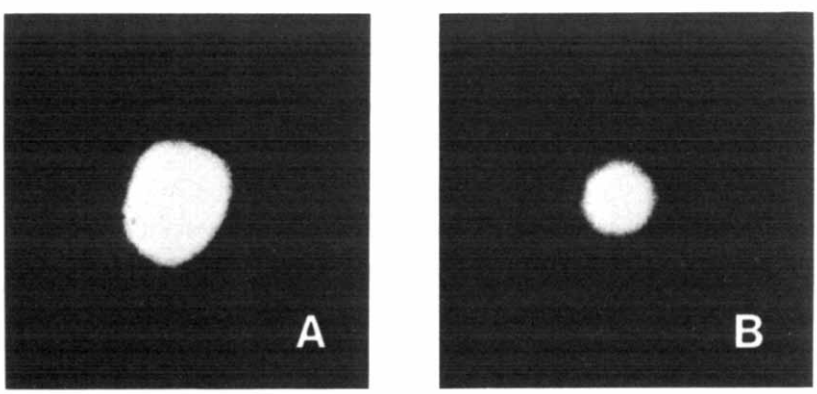

Fig. 3. Fluorescein injection as an indicator of cytoplasmic location. Two mouse macrophages that retained the dye for more than 15 min (A) and 10 min (B), respectively.

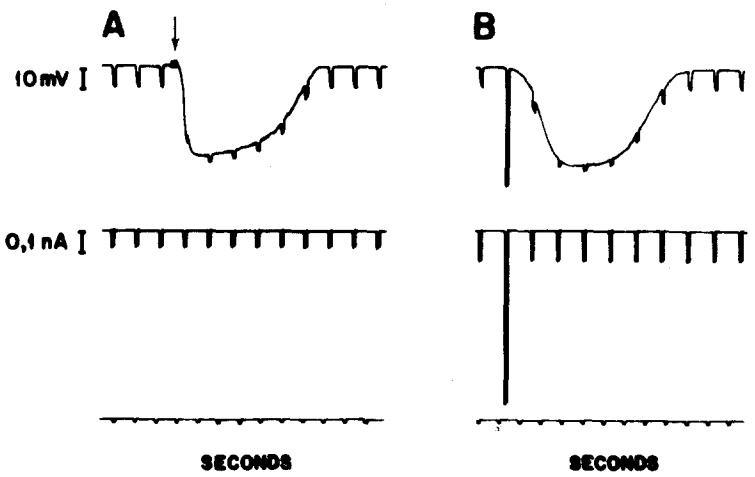

Fig. 4. Stimulation of slow hyperpolarizations in guinea-pig cells. A. A second empty electrode touches the cells surface (arrow) inducing firing in a quiescent cell. B. Hyperpolarizing current pulse initiates slow responses in another inactive cell. Upper traces: voltage recordings; lower trace: current monitoring.

