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Effect of Tumor Necrosis Factor on Epithelial Tight Junctions and Transepithelial Permeability[1]

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ABSTRACT

The serum factor inducing hemorrhagic necrosis of transplantable tumors [tumor necrosis factor (TNF)], and the macrophage hormone associated with cachexia in cancer and certain infectious diseases [cachectin] are known to be the same protein. Because an association may exist between TNF and the cachectic state, we wished to examine the effect of TNF on the permeability of epithelial barriers. We present data showing that TNF affects the tight junctional region between epithelial cells, lowering the transepithelial resistance and potential difference, and increasing the flow of solute between cells and across the epithelium. These effects are dose dependent, rapidly reversible, and inhibited by a monoclonal antibody to TNF-α. We suggest that the release of TNF at various sites throughout the body will cause a general breakdown in the barrier function of an epithelial cell sheet. This may relate to the cachexia observed in certain disease states. These findings are similar to our earlier published effects of phorbol esters and diacylglycerols on tight junctions, suggesting that protein kinase C activation may be involved.

INTRODUCTION

The human body is divided into series and parallel arrays of fluid-filled compartments which are delimited by epithelial or endothelial cell sheets. The cells dividing these compartments perform thermodynamic work by transporting solutes unidirectionally from one fluid compartment to the other, thereby defining and maintaining the unique composition of these compartments. The cells of the epithelial or endothelial sheet are, however, only one component of the barrier. Equally important is the junctional band surrounding each cell of the cell sheet. This "tight junction" or zonula occludens is a semipermeable barrier which allows certain solutes (depending upon their size and charge) to pass through this paracellular pathway between the cells from one fluid compartment to the other. The tight junction is composed of a network of protein strands the composition of which is just beginning to be understood (1). It is not static but appears to be highly regulated, producing permeability changes in response to a large number of changing physiological conditions (2), and playing a key role in transport processes previously thought to occur through the cells *per se* (3).

Protein kinase C, an ubiquitous signal transduction system which mediates many cellular functions, including cell division and carcinogenesis (4), also exerts control of permeability of tight junctions (5, 6). Relaxation of tight junctions by protein kinase C activation can dramatically increase the flow across the junctions (and thereby across the epithelium) of solutes as small as Na^+ or D-mannitol (M_r 182), or as large as epidermal growth factor (M_r 6100) (7).

In this study we expand upon our previous work on the regulation of transepithelial permeability, by examining the effect of the macrophage-secreted protein, tumor necrosis factor, on epithelial tight junctions.

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MATERIALS AND METHODS

The pig kidney epithelial (LLC-PK_1) cells used in these studies are from passages 185 to 200. Routine culturing entails seeding 1×10^5 cells in a 75-cm^2 culture dish containing 25 ml of α-minimum essential medium with 10% fetal bovine serum. After 1 week at 37°C and 5% CO_2, the culture reaches confluence, it is then trypsinized, and the passaging is repeated.

For measurements of transepithelial resistance and potential difference (voltage), a trypsinized suspension is seeded into Millicell HA filter cup assemblies (Millipore Corp.) containing 2 ml of culture medium with 10% fetal bovine serum and incubated at 37°C in a 5% CO_2 humidified atmosphere. These 30-mm-diameter assemblies have a filter base with 0.45-μm pores. Three such filter-cup assemblies are placed in a 100-mm Petri dish containing 15 ml of culture medium. When cells form a confluent monolayer, an intact epithelium exists across the filter base. Medium in the ring becomes the apical fluid compartment and medium in the Petri dish becomes the basolateral fluid compartment. Three days after seeding, the spontaneous transepithelial voltage is measured with 3 M KCl/agar bridges in series with calomel electrodes connected to a Fluk 8020B multimeter. Resistance measurements are performed by passing 40-μA current pulses via silver/AgCl electrodes connected to the culture medium via a second pair of 3 M KCl/agar salt bridges. Voltage deflections are measured with a third set of salt bridges connected to calomel electrodes, which in turn are connected to a Keithley model 197 autoranging digital multimeter.

