THE JOURNAL OF PREVENTIVE MEDICINE
2004; 12 (3-4): 43-50

IN VITRO EXPANSION OF *LISTERIA MONOCYTOGENES* - REACTIVE T LYMPHOCYTE SUBSETS IN HUMANS - A FLOW-CYTOMETRY APPROACH

Mihaela Zlei[1], Octăviţa Ailiesei[2], E. Carasevici[3]

1.Laboratory of Immunology and Genetics, "Sf. Spiridon" Hospital, Iaşi
2.Department of Biochemistry, Genetics and Microbiology, "Al.I.Cuza" University of Iaşi
3.Department of Immunology, Gr, T. Popa University of Medicine and Pharmacy, Iaşi

Abstract. The incidence of human listeriosis is sporadic, but we previously demonstrated that T lymphocytes with reactivity to *Listeria monocytogenes* are with a high frequency in healthy individuals. As kinetics of specific expansion within $CD4^+$ and $CD8^+$ T cell compartments is particular to a certain pathogen, we attempted to describe the peculiarities which define the T cell reactivity to *L. monocytogenes* in humans. Our results demonstrated that, although the proliferative response of the two categories of T cells was broadly synchronized, there was a striking difference in the magnitude of the two types of response, when compared. The differential maintenance of the antilisteric memory T cell subsets in the periphery, suggested by our study, generated a set of alternative issues which are discussed below.
Key words: *listeria*, humans, CD4/CD8 T cells, proliferation, flow-cytometry, CFSE

Rezumat. Deşi cazurile de listerioză întâlnite în populaţia umană sunt sporadice, în cadrul unui studiu anterior, efectuat într-un grup de subiecţi aparent sănătoşi, am pus în evidenţă existenţa reactivităţii limfocitare la antigene listeriene la un un numar crescut de subiecti din lotul analizat. Întrucât este cunoscut faptul că dinamica expansiunii celor două subtipuri limfocitare T majore, pe parcursul desfăşurării unui răspuns imun antiinfecţios, este particulară naturii agentului patogen, ne-am propus să caracterizăm în cultură, dinamica expansiunii limfocitelor T $CD4^+$ şi $CD8^+$ în prezenţa antigenelor listeriene. Rezultatele studiului de faţă arată faptul că, deşi răspunsul proliferativ al celor două categorii de limfocite a fost sincronizat, magnitudinea celor două tipuri de răspuns a fost considerabil diferită. Rezultatele obţinute au generat o serie de ipoteze, printre care şi existenţa unor mecanisme de menţinere diferenţială a celor două subtipuri limfocitare de memorie în periferie, consecutiv contaminării cu *Listeria monocytogenes*.
Cuvinte cheie: *listeria*, subiecţi umani, limfocite T CD4/CD8, proliferare, citometrie în flux, CFSE

INTRODUCTION

Listeriosis is an important zoonosis caused by the intracellular pathogen *Listeria monocytogenes*, a Gram-positive bacterium with particular growth characteristics and remarkable cell biology of infection. Cases of listeriosis mainly arise in man from the ingestion of highly contaminated food and by handling of new-born calves or other infected materials (1, 2). The infectious dose for humans depends on the condition of host, the virulence and infectivity of the pathogen,

the type and amount of contaminated food ingested, the concentration of pathogen in food, and the number of repetitive challenges (2).

Although the incidence of human listeriosis is sporadic, we previously demonstrated that persons with reactivity to *L. monocytogenes* present in healthy individuals with a high frequency. We concluded at that time that the T cell reactivity against killed *L. monocytogenes* may be of some concern, especially to the immuno-compromised individuals who are at greatest risk of developing the disease. Also, those results afforded to us to set up an experimental model aiming at assessing the functional interaction between macrophages and T cells involved in antilisterial immunity.

In the current study we used the same experimental model with the purpose to analyze the anti-listerial memory $CD4^+$ and $CD8^+$ T cells during their expansion following *in vitro* antigen-driven specific stimulation. The reason for designing such a study was dictated by the fact that $CD4^+$ and $CD8^+$ T cells recognize different classes of antigens and are responsible of fulfilling distinct effector functions. However, their contribution against infections is variable, depending on the nature of the pathogen.

