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Progression of Armed CTL from Draining Lymph Node to Spleen Shortly After Localized Infection with Herpes Simplex Virus 1[1]

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We have examined the generation of CTL immunity immediately after localized footpad infection with herpes simplex virus 1 (HSV-1) using three coordinated in vivo T cell tracking methodologies. Tetrameric MHC class I containing the immunodominant peptide from HSV-1 glycoprotein B (gB) showed that after infection the proportion of Ag-specific T cells peaked at day 5 within draining popliteal lymph nodes and 2 days later in the spleen. Preferential expression of the activation marker CD25 by tetramer-positive cells in draining popliteal nodes but not spleen suggested that gB-specific T cells were initially activated within the lymph node. In vivo cytotoxicity assays showed that Ag-specific effector cells were present within the draining lymph nodes as early as day 2 after infection, with a further 2-day lag before detection in the spleen. Consistent with the very early arming of effector CTL in the draining lymph node, adoptive transfer of CFSE-labeled gB-specific transgenic T cells showed that they had undergone one to four rounds of cell division by day 2 after infection. In contrast, proliferating T cells were first detected in appreciable numbers in the spleen on day 4, at which time they had undergone extensive cell division. These data demonstrate that HSV-1-specific T cells are rapidly activated and armed within draining lymph nodes shortly after localized HSV-1 infection. This is followed by their dissemination to other compartments such as the spleen, where they further proliferate in an Ag-independent fashion. ***The Journal of Immunology,*** **2002, 168: 834–838.**

Cytotoxic T lymphocytes kill virus-infected cells and thus limit the spread of infection. Naive CTL precursors are thought to primarily recirculate between the secondary lymphoid organs such as spleen and lymph nodes (1, 2). It is in these sites that CTL come into contact with the professional APCs, most likely dendritic cells (3) that are necessary for effective T cell priming. During localized infection, these APCs carry viral Ags from the site of replication to draining lymph nodes where specific T cells are primed. Once activated, these T cells emerge as a tissue-infiltrating effector pool that will ultimately limit the spread of pathogens from the site of infection. Cutaneous inoculation with herpes simplex virus (HSV-1)[3] represents an interesting model of a localized infection. HSV-1 replicates locally in skin or epithelial cells before entering peripheral nerve endings and traveling to the sensory ganglia by retrograde axonal transport where it establishes a relatively quiescent latent infection (4). $CD8^+$ T cells are protective against various forms of HSV-1 infection (5, 6). They appear to be important during the neuronal phase of virus replication (7) and can be found within ganglia as early as day 5 after infection (8, 9). Moreover, this same T cell subset has also been implicated in maintaining HSV-1 latency well after the resolution of the lytic phase of infection (9).

HSV-1 does not appear to replicate within the lymph nodes draining the site of cutaneous infection (10, 11). Early investigators found only low virus-specific precursor cell frequencies within these sites with little cytotoxic activity, suggesting that full effector arming required some form of migration of primed, but functionally immature, CTL (12, 13). This notion was reinforced by their finding that in vitro culture of lymph node cells in the absence of Ag resulted in the appearance of strong levels of specific cytotoxicity. However, armed virus-specific $CD8^+$ T cells can be found in lymph nodes that drain the site of cutaneous HSV-1 infection (10). Given these apparently contradicting observations, we were interested in determining the point at which these T cells were activated and armed before their migration to other sites. Using a combination of tetrameric class I-peptide complexes, in vivo cytotoxicity assays and fluorescently labeled TCR-transgenic T cell proliferation to address these issues, we have found that HSV-1-specific T cells are rapidly activated and armed within draining lymph nodes shortly after localized infection. Although they also proliferate within this location, they are found to undergo the bulk of their expansion outside the lymph node compartment.

Materials and Methods

Mice and virus

C57BL/6 mice and the glycoprotein B (gB) T-I-transgenic mice were obtained from the Department of Microbiology and Immunology, University of Melbourne Animal House and kept in specific pathogen-free conditions. The gBT-I mice express a transgenic TCR from a CTL clone that recognizes the HSV-1 gB498–505 determinant in complex with $H\text{-}2K^b$. The derivation of these animals will be described in more detail elsewhere (14).

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[3] Abbreviations used in this paper: HSV-1, herpes simplex virus 1; gB, glycoprotein B.