For measuring transepithelial fluxes of D-[^{14}C]mannitol, cell sheets in filter-cup assemblies are refed with normal medium or medium containing TNF.[2] Resistance values are measured prior to the above refeeding and then at 0.5-h intervals as described in "Results." After 2 h at 37°C, resistance values will have maximally decreased. The control set (of three cell sheets) and one set of TNF-treated cell sheets are then rinsed three times in (morpholinopropanesulfonic acid-buffered) saline at 25°C. Sets of three cell sheets are incubated in Petri dishes at 25°C with 2 ml of apical saline, and 15 ml of basolateral saline containing 1 mM D-[^{14}C]mannitol. Samples of apical saline (50 μl) are removed from each filter cup at 30-min intervals, and the radioactivity was determined by liquid scintillation counting. The radioactivity (dpm) is converted to μmol of D-mannitol, by dividing by the specific activity. The appearance of radiolabeled mannitol is then plotted as a function of time. The rate of D-mannitol flux is determined by linear regression.

The culture medium used in these studies is a product of Hazelton Research Products. The fetal bovine serum was purchased from HyClone Laboratories, Inc. The human recombinant tumor necrosis factor-α was obtained either from Boehringer Mannheim [2.2×10^7 units/mg protein (determined by a cell lytic assay with L929 cultures)] or Genentech [4.3×10^7 units/mg protein]. The anti-TNF-α monoclonal antibody was a product of Boehringer Mannheim. The D-[^{14}C]mannitol used in the tracer flux study was obtained from ICN Radiochemicals, Inc.

RESULTS

For our studies we chose the LLC-PK_1 renal epithelial cell line because of its close parallel in physiological properties to the proximal tubule of the kidney (8), one of the most extensively investigated epithelial preparations. By culturing LLC-PK_1 cells on permeable filters we can monitor the transepithelial voltage and resistance across the cell sheets, as well as the

[2] The abbreviation used is: TNF, tumor necrosis factor.

transepithelial flux of radiolabeled solutes. We can then examine the effects of TNF on these properties, as we previously had done with phorbol esters and diacylglycerols (5, 6).

Unlike the near immediate effects of phorbol esters, an effect of TNF on transepithelial resistance does not occur until almost 90 min after TNF exposure to cell sheets (Fig. 1). The effect on resistance is mirrored in an identical effect on the potential difference (voltage) across the cell sheets (data not shown). The sharp drop in resistance [and voltage] reverses within 60 min [hence this effect is not due to general cytotoxicity] and is also dose dependent [a strong response in the 5–50-ng/ml range, as has been reported for other TNF effects in other cell types (9, 10)]. This same effect, and the same time course of fall and recovery of resistance, occurs whether the TNF is removed from the culture medium 30 min after application or is left in contact with the cells throughout the study. This suggests that once in contact with TNF, the cell sheet is committed to the drop in resistance. Since the continued presence of TNF does not block the recovery of resistance, the original intracellular signal which causes transepithelial resistance to drop is attenuated, or a second opposing signal is generated. We characteristically observe that resistance not only returns to original values but also normally rises above control values (Fig. 2). This "overshoot" is transient and resistance values return to initial levels usually before 12 h. The observed gradual decrease in control resistance levels is characteristic of all of our experiments. It is always a slow, graded decrease which rarely falls below 80% of initial resistance values.

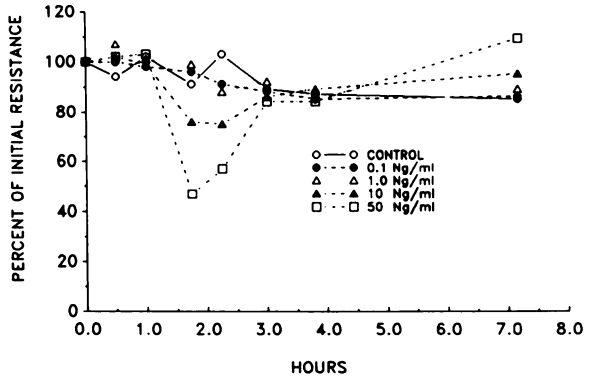

Fig. 1. Effect of various concentrations of TNF on transepithelial resistance across LLC-PK$_1$ cell sheets. Resistance measurements were taken at time zero across cell sheets refed with fresh control medium 4 h previously. Five sets of cell sheets (in filter cups) were then refed with control medium or medium containing various concentrations of human recombinant TNF-α. Resistance measurements were taken at the 0.5-h intervals shown, for a period of 7 h. Results are expressed as the percentage of the time zero reading (100%) of each cell sheet. One representative cell sheet (of a set of three) is shown for each condition. This experiment was repeated with cells of a later passage and similar results were obtained. Spontaneous transepithelial voltage readings were taken before each resistance reading and similar traces to the resistance data were obtained.