MATERIAL AND METHODS

Bacterial antigens. *L. monocytogenes* EGD was grown in trypticase soy broth at 37°C, overnight, then washed and resuspended in phosphate buffered saline (PBS). Bacterial numbers were determined by plating aliquots of 1:10 dilutions on trypticase soy agar plates. Bacteria were killed at 65°C, 120 min, in a water bath. The suspension named HKL (from heat killed *Listeria*) was then lyophilized and redissolved in DMSO (dimetil-sulf-oxid, Sigma) resulting a complex mixture of listeric antigen containing $2x10^9$ colony forming units (CFUs)/ ml or 8 mg proteins/ml. The suspension was stored at -70°C as 100 µl aliquots until used.

Isolation and cultivation of peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from samples of 40 ml heparinized peripheral blood of 15 apparently healthy donors by Ficoll Hipaque (Sigma) density gradient centrifugation (300xg, 25 minutes, room temperature). PBMCs were plated on round bottomed 96-well tissue culture plates (Nunc, Denmark), at a density of $1x10^6$ cells/ml RPMI 1640 medium (Sigma), supplemented with L-glutamine (2mM), 10% autologous human plasma, and antibiotic. Cultures were performed at 37°C, 5% CO_2 with/ without stimulation. Only the subjects whose T lymphocytes manifested clear reactivity for listerial antigens (responders) were considered for the analysis of HKL-specific T cell subsets.

CFSE labeling. Prior to culture 1-3 $x10^7$/ml PBMCs were labeled with 1.25-5 µM carboxifluorescein diacetate succinimydil ester (CFSE, Molecular Probes, USA), 10 minutes, 37°C. The labeling was quenched by adding one volume of cold human autologous plasma and cells were washed twice

in cold RPMI 1640. Sequential measurements of cellular CFSE levels were performed at day 3, 5, 7, 9, and 11 of culture. Proliferation was assessed by calculating the percent of T cells ($CD4^+$ or $CD8^+$) which had a diminished amount of intracellular CFSE, due to the mitotic activity.

***In vitro* stimulation**. To assess the presence of *Listeria*-specific lymphocytes in the PBMCs from healthy donors, $2x10^5$ CFSE-labeled cells per well were plated with 50 μg/ml HKL. In parallel, cells were incubated only with RPMI medium (the negative control).

Flow-cytometry analysis of cellular CFSE levels. Beginning with day 5 of culture, cells were collected, stained with anti-$CD4^+$ and anti-$CD8^+$ monoclonal antibodies and analyzed on a FACSCalibur flow cytometer (Becton Dickinson) every 2 days. Shortly, 2-$5x10^5$ cells/50μl suspension/ tube were stained at room temperature, 30 minutes, with 1-2μl anti-CD4-phycoerithrin (PE) or anti-CD8-phycoerithrin-cyan-5 (PE-Cy5)- conjugated monoclonal antibody (Pharmingen). The fluorescence level of CFSE was detected also by flow-cytometry. All experiments were performed in triplicate.

RESULTS

PBMCs of 11 from the 15 subjects tested for the *in vitro* reactivity to HKL/derived antigens responded significantly to listerial antigens compared to the same cells incubated only with RPMI medium. As depicted by figure 1, our results, estimated as average values and standard deviation, demonstrated the existence of a synchronized dynamics between the two categories of T lymphocytes following *in vitro* exposure to HKL-derived antigens. For all responders, the HKL-reactive $CD4^+$ and $CD8^+$ T lymphocytes could be already detected five days from the beginning of culture, although the number of detectable cells was low (10% ± 4.5 $CD4^+$, and 1.8% ± 0.6 $CD8^+$). The expansion of both HKL-reactive T lymphocyte subtypes followed an abrupt increase at day 7 (18.6% ± 7 $CD4^+$, and 2.1 ± 0.8 $CD8^+$). The peak of proliferation was detected in our study at day 9 from the beginning of culture (42.9% ± 12 $CD4^+$, and 3.2% ± 1.1 $CD8^+$), followed by a marked decrease at day 11 (28.5% ± 9 $CD4^+$, and 2.7% ± 0.9 $CD8^+$) .

