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Listeria monocytogenes, but Not *Salmonella typhimurium*, Elicits a CD18-Independent Mechanism of Neutrophil Extravasation into the Murine Peritoneal Cavity

J. WAYNE CONLAN* AND ROBERT J. NORTH

Trudeau Institute, Inc., Saranac Lake, New York 12983

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This study shows that extravasation of neutrophils into the peritoneal cavities of mice in response to intraperitoneal (i.p.) inoculation of wild-type *Listeria monocytogenes* requires the participation of leukocyte adhesion molecules that are different from those involved in neutrophil recruitment in response to i.p. inoculation of *Salmonella typhimurium*. In the case of *S. typhimurium*, extensive neutrophil influx could be essentially abolished by treating mice with either anti-CD11b or anti-CD18 monoclonal antibodies, whereas the same monoclonal antibodies failed to prevent neutrophil accumulation in the peritoneal cavity in response to inoculation of *L. monocytogenes*. On the other hand, i.p. inoculation of a listeriolysin-negative strain of *L. monocytogenes* induced a CD11b-dependent neutrophil influx. The possibility that wild-type *L. monocytogenes*, by virtue of its ability to inhabit the cytosol of the cells it infects, induces the expression of endothelial cell adhesion molecules in the microvasculature of the peritoneal cavity to which neutrophils adhere via leukocyte adhesion molecules distinct from β-2 integrins is discussed.

Circulating neutrophils are the first leukocytes to extravasate in large numbers from the blood into sites of inflammation. Extravasation of neutrophils is preceded by their selective irreversible adhesion to the endothelium of blood vessels that traverse inflamed tissue, and it is now established that this adhesion is predominantly dependent on interactions between the surface β-2 integrin molecule (CD18/CD11b) of neutrophils and intercellular adhesion molecule 1 inducibly expressed on the surface of vascular endothelial cells (reviewed in references 2, 10, and 18). Neutrophils can, additionally, adhere to endothelium via their other β-2 integrins, namely, CD18/CD11a and CD18/CD11c (17, 19). Therefore, adhesive interactions involving β-2 integrins are referred to as being CD18 dependent. However, both CD18-independent (6) and intercellular adhesion molecule 1-independent (22) mechanisms of neutrophil adhesion to endothelium have been reported. Indeed, the finding (6) that leukocyte adhesion receptor and endothelial cell counterreceptor usage can vary from tissue to tissue following inoculation of the same inflammogen and within the same tissue following inoculation of different inflammogens strikingly illustrates the molecular complexity involved in the extravasation of leukocytes. The proinflammatory mediators that induce the expression of counteradhesion molecules on vascular endothelium prior to the margination of neutrophils to the endothelium are presumed to originate from nearby extravascular cells stimulated by the inflammogen (10). Clearly, to induce leukocyte extravasation by different adhesion and counteradhesion molecules, extravascular host cells resident in the inflamed tissues must have the capacity to release different inflammatory mediators in response to different inflammogens.

The purpose of this study was to determine whether different facultative intracellular bacteria, possibly because they grow in different compartments in host cells, elicit different mechanisms of neutrophil recruitment. The results show, under the experimental conditions used, that whereas *Salmonella typhimurium* induces exclusively a CD18-dependent mechanism of neutrophil recruitment into the peritoneal cavity, *Listeria monocytogenes* induces exclusively a CD18-independent pathway. They show, in addition, that the ability of *L. monocytogenes* to induce a CD18-independent pathway of neutrophil migration reflects the ability of this organism to produce a molecule (listeriolysin) that enables it to escape from the phagocytic vacuole of host cells.

* Corresponding author. Mailing address: Trudeau Institute, Inc., P.O. Box 59, Saranac Lake, NY 12983. Phone: (518) 891-3080. Fax: (518) 891-5126.

MATERIALS AND METHODS

Mice. Male CB6/F1 mice were obtained from the Trudeau Institute Animal Breeding Facility, Saranac Lake, N.Y. They were used when they were 9 to 13 weeks old.