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Six- to 8-wk-old mice were infected in the hind foot with 5×10^4 PFU of HSV-1 KOS strain, and the spleen and popliteal and axillary lymph node cells were examined at the times described.

mAbs and K^b-gB tetramer

The following mAbs were purchased from BD PharMingen (San Diego, CA): anti-CD8α-APC (53-6.7), anti-CD25-FITC (7D4), anti-CD44-FITC (IM7), and anti-CD62L-FITC (MEL-14). The K^b-gB tetramers containing the immunodominant gB peptide, gB498–505, were prepared essentially using the protocol by Altman et al. (15) as described elsewhere (10).

Flow cytometric analysis

Briefly, 1×10^6 cells were stained with an excess of Abs on ice for 20 min. Cells were then washed with PBS containing 1% BSA. Finally, the cells were stained with the K^b-gB tetramers for 20 min at 37°C and washed as previously described (10). Propidium iodide was added before analysis and used to exclude dead cells. Data were collected on 1×10^5 live cells on a FACScan flow cytometer using CellQuest software (BD Biosciences, San Jose, CA)

In vivo CTL assay

To prepare target cells to detect in vivo cytotoxic activity, erythrocytes were removed from naive C57BL/6 spleen and lymph node cell suspensions by osmotic lysis. The cells were then washed and split into two populations. One population was pulsed with 10^{-6} M gB peptide, incubated at 37°C for 45 min, and labeled with a high concentration of CFSE (2.5 μM) ($CFSE^{high}$ cells). The second control target population was left without peptide and was labeled with a low concentration of CFSE (0.25 μM) ($CFSE^{low}$ cells). For i.v. injection, an equal number of cells from each population was mixed together, such that each mouse received a total of 20×10^6 cells in 150 μl of PBS. Cells were injected into mice that had previously been infected with HSV-1 a number of days earlier as described and 4 h later sacrificed for their lymph nodes and spleens. Cell suspensions were analyzed by flow cytometry, and each population was detected by their differential CFSE fluorescence intensities. Up to 1×10^4 CFSE-positive cells were collected for analysis. To calculate specific lysis, the following formula was used: ratio = (percentage $CFSE^{low}$/percentage $CFSE^{high}$). Percentage specific lysis = [1 − (ratio unprimed/ratio primed) × 100].

In vivo proliferation assay

Naive gBT-I lymph nodes were removed and the resulting single-cell suspension was incubated with CFSE (2.5 μM) at 37°C for 10 min. The cells were then washed and resuspended in HBSS so that each mouse received 2×10^6 cells i.v. At various times after the transfer of the CFSE-labeled gBT-I cells, mice were injected in the hind foot with 5×10^4 PFU of HSV-1 as described previously. After a maximum of 96 h since the first infection, all mice were sacrificed and their popliteal lymph nodes and spleens were taken and single-cell suspensions were made. Cells were stained with anti-CD8α-APC Ab as described and the resulting suspensions were run on a FACScan flow cytometer. Data were collected on between 2×10^3 and 1×10^4 CFSE-positive CD8-positive cells and analyzed using CellQuest software (BD Biosciences). As the cells divide the CFSE intensity is halved, giving peaks of different intensities that correlate with the number of cell divisions.

Results

Tracking gB-specific T cell accumulation in lymph nodes and spleen after footpad infection using tetrameric class I complexes

Footpad infection with HSV-1 results in a localized infection that does not spread to the draining popliteal lymph node (10). We wanted to compare the kinetics of CTL accumulation and activation within the draining lymph nodes with that observed in the remainder of the lymphoid compartment. The $CD8^+$ T cell response to HSV-1 in C57BL/6 mice is largely directed to the immunodominant determinant from the gB, gB498–505 (16, 17). To identify the gB-specific $CD8^+$ T cells responding to HSV-1, MHC class I tetrameric complexes were constructed, incorporating the gB epitope. C57BL/6 mice were immunized with HSV-1 in the hind feet, the popliteal lymph nodes and spleen were removed, and the cells were stained with anti-CD8 Ab and tetramer over a period of 9 days.

Given the localized nature of the infection, we were surprised that tetramer-positive cells appeared almost simultaneously in both lymph node and spleen at around day 5 after infection (Fig. 1). The frequency of gB-specific T cells rapidly increased from near background levels at day 4 after infection to ~5% of both spleen and lymph node $CD8^+$ T cells by day 5 after infection. Although this time represented the peak of gB-specific T cells found in the lymph node, the levels continued to increase within the spleen for another 2 days, reaching ~13% of all $CD8^+$ T cells in this site 7–8 days after infection. Interestingly, the proportion of gB-specific T cells declined rapidly within the draining lymph nodes, especially compared with the spleen, dropping to levels of <0.7% of $CD8^+$ T cells in that site by day 9 after infection (Fig. 1). Moreover, gB-specific T cells appeared to be selectively excluded from non-draining lymph nodes throughout the course of the response (Fig. 1). These data indicate that having left the draining popliteal lymph nodes, HSV-1-specific T cells do not readily re-enter peripheral lymph node tissues, at least within the first 9 days after infection. Finally, while both splenic and lymph node T cells have down-regulated CD62L expression and express stable activation markers like CD44, there is differential expression of CD25 (Fig. 2). Only lymph node gB-specific T cells express this molecule at appreciable levels. This is consistent with a scenario where only T cells within the draining popliteal lymph nodes are undergoing antigenic stimulation while those found within the spleen represent a postmigratory population.