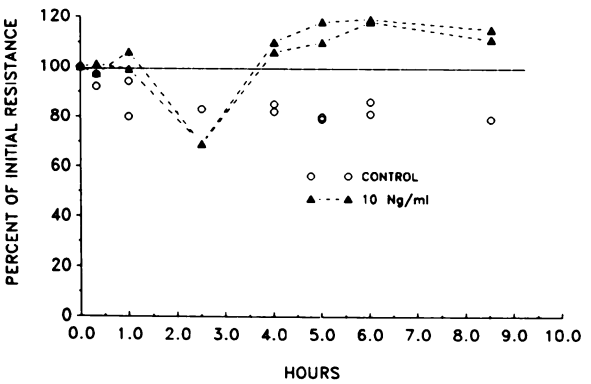

Fig. 2. Secondary enhancement of transepithelial resistance caused by TNF. The transepithelial resistance across pairs of LLC-PK$_1$ cell sheets was measured following the procedures outlined in "Materials and Methods." At time zero one set of cell sheets (control) in filter-cup assemblies was refed with normal medium. The other set was refed with medium containing 10 ng/ml TNF. The readings of two cell sheets for each condition are reported here. The control resistances show a slight (15%) but steady decline of resistance values during the course of the experiment. Cell sheets treated with TNF have the characteristic reversible resistance drop at approximately 2 h. After the recovery, resistance values not only rise above control levels but eventually exceed initial levels before slowly returning to control levels. This experiment was repeated with cells of a later passage and similar results were obtained.

Table 1 *Transepithelial flux of 1 mM D-[^{14}C]mannitol across control and TNF-treated LLC-PK$_1$ cell sheets*

Cell sheets in filter-cup assemblies were refed with normal medium or medium containing 40 ng/ml TNF. Resistance values were measured at 0.5-h intervals, until maximally decreased (2 h), and then cell sheets (Sets A and B) were rinsed in saline and a flux experiment was performed as described in "Materials and Methods." The rate of D-mannitol flux was then determined by linear regression of the plots of appearance of D-[^{14}C]mannitol *versus* time. The third set of cell sheets (Set C) was incubated for 3 h at 37°C, by which time resistance recovered to 72% of the initial readings. The above flux procedure was then performed on these cell sheets.

Set	Condition	Time (h)	Resistance (% of initial)	D-Mannitol flux (nmol/h × cm^2)
A	Control	2	88 ± 2[a]	3.44 ± 0.27[b]
B	40 ng/ml TNF	2	59 ± 6[a]	4.75 ± 0.15[b]
C	40 ng/ml TNF	3	72 ± 7[a]	4.06 ± 0.09[b]

[a] Average resistance of three cell sheets ± range of values.
[b] Average of linear regression determinations for three cell sheets ± SE.

To confirm that the TNF-induced sharp decrease in transepithelial resistance is due to induced leakiness in the tight junctions, we added 1 mM D-[^{14}C]mannitol to the basolateral fluid compartment and then measured the rate of appearance of D-[^{14}C]mannitol in the apical compartment. In previous work we have shown that D-mannitol has negligible affinity for any membrane transport system in LLC-PK$_1$ cells and must cross the cell sheets by passing across the tight junctions and moving between the cells (11). When cell sheets were treated with TNF for 2 h at 37°C (transepithelial resistance had fallen to approximately 60% of initial values), the unidirectional flux of 1 mM D-[^{14}C]mannitol across the TNF-treated cell sheets (Table 1), had risen to 138% of control flux values. By 3 h (by which time resistance had recovered to over 70% of initial values) the D-mannitol flux rate had returned to 118% of control values. We believe that these effects on transcellular mannitol flux would be more dramatic [as in the phorbol ester studies (5, 6)], but with TNF there is a relatively rapid recovery of tight junction resistance, which was not true with phorbol esters. This recovery is sufficiently rapid that it is near completion while we are still measuring the mannitol flux. Therefore our flux value is actually a reflection of an average permeability of the junctions when resistance fell to its lowest value, and at a later stage when resistance is recovering.