Bacteria. Log-phase cultures of virulent *S. typhimurium* (strain C5R), virulent *L. monocytogenes* (strain EGD), and listeriolysin-negative avirulent *L. monocytogenes* (strain DPL-1044) were prepared as described previously (3, 5) and stored frozen at −70°C. For experimental use, they were thawed, pelleted by centrifugation, resuspended, and diluted in sterile saline to the required inoculum. Dead *L. monocytogenes* (EGD) and *S. typhimurium* cultures were prepared by exposure to UV irradiation as described elsewhere (8) and stored at −70°C. The sterility of UV-killed bacteria was confirmed by plating on Trypticase soy agar and incubation at 37°C for 24 h. The bacterial concentrations of these UV-killed preparations were assumed to be the same as their CFU immediately before irradiation. Viable and dead bacteria were inoculated intraperitoneally (i.p.) in a volume of 0.2 ml.

Sterile inflammogens. A 4% (wt/vol) solution of casein (Difco Laboratories, Detroit, Mich.) was prepared in saline, buffered to pH 7 with NaOH, and autoclaved. Solutions (10%, wt/vol) of brewer's thioglycolate (Difco) and Proteose Peptone (Difco) were prepared in distilled water and sterilized by autoclaving. *S. typhimurium* smooth lipopolysaccharide (LPS) (Sigma Chemical Co., St. Louis, Mo.) was prepared in saline and sterilized by membrane filtration (0.22-μm pore size). All sterile inflammogens were inoculated i.p. in a volume of 0.2 ml.

TABLE 1. Effect of anti-CD11b treatment on neutrophil accumulation in the peritoneal cavities of mice following i.p. inoculation of sterile inflammogens[a]

Inflammogen	No. (10^4) of neutrophils ± SD recovered in peritoneal lavages	
	Control mice	NIMP-R10-treated mice
Saline	5 ± 4	
Sodium caseinate	423 ± 131	50 ± 22
Proteose Peptone	495 ± 127	286 ± 39
Thioglycolate	1,060 ± 130	376 ± 118

[a] Anti-CD11b MAb NIMP-R10 was administered intravenously in a dose of 0.25 mg 4 h before i.p. injection of inflammogens. PC were harvested by lavage 12 h postinjection, and total and differential counts were performed. Five mice per group were used.

MAbs. Monoclonal antibody (MAb) NIMP-R10 is a rat immunoglobulin G subclass 2b MAb against murine CD11b (11). The hybridoma secreting NIMP-R10 was generously provided by A. Lopez, Institute of Medical and Veterinary Sciences, Adelaide, Australia. MAb 2E6 is a hamster MAb against murine CD18 (12). The hybridoma secreting this MAb was a gift from R. Steinman, Rockefeller University, New York, N.Y. MAb YN1.17 is a rat immunoglobulin G subclass 2b MAb against murine intercellular adhesion molecule 1 (9), and MAb MK1.9 is a rat immunoglobulin G subclass 1 MAb against murine vascular cell adhesion molecule 1 (13). The hybridomas secreting these MAbs were obtained from the American Type Culture Collection, Rockville, Md. All MAbs were grown as ascites and partially purified by ammonium sulfate precipitation. MAbs NIMP-R10 and 2E6 were given intravenously in a dose of 0.25 mg, whereas MAbs YN1.17 and MK1.9 were given in a dose of 0.5 mg. All MAbs were given 4 h before i.p. inoculation of bacteria or sterile inflammogens.

Peritoneal exudates. Peritoneal cells (PC) were collected at various times up to 12 h after i.p. inoculation of bacteria or sterile inflammogens. The mice were killed by cervical dislocation, and cells were harvested by lavaging the peritoneal cavity twice with 3-ml volumes of phosphate-buffered saline containing 20 U of heparin per ml, using a syringe fitted with a 20-gauge needle. Harvested cells were kept on ice. Total cell counts were determined with a hemocytometer, and viability was determined by trypan blue exclusion staining. Cell viability was always >95%. Differential counts were done on cytosmears. This involved diluting the cells to 10^5 per ml in phosphate-buffered saline containing 3% (vol/vol) fetal calf serum and cytocentrifuging them at 550 rpm for 5 min in a Shandon Cytospin II centrifuge. The cytosmears were air dried and stained with Diff Quik (Baxter Corp., Miami, Fla.). Differential counts were obtained by counting 500 consecutive cells in each smear. To calculate the number of viable bacteria recovered in lavages, 1 ml of PC was added to 4 ml of sterile distilled water, vortex mixed, and sonicated. The sonicate was plated on Trypticase soy agar, and CFU were counted after incubation at 37°C for 24 h. To determine the number of host cell-associated viable bacteria, PC were pelleted by low-speed centrifugation (300 × *g* for 5 min) and the cell-free supernatants were plated as described above; the resulting CFU were subtracted from the viable count obtained by plating uncentrifuged PC. To determine the total cell-associated bacterial count, the number of PC with bacteria associated and the number of bacteria per PC were calculated by microscopic examination of cytosmears. By 12 h, 20-fold more *S. typhimurium* bacteria and 5-fold more *L. monocytogenes* bacteria were recovered in peritoneal lavages than in the livers and spleens of mice inoculated i.p., respectively, with one or the other organism (data not shown).