Despite the evident synchrony of the two types of response, our results demonstrated a striking difference in the magnitude of the response when compared. Following the exposure to listeric antigen, at the moment of the proliferative peak (day 9), the average proportion of responding $CD4^+$ T lymphocytes (42.9%±12) was remarkable higher than the percentage of specific $CD8^+$ T lymphocytes (3.2%±1.1) expanded in culture as a response to antigenic stimulation.

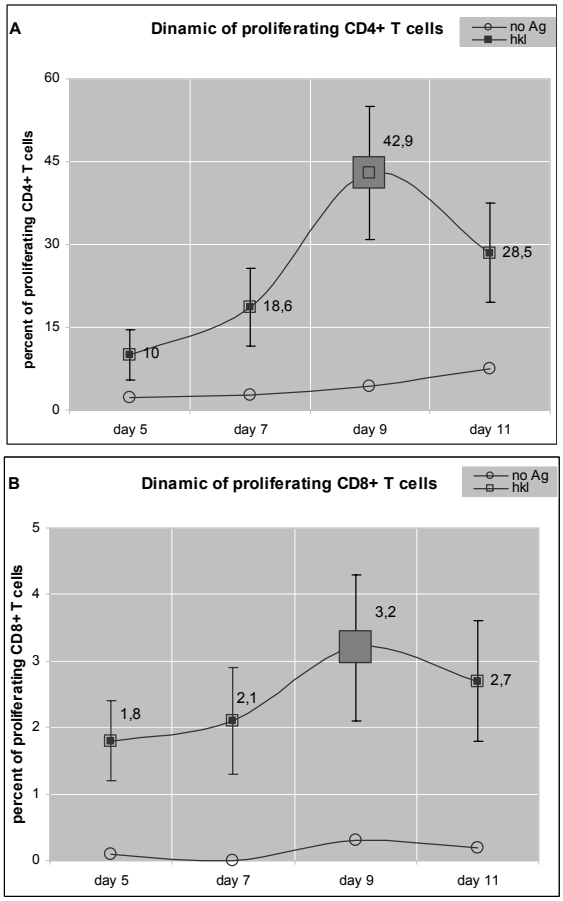

Fig. 1: **Dynamics of proliferation of *L. monocytogenes*-specific $CD4^+$ (A) and $CD8^+$ (B) T lymphocytes during *in vitro* restimulation with HKL**

In the attempt to find an explanation for this discrepancy, we considered the possibility that *Listeria* - specific $CD8^+$ T cells, which were detected beginning with day 5, would be triggered to expansion later during culture, in a manner comparable to that of their $CD4^+$ counterparts. For this purpose, we analyzed the CFSE - expression profile 11 and 13 days after the initiation of culture. As it can be seen in table 1, our results showed a constant CD4/CD8 ratio amongst the specific T cells, with a gradual decrease in the proportion of both T cell subtypes, beginning with day 11 of culture.

Tabel 1. Averages of HKL-specific CD4/CD8 ratio at day 9 of culture

CD4/CD8 ratio	Day 5	Day 7	Day 9	Day 11	Day 13
	5,56	12,24	13,41	5,56	4,30

DISCUSSIONS

The fact that listeric proteins can be presented both, by the MHC class I, and the MHC class II molecules, triggering $CD4^+$ and $CD8^+$ T cell mediated immune responses, is widely accepted.

Help provided by $CD4^+$ T cells in infections for the development of $CD8^+$ T cell – mediated responses is variable and depends especially on the nature of the pathogen. Effective control of *Mycobacterium tuberculosis*, *Toxoplasma gondii*, and *Plasmodium* ssp. requires $CD4^+$ T cell help, for the generation of effective $CD8^+$ T cell responses. In contrast, $CD8^+$ T cell responses to other infections, such as influenza virus, and LCMV occur in the absence of $CD4^+$ T cells (3,4,5, 6,7). This differential requirement for $CD4^+$ T cell help reflects the presence of many variables in the context of different infections, including the type and tropism of the pathogen, the level of costimulation delivered by the antigen presenting cells, such as macrophages, the affinity and level of presentation of $CD8^+$ T cell epitopes, the cytokine milieu, and the frequency of naïve $CD8^+$ T cell precursors (8,9,10,11).

As kinetics of specific expansion within $CD4^+$ and $CD8^+$ T cell compartments are frequently described as being particular to a certain pathogen, we attempted to describe the peculiarities which define the T cell reactivity to *L. monocytogenes* in humans (12).