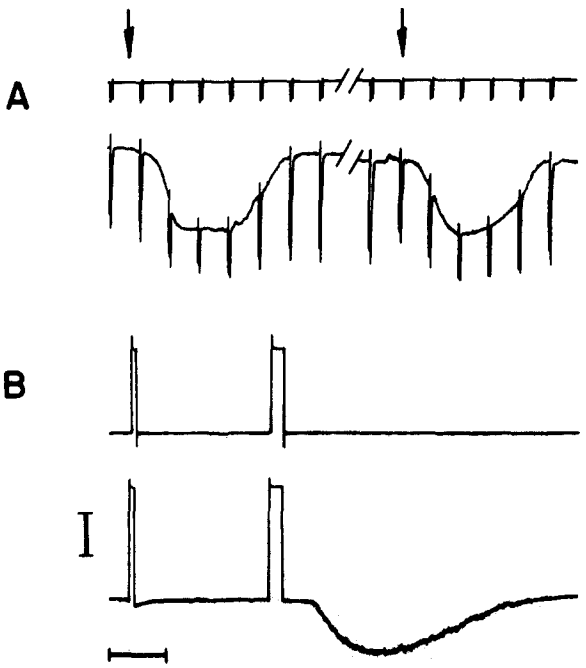

Fig. 5. Threshold for slow hyperpolarizations. Two records of the typical patterns from mechanically induced (tap indicated by arrows) hyperpolarizations in guinea-pig macrophage. Current $5 \cdot 10^{-10}$ A. B. Current pulse ($1 \cdot 10^{-9}$ A) of small duration fails to induce response in mouse microphage. With longer duration a slow hyperpolarization is induced. Upper traces correspond to current recordings, lower traces to voltages recordings. Calibration: vertical bar, 15 mV and 20 mV for B; horizontal bar, 2 s for A and 1 s for B.

previously studied gave the authors the impression of sensitivity to mechanical stimulation but no further attempt was made to clarify the phenomenon. Electrical stimulation evokes such a variety of responses in different cells and in some instances within the same cell that no threshold condition can be defined precisely.

In some instances, cell penetration was followed by successive spontaneous hyperpolarizing waveforms fairly constant in shape, amplitude and duration. The variety of the hyperpolarizations is considerable, so that the only way in which the effect of a substance on the triggering of electrical responses can be validated is to follow the changes induced in the same cell [7]. A further check of other cells in the same culture dish gives additional support to the data.

We have shown that the slow hyperpolarization results from an increase in K^+ permeability. This mechanism seems to be due to a Ca^{2+}-sensitive potassium channel as described for the red blood cell membrane and found also in several other cells (cat cortical neurons, cultured dorsal root ganglion cells, moluscan neurons, frog muscle fibers, rat liver cells). As Lew and Ferreira propose in their excellent review of the subject [12] "it is clear that one is dealing with a widespread mechanism present in evolutionary distant cells".

In the case of our cells it is possible to correlate the slow hyperpolarizations with this channel because; (a) it is a K^+ permeability change [6,7] with a time course compatible with the red blood cell effect [10]; (b) quinine, the most specific blocking agent for the channel, also blocks the macrophage response (experiments in progress); (c) Verapamil, the potent calcium antagonist, is also effective in blocking the response [19].

Gallin and Gallin [8] have shown that membrane potential changes in macrophages precede the membrane spreading and pseudopod formation that follows chemotactic stimulation. Changes in cytoplasmic calcium concentration seem to be closely related to the advancement or retraction of pseudopods in amoebas, where a local flow of current has been detected by means of an ultrasensitive extracellular vibrating probe [20]. These observations are compatible with the possibility that a Ca^{2+}-dependent potassium channel could trigger local changes involved in particle ingestion and secretion, besides mediating the chemotactic response. Localized membrane changes are of a great physiological importance and the electrical changes may either trigger or modulate mechanisms related to cell locomotion and adhesiveness. In phagocytic cells which are evolutionarily distant from the above mammalian system, such as the amoeba [21] and paramecium [22] a very similar mechanism of Ca^{2+}-dependent slow hyperpolarization has also been demonstrated.

The exact physiological role of the slow hyperpolarization remains unclear but it should be remarked that almost all motile cells have these membrane responses. It is tempting to speculate that there is a common event in cells from different sources, including macrophages. This may involve a common change in membrane permeability similar to that which we have described [7]. Megakaryocytes represent a new addition to the list of these cells [23]. There might also be a common event involved in the contractile systems of the different kinds of cell so far studied. There is increasing evidence from a number of different cell sources [24] including macrophages [25,26] that the contractile cytoplasmic proteins are involved both in delicate segmental or in more gener-

alized movements of the cell membrane. Free Ca^{2+} may play a major role in regulating these state changes of the cytoplasmic matrix. Since slow membrane electrical responses seem to be dependent upon Ca^{2+} entry into the cytoplasm it is of interest to explore the possible correlations of the electrical changes with the 'Gardos effect' [10] described for the red blood cell membrane.

We believe that the peculiarities of phagocytic cells described here explain the difficulties that have led several laboratories to cease electrophysiological observations on macrophages. The slow hyperpolarization awaits further characterization both of its subcellular mechanisms and their significance to the biology of the macrophage.

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