TABLE 2. Effects of treatment with anti-CD11b or anti-CD18 MAbs on neutrophil migration into the peritoneal cavities of mice inoculated i.p. with *S. typhimurium*[a]

MAb treatment	No. (10^4) of neutrophils ± SD recovered in peritoneal lavages	Log_{10} bacteria ± SD in peritoneal lavage
None	1,320 ± 420	5.71 ± 0.37
NIMP-R10 (anti-CD11b)	18 ± 10	5.87 ± 0.22
2E6 (anti-CD18)	61 ± 20	5.17 ± 0.35

[a] PC were collected by lavage 12 h after i.p. inoculation of *S. typhimurium*. MAbs were administered intravenously 4 h before inoculation of bacteria. Five mice per group were used.

TABLE 3. Accumulation of neutrophils in the peritoneal cavities of mice inoculated i.p. with various doses of UV-killed *S. typhimurium* or LPS[a]

Inoculum	No. of neutrophils (10^4) ± SD recovered in peritoneal lavages
UV-killed *S. typhimurium*	
10^9	140 ± 55
10^8	114 ± 34
10^7	275 ± 34
10^6	314 ± 278
10^5	106 ± 20
S. typhimurium LPS (ng)	
50,000	102 ± 26
5,000	75 ± 19
500	34 ± 5
50	115 ± 49
5	101 ± 45

[a] PC were collected 12 h after i.p. inoculation of inflammogens. Three mice per group were used.

RESULTS

Different sterile inflammogens induce different mechanisms of neutrophil migration into the peritoneal cavity. To determine whether anti-CD11b MAb (NIMP-R10) as prepared in this laboratory could inhibit neutrophil extravasation, preliminary experiments were performed to gauge the ability of this MAb to bind to neutrophils and prevent their recruitment into

TABLE 4. Effects of treatment with anti-CD11b or anti-CD18 MAbs on neutrophil extravasation into the peritoneal cavities of mice inoculated i.p. with *L. monocytogenes*[a]

MAb treatment	No. (10^4) of neutrophils ± SD recovered in peritoneal lavages	Log_{10} bacteria ± SD in peritoneal lavage
None	500 ± 64	4.58 ± 0.14
NIMP-R10 (anti-CD11b)	363 ± 86	6.06 ± 0.16
None	318 ± 95	4.21 ± 0.25
2E6 (anti-CD18)	769 ± 263	5.84 ± 0.11

[a] PC were harvested by lavage 12 h after i.p. inoculation of 5 × 10^6 CFU of *L. monocytogenes* bacteria. MAbs were administered intravenously 4 h before inoculation of bacteria. Four or five mice per group were used.