Our results, apparently suggesting a differential maintenance of the anti-listeric memory T cell pools in the periphery, when $CD4^+$ and $CD8^+$ subtypes were compared, also generated the following alternative explanations to be considered: 1. these difference are indeed consistent with the more rapid contraction of the $CD8^+$ memory pool for *L. monocytogenes; 2.* these discrepancies are attributed to the differences in MHC class I and II – mediated antigen processing and presentation, 3. the relatively reduced peripheral frequency of $CD8^+$ T cells specific for *L. monocytogenes* is the consequence of the previous exposure to a probably reduced infectious inoculum; 4. these differences are the result of a preferential migration of the two subtypes of T cells from the periphery to different lymphoid and non-lymphoid organs.

1. If the differences detected in the magnitude of the response between the two T cell compartments reflect the more rapid contraction of the $CD8^+$ memory pool, it is useful to figure out for what purpose these differences are necessary in the response against *L. monocytogenes*. A possible explanation may be the fact that intracellular pathogens, like *L. monocytogenes*, require more elaborated and more efficient protective mechanisms. To ensure such a protection, the acquisition

and maintenance of $CD4^+$ memory, specialized in providing rapid assistance and help for antibacterial immune mechanisms, may be more advantageous than the maintenance of a numerous $CD8^+$ memory pool, directly responsible for the accomplishment of such mechanisms. On the other hand, the fact that $CD8^+$ T cell compartment has an undisputedly major role in the anti-listerial protection was repeatedly proved by others (11). Why, then, there is such a weak peripheral persistence of the very T cell compartment with the most important contribution to the anti- listerial protection? One hypothesis may be that, despite the low number, $CD8^+$ T cells are capable to rapidly expand *in vivo* when needed, which rules out the necessity for the maintenance of a high number of cells.

Therefore, it is possible that, in the case of contamination with *L. monocytogenes*, a prompt and efficient immune intervention requires different conditions for the activation and expansion of T cells.

2. Our experimental system is based on the incubation of PBMCs with heat processed *listeria* (HKL), conditions which probably does not provide the optimal access to the MHC class I pathway. In the absence of listeriolysin, which normally helps *Listeria* to escape from the phagolysosomal vacuole into the cytosol of the host cell (13), and which may be lost during bacterial heating, listerial antigens may be prevented from gaining access to the cytosol. However, in all responsive subjects investigated in our study, there has been detected a proportion, though small, of proliferating $CD8^+$ T lymphocytes. Such a phenomenon may be attributed to the frequently mentioned "cross-priming" mechanism (14).

3. Our study has been focused on the investigation of anti-listeric immune memory and protection in humans. The subjects included in the study group, despite their obvious responsiveness to listerial antigen following *in vitro* stimulation, had been probably exposed to relatively low doses of bacteria, as none of them had a known history of *listeria*-associated symptoms. There are numerous studies based on the murine experimental model of infection, which describe a direct correlation between the infectious inoculum and the magnitude of $CD8^+$ response (2). This information may also provide an explanation for our results, which demonstrated a reduced *in vitro* expansion of HKL-specific $CD8^+$ T cells.

4. The much lower frequency of proliferating $CD8^+$ T cells, compared to $CD4^+$ cells, following PBMCs exposure to HKL may be also attributed to the preferential migration of the two subtypes of T cells from the periphery to different lymphoid and non-lymphoid organs. It is well possible that $CD4^+$ T lymphocytes with specificity against listeric antigens are preferentially maintained in the peripheral circulation, whilst memory $CD8^+$ T cells migrate to the lymphoid and non-lymphoid organs (12,15,16).

More likely, the re-exposure to listerial antigens, on the occasion of a repeated contamination, would rather take place at these "non-peripheral" sites, and not in the periphery. On the other hand, the persistence of a consistent $CD4^+$ T pool in the blood, specialized for providing assistance and help for the $CD8^+$ T cell- mediated effector mechanisms, it is probably required for the ensuring of a prompt antilisterial protection.

CONCLUSIONS

Generation and maintenance of memory $CD4^+$ and $CD8^+$ T lymphocytes have a distinct pattern following contamination with *L. monocytogenes* and the understanding of this pattern is essential for defining the intercellular interactions during the development of an antilisteric immune response. Our study suggested a possible differential maintenance of the antilisteric memory T cell subsets in the periphery.