gB-specific cytotoxicity appears in the draining lymph nodes before other secondary lymphoid organs

Originally we expected a much clearer lag in the initial appearance of tetramer-positive cells within the splenic compartment given that the immune response was expected to start within the draining popliteal lymph nodes. It could be argued that some virus-specific T cells may have already left the priming site and expanded within the spleen by the time tetramer-detectable levels were reached, and this might explain the near simultaneous appearance of tetramer-staining CTL. In an attempt to detect the presence of gB-specific T cells within draining nodes before day 5 after infection and before significant effector migration, we directly determined whether HSV-1-specific cytotoxic effector cells were present using an in vivo cytotoxicity assay (18, 19). This method involves the in vivo

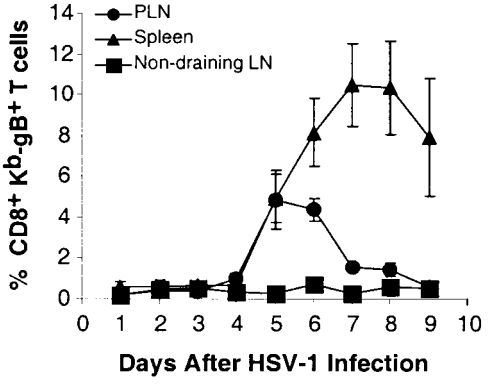

FIGURE 1. Expansion of gB-specific $CD8^+$ T cells following HSV-1 infection. Cells were obtained from popliteal lymph nodes (PLN), spleen, and nondraining axial lymph nodes (LN) of mice 1–9 days following footpad infection with HSV-1, and stained directly ex vivo with anti-CD8 Ab and K^b-B tetramer. The numbers represent the percentage of $CD8^+$ T cells that are stained by the K^b-B tetramer. A minimum of 4 and a maximum of 11 data points are shown, with error bars representing SD.

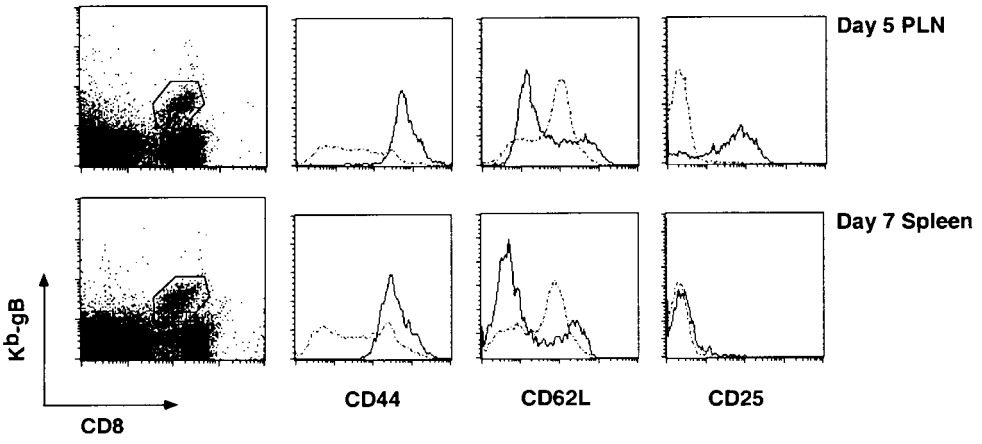

FIGURE 2. Phenotype of the gB-specific T cells at the peak of the response within the draining popliteal lymph node and the spleen. Cells were obtained from popliteal lymph nodes (day 5 after infection) and spleen (day 7 after infection) and stained with K^b-gB tetramer, anti-CD8, and either anti-CD44, anti-CD62L, or anti-CD25 Abs. The solid line histogram indicates the phenotype of the cells falling within the gated population (*left panel*), while the dotted line histogram is the phenotype of the $CD8^+$ population found within an uninfected control mouse.

selective lysis of fluorescently labeled gB peptide-pulsed splenocytes. We chose a fairly rapid 4-h assay period with the reasoning that it would minimize migration of dying target cells from other sites and more likely measure the presence of CTL effector cells in the organs under investigation.