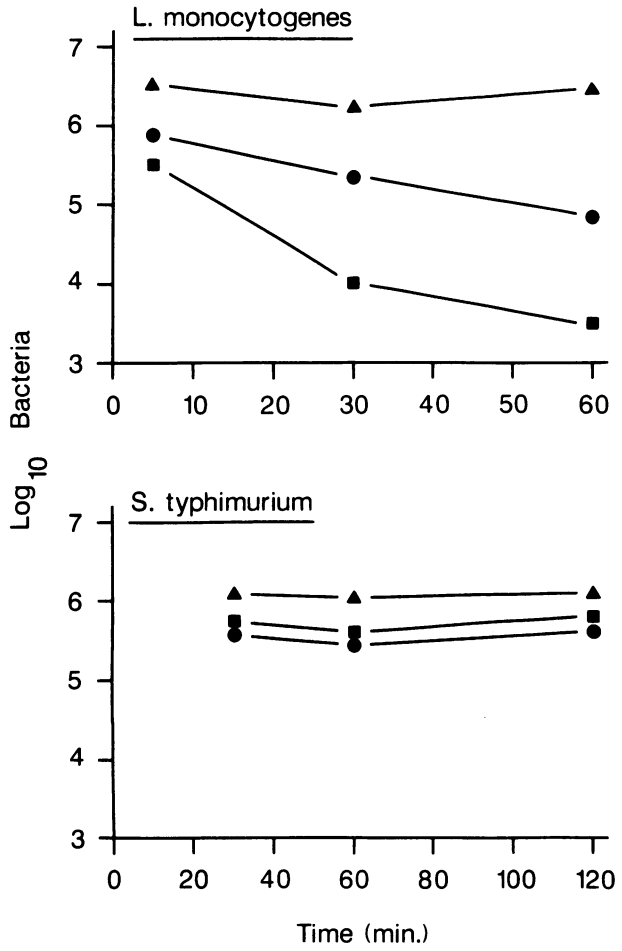

FIG. 1. Early fate of i.p. inocula of *L. monocytogenes* and *S. typhimurium*. Mice were inoculated with 5×10^6 CFU of one or the other organism, and peritoneal lavages were collected at 5, 30, 60, and 120 min postinoculation. Cell-associated (●) and cell-free (■) CFU and total cell-associated bacteria (▲) were determined as described in Materials and Methods. The means of four mice per group are shown. Standard errors of the means were <0.25 $\log_{10}$ unit.

the peritoneal cavities of mice in response to i.p. inoculation of several commonly used sterile inflammogens. The results presented in Table 1 show that in mice treated with NIMP-R10, neutrophil recruitment into the peritoneal cavity in response to i.p. inoculation of casein was almost completely inhibited. By contrast, the same treatment was less effective in preventing neutrophils from accumulating in the peritoneal cavity in response to i.p. inoculation of thioglycolate or Proteose Peptone. In the last circumstances, the failure of NIMP-R10 to completely inhibit neutrophil migration was not due to inadequate availability of this MAb in circulation, because similar results (not shown) were obtained with mice given twice the dose (0.5 mg) of this antibody. The results indicate, instead, that neutrophils are recruited into the peritoneal cavity in response to thioglycolate or Proteose Peptone partly by a CD11b-independent mechanism that was not inhibited by NIMP-R10 and partly by a CD11b-dependent mechanism that was inhibited by this MAb.

***S. typhimurium* inoculated i.p. elicits a CD18/CD11b-dependent mechanism of neutrophil extravasation into the peritoneal cavity.** In view of the foregoing findings, experiments were performed to determine whether *S. typhimurium* inoculated i.p. induces either a CD18/CD11b-dependent or -independent mechanism of neutrophil recruitment into the peritoneal cavity. Table 2 shows that injecting mice i.p. with 5×10^5 CFU of viable *S. typhimurium* cells caused a large influx ($>10^7$) of neutrophils into the peritoneal cavity by 12 h which was essentially inhibited by treatment with MAb NIMP-R10. Thus, in this situation neutrophil migration into the peritoneal cavity is mediated almost exclusively by a CD11b-dependent mechanism. Therefore, as expected, MAb 2E6, which binds the CD18 polypeptide chain of the CD18/CD11b adhesion receptor complex of neutrophils, also almost completely inhibited neutrophil recruitment. Because similar numbers of viable *S. typhimurium* bacteria were recovered in the peritoneal lavages from control, NIMP-R10-treated, and 2E6-treated mice, it is apparent that the peritoneal cavities of all three groups of mice were exposed to similar levels of inflammatory stimulation by the organism during the first 12 h of inflammation.

The stimulus for inflammation was unlikely due to extracellular LPS because i.p. inoculation of as many as 10^9 UV-killed *S. typhimurium* bacteria or as much as 50,000 ng of *S. typhimurium* LPS caused a 5- to 10-fold-smaller influx of neutrophils into the peritoneal cavity than did live organisms (Table 3). This suggests that the neutrophil influx elicited by viable *S. typhimurium* is not solely due to LPS.