When LLC-PK$_1$ cells are cultured on plastic or glass, as opposed to a permeable filter, the confluent monolayer is highlighted by the appearance of fluid-filled "domes" (Fig. 3). These arise from the unidirectional transport of solutes and water from the culture medium (apical compartment) to the space between cell sheet and dish (basolateral compartment) (12). When 50 ng/ml TNF were added to the culture medium there was initially no effect, but by 2 h many domes began to collapse. This dome collapse is another manifestation of the decrease in tight junctional resistance caused by TNF, because domes collapse when tight junctions allow backleak of the solutes and water transported across to the undersurface. Similar effects were observed when phorbol esters and diacylglycerols were applied to these cells (5, 6). However, using TNF we observe a

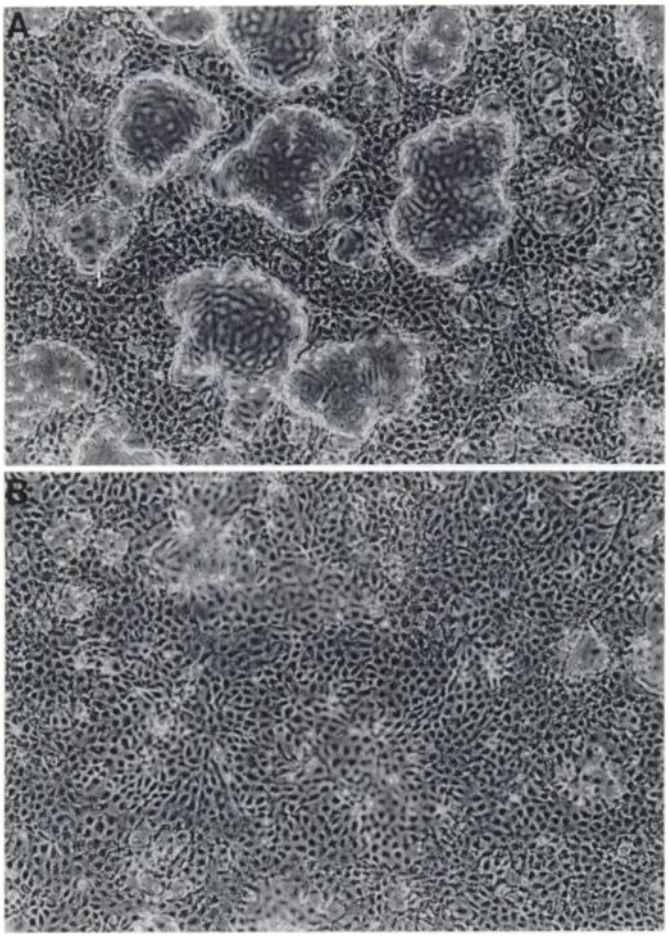

Fig. 3. Effect of 50 ng/ml TNF on domes of confluent LLC-PK1 monolayers. Confluent LLC-PK$_1$ cultures on solid subsurfaces, such as plastic culture dishes, characteristically form three-dimensional fluid-filled blister formations termed domes. These structures arise because the uniformly polarized cells vectorially transport solutes from the apical compartment (culture medium overlaying cells) to the basolateral compartment (space between cells and dish). *A*, monolayer in normal medium; *B*, monolayer in medium containing 50 ng/ml TNF for 2 h at 37°C. Domes are collapsing with the same time course as the drop in transepithelial resistance.

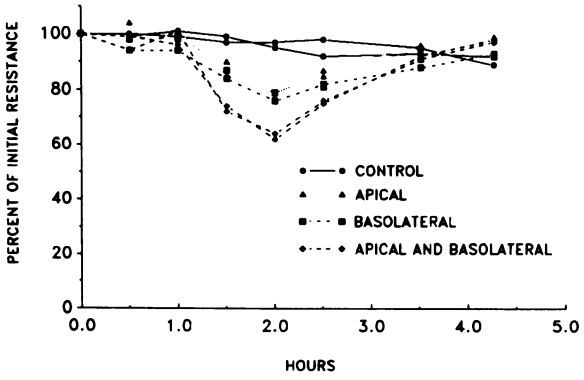

Fig. 4. Effect of apical *versus* basolateral exposure of LLC-PK$_1$ cell sheets to TNF. After initial resistance readings as described in Fig. 1, cell sheets were refed with normal medium in both apical and basolateral compartments, medium containing 10 ng/ml TNF in both compartments, or normal medium in one compartment and TNF-containing medium in the other compartment. Resistances were recorded at the times indicated as described in "Materials and Methods." The readings of two cell sheets for each condition are shown here. The characteristic resistance drop at 2 h occurred whether the TNF was in the apical or basolateral compartment. When placed in both compartments, the effect was additive. This experiment was repeated with cells of a later passage with similar results.