Fig. 3*A* shows the extent of lysis of peptide-pulsed splenocytes at the peak of the response in the popliteal lymph nodes (day 5) 4 h after they had been transferred into the infected host. There was near complete loss of the peptide-pulsed peak, whereas the peak corresponding to targets labeled only with CFSE is readily detected. This selective loss of peptide-pulsed target cells translates to a specific lysis of >95%. We evaluated the development of this response over the course of infection (Fig. 3*B*). Low but reproducible levels of killing were seen within the draining popliteal lymph nodes as early as day 2 after infection (15%). This killing is T cell mediated since no detectable lysis could be observed in RAG knockout mice (data not shown). Lysis of peptide-pulsed targets was first seen in the spleen at day 4 after infection, with an average specific lysis of 12% (Fig. 3*B*). From these early points and also the half-maximal values, there was an approximate 48-h lag in appearance of killing between lymph node and spleen. Moreover, the in vivo cytotoxicity assay revealed the presence of specific effector cells 2–3 days before they were detectable using K^b-gB tetramer complexes (Fig. 1).

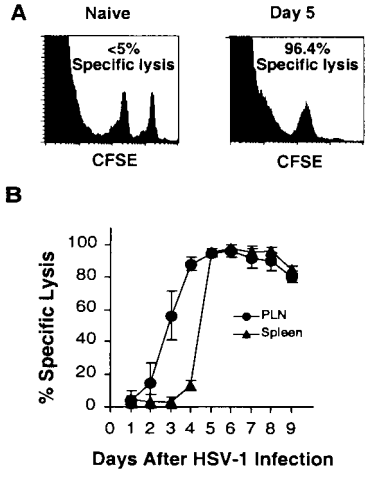

FIGURE 3. gB-specific cytotoxicity appears in the popliteal lymph nodes before other secondary lymphoid organs. To analyze gB-specific cytotoxicity in vivo, syngenic spleen cells were pulsed with gB peptide and labeled with CFSE to give a high level of fluorescence intensity ($CFSE^{high}$). To control for Ag specificity, unpulsed syngenic spleen cells were labeled with CFSE to give lower fluorescence intensity ($CFSE^{low}$). A 1:1 mixture of 1×10^7 cells of each target cell population was injected i.v. into naive C57BL/6 controls along with mice that had been infected with HSV-1 1–9 days before. After 4 h, the mice were killed and the draining popliteal lymph node and spleen cells were analyzed for the presence of $CFSE^{high}$ (gB-pulsed) and $CFSE^{low}$ (unpulsed) target cell populations. To quantify in vivo cytotoxicity, the elimination of the gB-pulsed $CFSE^{high}$ population was monitored and the ratio between the percentage of unpulsed and gB-pulsed target cells was calculated. The percentage of specific lysis was then determined as described in *Materials and Methods*. *A*, Data show a representative histogram plot of lymph node cells obtained from a naive control and day 5-infected mouse 4 h after transfer of CFSE-labeled target cells. *B*, The kinetics of the in vivo gB-specific cytotoxic response in the popliteal lymph nodes (PLN) and spleen is shown from mice infected 1–9 days previously. A minimum of 4 and a maximum of 14 data points are shown, with error bars representing SD.

Activated gB-specific T cells can be found in the popliteal lymph nodes early after infection and only in the spleen after they undergo a number of cell divisions

The flow cytometry data showed that although maximal gB-specific cell numbers were found in the draining lymph nodes at day 5 after infection, these T cells were first stimulated at least 3 days earlier since low, but consistent, cytotoxicity was measurable 2 days after infection. To confirm that gB-specific T cells were activated so soon after infection, we made use of gB-specific T cells from the TCR-transgenic mouse, gBT-I, which encodes a transgenic receptor for this determinant. When labeled with the dye CFSE, these cells halve their fluorescence intensity on every cell division (20–22). Fig. 4 shows that by 48 h after infection, gB-specific transgenic T cells found within popliteal lymph nodes have already undergone between one and four rounds of cell division. Interestingly, examination of the spleen showed that gB-specific T cells remained largely undivided at this time, highlighting that there was little Ag-specific stimulation in this compartment, at least within the first few days after infection. Proliferating T cells were first seen in the spleen no earlier than day 3 after infection when they appeared in small numbers as a broad peak with low fluorescence intensity relative to undivided cells. Unlike the lymph node where early cell division was clearly evident, the bulk of splenic T cells had already undergone considerable cell division at first detection. More dramatically, there was a