Virulent, but not listeriolysin-negative or UV-killed, *L. monocytogenes* elicits a CD18-independent mechanism of neutrophil recruitment into the peritoneal cavity. While there is ample evidence (14–16, 21) that *L. monocytogenes* rapidly escapes the phagocytic vacuole and grows in the cytoplasm of the host cell, there appears to be no evidence that *S. typhimurium* does so. Therefore, experiments were carried out to determine whether *L. monocytogenes* induces a different mechanism of neutrophil extravasation than does *S. typhimurium*. Table 4 shows that, in contrast to the situation with *S. typhimurium*, treatment with MAb NIMP-R10 or 2E6 failed to inhibit recruitment of neutrophils into the peritoneal cavity in response to i.p. inoculation of 5×10^6 CFU of wild-type virulent *L. monocytogenes*. This was not due to exhaustion of MAb because similar results were obtained when mice were treated with a fourfold-greater amount of MAb NIMP-R10 (not shown). Moreover, the same quantities of these MAbs were able to inhibit *S. typhimurium*-induced extravasation of neutrophils (Table 2). Thus, with *L. monocytogenes*, neutrophil migration was not only independent of CD11b involvement but also independent of CD18. This suggests that neutrophil extravasation into the peritoneal cavity in response to i.p. inoculation of *L. monocytogenes* cells is not mediated via adhesive interactions involving any of the three β-2 integrins, CD18/CD11a, CD18/CD11b, and CD18/CD11c.

However, despite their failure to interfere with neutrophil accumulation, MAbs NIMP-R10 and 2E6 did cause exacerbation of listeriosis, as evidenced by an approximately 30- to 40-fold increase in the number of bacteria recovered in peritoneal lavages from MAb-treated mice over the number recovered in those from control mice. Perhaps these MAbs interfered with the ability of peritoneal macrophages to ingest and kill *L. monocytogenes*, as has been shown to be the case in vitro (7). Thus, unlike the situation with *S. typhimurium*, CD18-dependent bactericidal mechanisms are crucial for restricting the early growth of *L. monocytogenes* in the peritoneal cavity. In NIMP-R10-treated mice, the increased bacterial burden did not result in an increase in the number of neutrophils migrating into their peritoneal cavities, but in 2E6-treated mice, it did. The reason for this difference is not known.

To determine whether the ability of *L. monocytogenes* to

elicit a CD18-independent mechanism of neutrophil extravasation into the peritoneal cavity was a reflection of the viability and virulence of the wild-type organism, experiments were performed to examine neutrophil recruitment in response to i.p. inoculation of dead or avirulent organisms. Neutrophil migration into the peritoneal cavity after i.p. inoculation of 5×10^6 CFU of listeriolysin-negative avirulent *L. monocytogenes* bacteria was mediated almost exclusively via a CD11b-dependent mechanism, in that it was almost completely inhibited in mice treated with anti-CD11b MAb NIMP-R10 [$(66 \pm 17) \times 10^4$ neutrophils versus $(430 \pm 55) \times 10^4$ neutrophils in controls]. When 5×10^6 UV-killed *L. monocytogenes* bacteria were inoculated into mice, there were $(410 \pm 99) \times 10^4$ and $(27 \pm 14) \times 10^4$ neutrophils, respectively, recovered in peritoneal lavages in control and NIMP-R10-treated mice. It will be noted that these findings are almost the complete opposite of that obtained with virulent *L. monocytogenes* (Table 4) in which neutrophil recruitment was mediated by a CD18-independent mechanism. This was the case, in spite of the fact that the dead and listeriolysin-negative organisms caused the same number of neutrophils to extravasate as wild-type organisms.

***L. monocytogenes* and *S. typhimurium* preferentially associate with resident macrophages following their i.p. inoculation.** One potential source of proinflammatory mediators of neutrophil extravasation into the peritoneal cavity are infected resident PC. Therefore, to determine whether i.p. inoculated bacteria are predominantly ingested by resident macrophages, peritoneal lavages were collected from mice 5, 30, 60, and 120 min after i.p. inoculation of 5×10^6 CFU of *S. typhimurium* or *L. monocytogenes*. Viable cell-associated and viable cell-free bacteria were enumerated following low-speed centrifugation of lavages. Total cell-associated bacteria were enumerated by microscopic examination of stained cytosmears. The results are shown in Fig. 1. It shows that an i.p. inoculum of *L. monocytogenes* rapidly became cell associated and that approximately 95% of the inoculum was inactivated within 1 h. When stained smears of PC obtained 1 h postinoculation were examined by microscopy, cell-associated *L. monocytogenes* bacteria were found to be exclusively associated with macrophages. In the case of *S. typhimurium*, there was no inactivation of the inoculum during the first 2 h, by which time approximately 50% of the bacteria were cell associated, exclusively with macrophages.