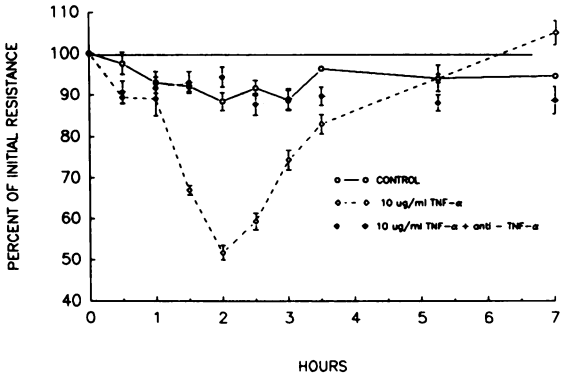

Fig. 5. Anti-TNF-α monoclonal antibody inhibits TNF-induced decrease in transepithelial resistance. Preincubating 10 ng/ml TNF-α (330 units/ml) with anti-TNF-α (150 units/ml) in culture medium for 30 min at room temperature completely blocked the resistance decrease caused by TNF-α. At time zero, culture medium (control), culture medium with TNF-α, or culture medium with THF-α preincubated with anti-TNF-α antibody was added to the apical compartment of sets of three cell sheets. Control medium was added to the basolateral compartment. Resistance was measured at the times indicated for a period of 7 h. Results are expressed as the average of three cell sheets (percentage of initial resistance) ± SE. Cell sheets incubated with antibody but without TNF-α showed a tracing very similar to that of control.

more rapid recovery of the domes on the monolayer, with near total recovery of domes by 4 h. This time course of TNF inducing dome collapse and recovery is very similar to the time course of the TNF-induced resistance changes described in Fig. 1. We also note that there is no indication of any cell degeneration accompanying the dome collapse, even at TNF levels of 100 ng/ml for 24-h incubations at 37°C.

In marked contrast to the morphological changes of subconfluent LLC-PK$_1$ cells caused by protein kinase C activators (6), TNF had no effect on subconfluent LLC-PK$_1$ morphology. Additional experiments are required, but this may signify that the relatively undifferentiated, subconfluent LLC-PK$_1$ cultures lack the receptor and/or signal transduction system required for TNF effects.

Our previous work with epidermal growth factor (EGF) showed that its receptors were localized to the basolateral side of the LLC-PK$_1$ cell sheets (12). Expecting similar localization for cell surface-mediated TNF effects, we presented TNF to either the apical or the basolateral surface of LLC-PK$_1$ cell sheets and monitored transepithelial voltage and resistance. In contrast to results with EGF, TNF induced a decrease in resistance when presented to either the apical or the basolateral cell surface (Fig. 4). The drop in resistance was of equal magnitude and identical time course in each case. When presented simultaneously to both cell surfaces, the time course was again the same but the effect was additive. It is unlikely that TNF is simply leaking across the cell sheet to the surface containing its receptors because it is a relatively large molecule. TNF monomers have molecular weights of 17,000, and the active form of TNF may be a trimer with a molecular weight of 55,000 (13). Additional work is required to understand how TNF is equally effective from either side of a polar cell sheet and why addition to both surfaces is synergistic.

To test whether TNF-α itself is causing the observed changes in transepithelial resistance, TNF-α was preincubated with a monoclonal antibody to TNF-α for 30 min at 25°C in culture medium. This resulted in a complete inhibition of the effect of TNF-α on tight junctional resistance (Fig. 5). When incubated with cell sheets by itself, the monoclonal antibody produced no effect on transepithelial resistance.