Insights into the understanding of the particular manner of immune response to *L. monocytogenes* can be helpful in designing either preventive antilisterial, or antitumor and antiviral vaccines.

REFERENCES

1. Fenlon DR, Wilson J, Donachie W: *The incidence and level of Listeria monocytogenes contamination of food sources at primary production and initial processing*. J Appl Bacteriol. 1996; 81 (6): 641-50.
2. Barbuddhe SB, Malik SV, Kumar P: *High seropositivity against listeriolysin O in humans*. Ann Trop Med Parasitol. 1999; 93 (5): 537-9.
3. Serbina NV, Lazarevic V, Flynn JL: *CD4(+) T cells are required for the development of cytotoxic CD8(+) T cells during Mycobacterium tuberculosis infection*. J Immunol. 2001; 167 (12): 6991-7000.
4. Casciotti L, Ely KH, Williams ME, Khan IA: *CD8(+)-T-cell immunity against Toxoplasma gondii can be induced but not maintained in mice lacking conventional CD4(+) T cells*. Infect Immun. 2002; 70 (2): 434-43.
5. Carvalho LH, Sano G, Hafalla JC, Morrot A, Curotto de Lafaille MA, Zavala F: *IL-4-secreting $CD4^+$ T cells are crucial to the development of $CD8^+$ T-cell responses against malaria liver stages*. Nat Med. 2002; 8 (2): 166-70.
6. Riberdy JM, Christensen JP, Branum K, Doherty PC: *Diminished primary and secondary influenza virus-specific CD8(+) T-cell responses in CD4-depleted Ig(-/-) mice*. J Virol. 2000; 74 (20): 9762-5.
7. Matloubian M, Concepcion RJ, Ahmed R: *$CD4^+$ T cells are required to sustain $CD8^+$ cytotoxic T-cell responses during chronic viral infection*. J Virol. 1994; 68 (12): 8056-63.
8. Zhan Y, Corbett AJ, Brady JL, Sutherland RM, Lew AM: *$CD4^+$ help-independent induction of cytotoxic $CD8^+$ cells to allogeneic P815 tumor cells is absolutely dependent on costimulation*. J Immunol. 2000; 165 (7): 3612-9.
9. Franco A, Tilly DA, Gramaglia I, Croft M, Cipolla L, Meldal M, Grey HM: *Epitope affinity for MHC class I determines helper requirement for CTL priming*. Nat Immunol. 2000; 1 (2): 145-50:
10. Mintern JD, Davey GM, Belz GT, Carbone FR, Heath WR: *Cutting edge: precursor frequency affects the*

helper dependence of cytotoxic T cells. J Immunol. 2002; 168 (3): 977-80.

11. Shedlock DJ, Whitmire JK, Tan J, MacDonald AS, Ahmed R, Shen H: *Role of $CD4^+$ T cell help and costimulation in $CD8^+$ T cell responses during L. monocytogenes infection*. J Immunol. 2003; 170 (4): 2053-63.
12. Schiemann M, Busch V, Linkemann K, Huster KM, Busch DH: *Differences in maintenance of $CD8^+$ and $CD4^+$ bacteria-specific effector-memory T cell populations*. Eur J Immunol. 2003; 33 (10): 2875-85.
13. Bonnemain C, Raynaud C, Reglier-Poupet H, Dubail I, Frehel C, Lety MA, Berche P, Charbit A: *Differential roles of multiple signal peptidases in the virulence of Listeria monocytogenes*. Mol Microbiol. 2004; 51 (5): 1251-66.
14. Ackerman AL, Cresswell P: *Cellular mechanisms governing cross-presentation of exogenous antigens*. Nat Immunol. 2004; 5 (7): 678-84.
15. Kursar M, Bonhagen K, Kohler A, Kamradt T, Kaufmann SH, Mittrucker HW: *Organ-specific $CD4^+$ T cell response during Listeria monocytogenes infection*. J Immunol. 2002; 168 (12): 6382-7.
16. Pope C, Kim SK, Marzo A, Masopust D, Williams K, Jiang J, Shen H, Lefrancois L. *Organ-specific regulation of the $CD8^+$ T cell response to Listeria monocytogenes infection*. J Immunol. 2001; 166 (5): 3402-9.