DISCUSSION

According to the results of this study, wild-type *L. monocytogenes* and *S. typhimurium* inoculated i.p. into mice each elicit a different mechanism of neutrophil recruitment into the peritoneal cavity. Neutrophils accumulate via a CD18-independent mechanism in response to i.p. inoculation of *L. monocytogenes*, whereas they accumulate by a CD18/CD11b-dependent mechanism in response to *S. typhimurium*. This indicates that each organism induces the expression of different neutrophil-binding adhesion receptors on the surface of endothelial cells of the microvasculature that serves the peritoneal cavity. This finding contradicts the notion that neutrophil migration into the peritoneal cavity is exclusively mediated via CD18, as has been suggested by others using different inflammatory stimuli (6). Moreover, the failure of anti-CD11b treatment to prevent neutrophil accumulation in the peritoneal cavity following i.p. inoculation of *L. monocytogenes* is in contrast to previous findings from this laboratory showing that the same MAb-treatment prevented neutrophils from focusing to sites of *L. monocytogenes* infection in the liver (4). This suggests that the mechanisms of neutrophil migration induced by *L. monocytogenes* depend on the tissue it infects. In this regard, *L. monocytogenes* behaves like some other inflammogens that can induce either CD18-dependent or -independent mechanisms of neutrophil influx (6) depending on the site at which they are injected.

The source of proinflammatory mediators (cytokines and chemokines) responsible for attracting neutrophils into the peritoneal cavity in response to i.p. inoculation of *L. monocytogenes* or *S. typhimurium* was not revealed by the present study. One possibility is that the mediators are generated by resident peritoneal macrophages in response to having ingested the i.p. inoculated *L. monocytogenes* or *S. typhimurium* bacteria. Reports that these two organisms localize to different compartments of host macrophages (1, 15, 21) could explain why they induce different neutrophil recruitment pathways. For instance, the ability of *L. monocytogenes* to induce a CD18-independent mechanism of neutrophil migration into the peritoneal cavity as reported here requires that the organism be viable and capable of producing listeriolysin. By contrast, listeriolysin-negative or dead *L. monocytogenes* induced a CD11b-dependent neutrophil influx into this compartment. Because virulent, but not listeriolysin-negative or dead, *L. monocytogenes* can escape from the phagocytic vacuole of macrophages (20), it seems reasonable to propose that virulent organisms induce the production of different inflammatory mediators by virtue of their being located in the macrophage cytosol.

On the other hand, virulent *S. typhimurium*, like listeriolysin-negative *L. monocytogenes*, reportedly remains confined to phagocytic vacuoles following ingestion by macrophages (1). This might explain why *S. typhimurium*, like listeriolysin-negative *L. monocytogenes*, induces a CD18-dependent recruitment of neutrophils into the peritoneal cavity. However, unlike listeriolysin-negative *L. monocytogenes*, *S. typhimurium* can survive in phagocytic vacuoles (1), and this ability might explain why viable *S. typhimurium* is a more potent inflammogen than listeriolysin-negative or dead *L. monocytogenes*, dead *S. typhimurium*, or LPS.

Lastly, attempts to block neutrophil extravasation into the peritoneal cavity by treating mice with anti-intercellular adhesion molecule 1 or anti-vascular cell adhesion molecule 1 MAbs (results not reported) were unsuccessful following i.p. inoculation of *L. monocytogenes* or *S. typhimurium*. This might indicate that under these circumstances either neutrophil extravasation did not involve the vascular endothelial cell adhesion receptors recognized by these MAbs or the MAbs failed to bind effectively to their respective target molecules. The present study could not discriminate between these possibilities. The role of endothelial cell adhesion receptors is under investigation.

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