DISCUSSION

We present evidence that TNF can induce decreased resistance in epithelial tight junctions, leading to leakage of solutes

across the epithelial barrier and consequent dissipation of electrical and chemical gradients. Because of (*a*) the physiological importance of biological transport across epithelial and endothelial barriers *in vivo,* (*b*) the amount of energy consumed in such transport, and (*c*) the significant role of the tight junctional barrier in these vectorial transport processes, we propose that TNF-mediated disruption of tight junctional barriers can result in greatly increased expenditure of energy, as transepithelial transport becomes less efficient. This may then contribute to cachexia.

Unidirectional transport of solutes from one fluid compartment to another frequently proceeds against a concentration and/or electrical gradient and therefore requires energy. The active transport of protons into the gastric lumen or the reabsorption of glucose from the urine are examples. The dependence of transport upon metabolism arises from dependence of ATPases on ATP availability. Less apparent is the fact that transport, by consuming ATP (and thereby altering the intracellular ATP/ADP ratio) can feed back and control metabolism. This has been observed in renal epithelia by effects of ouabain (14) and phloridzin (15) on oxygen consumption. If epithelial barriers throughout the body are made nonspecifically leaky through a decreased resistance in tight junctional (paracellular) pathways, solutes will flow back down their electrochemical gradients and energy (and metabolic fuels) will be wasted.

A vivid example of caloric waste is cachexia, a catabolic state leading to pronounced weight loss and fatigue even with normal caloric intake. Once thought to arise from lipopolysaccharide endotoxin, the cachectic state may arise in part from the action of the macrophage hormone, TNF (cachectin) (16), although there is still considerable disagreement on the association of elevated serum levels of TNF and diseases such as cancer, which are associated with cachexia (17, 18).

TNF is but one member of a family of cytokines released by cells of the immune system in response to various invasive stimuli. TNF has been observed to act synergistically with other cytokines such as the interleukins to modulate a variety of cellular activities (19–21). This synergism may play a role in the tight junction effects described here, specifically by TNF inducing the synthesis of a specific interleukin by the LLC-PK_1 cells, which may then be directly responsible for the tight junction permeability change. This possibility will be tested in future experiments, especially since it may help to explain the 2-h delay in TNF effects on tight junctions, as well as the reversibility of the phenomenon.

The rapid reversibility of the permeability changes does not necessarily lessen the significance of this effect of TNF for epithelial barriers. The cultured epithelial monolayer *in vitro* has all epithelial cells exposed to TNF simultaneously. However, an epithelial cell layer *in vivo* will receive exposure to TNF by its release from macrophages in the microvasculature. It is unlikely that all cells of the epithelium would receive threshold levels of TNF at the same time. Therefore discrete groups of cells in the epithelium may experience junctional collapse at different times. Each group will represent, however, a low resistance shunt pathway across the epithelium at any given time. The overall net effect would be a prolonged dissipation of transepithelial gradients and transepithelial solute leaks, even though different regions of epithelial cells, at varying times, are undergoing increased tight junctional leakiness or recovery from this state.

In summary, although the action and interactions of TNF are just beginning to be understood, it has demonstrated ability to systemically alter intermediary metabolism as evidenced by induced changes in lactate production and glucose oxidation (22). The high levels of TNF appearing in the circulation of some cancer patients (23) and acquired immunodeficiency syndrome patients (24) who are manifestly cachectic suggest that this cytokine may have a role in the systemic catabolism observed in widely different disease situations. These various diseases have in common the phenomenon of disruptive invasion, either viral, bacterial, or metastatic, at the interfaces between fluid compartments. As stated above, these interfaces are normally epithelial barriers and hence the sites of large expenditures of energy through biological transport. If TNF compromises these barriers, energy may be significantly wasted.

This is, however, certainly not the only catabolic action of TNF, since it has been shown to alter 3T3 preadipocyte differentiation and glycerophosphate dehydrogenase synthesis (25), examples of TNF effects on intermediary metabolism which are fully independent of effects on transepithelial permeability. However, an effect of TNF on epithelial and endothelial barriers seems well established. TNF administration results in increased leukocyte infiltration into the lung (16) and neutrophil penetration of endothelial junctions (26). By temporarily disrupting tight junctions, TNF may facilitate the access of white blood cells to sites of invasion, but a consequence of this immune response may be the backflow of solutes through the permeabilized junctions and the ensuant bioenergetic cost to the organism.

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