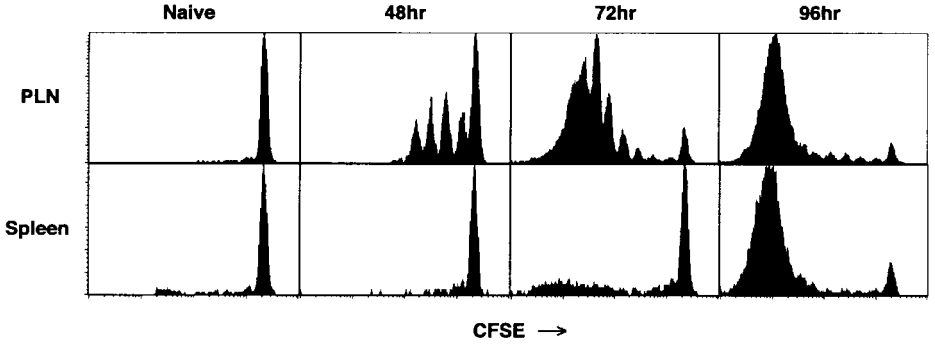

FIGURE 4. Proliferation of gBT-I-transgenic T cells occurs early in the popliteal lymph node following HSV-1 infection. CFSE-labeled transgenic gB-specific T cells were adoptively transferred into naive mice that were subsequently infected with HSV-1 in the hind feet. At various times after infection, the mice were sacrificed and cells from the draining popliteal lymph node (PLN) and spleen were analyzed by flow cytometry. Proliferation of gB-specific cells was determined by assessing the reduction in CFSE-associated fluorescence intensities of CD8-positive T cells.

massive influx of gB-specific transgenic T cells that had undergone multiple rounds of proliferation at 4 days after infection. Significantly, the division profile in both spleen and lymph node appears identical at this time point. This suggests that the splenic gB-specific T cells were activated at approximately the same time as those found in the popliteal lymph node, that they were most likely derived from the latter population, and that they were released as a burst at around day 4 after infection.

Discussion

gB-specific CTL are activated in the popliteal lymph nodes soon after infection with HSV-1. The first hint of killing was seen 2 days after infection, by which time the transgenic T cells had undergone between one and four rounds of cell division. Other investigators have reported that CTL show some level of killing within a few rounds of cell division, although they require at least five divisions to reach full effector capability (19, 23). This is consistent with both the timing and strength of killing seen in the popliteal lymph nodes after footpad infection, with maximal values not seen until day 4 after infection, by which time the cells had undergone at least six rounds of cell division. The results also agree with our earlier demonstration that at least at the peak of the response, gB-specific T cells are fully armed within draining lymph nodes (10) and reinforce that the acquisition of effector activity does not require any level of extralymphoid maturation as originally proposed (12, 13).

Given the early appearance of specific lysis as well as cell proliferation in draining lymph nodes, it was at first surprising that we could only detect tetramer-staining T cells from day 5 after infection and then with similar proportions in both lymph nodes and spleen. However, from the CFSE proliferation data we calculate that gB-specific cells divide every 5–6 h (data not shown). This means that the 5% of tetramer-staining $CD8^+$ T cells in the lymph node (Fig. 1) at this time could have been expanded from levels as low as 0.2% of the $CD8^+$ population over the prior 24-h period. The simultaneous appearance of tetramer-positive cells in lymph node and spleen could also signify a sudden release of specific CTL from the responding node rather than a steady trickle of cells from the beginning of the response. It is known that specific lymphocytes suddenly appear in efferent lymphatics draining responding lymph nodes after a refractile period during which these cells are thought to be undergoing Ag-specific stimulation (24, 25). Therefore, CTL might not leave the lymph node until the increasing CTL frequency exceeds some threshold such as the Ag presentation capacity of this compartment. Consistent with this, proliferating gB-specific transgenic T cells did not appear in the spleen in appreciable numbers until a sudden burst around day 4 after infection. This burst coincided with the first time that cytotoxicity was seen in this compartment and before tetramer levels appeared in either site. There was little difference in the proliferation profile of CFSE-labeled gBT-I cells in lymph node or spleen, suggesting that both populations were subjected to the same stimulation event, most likely within the former location relatively shortly after infection, and that cell division per se did not determine release from this site.

A progressive migration of activated T cells from the draining lymph nodes to the spleen is consistent with the finding that CD25 was expressed by the lymph node T cells but already down-regulated by the time those cells had reached the spleen. Interestingly, CD25 up-regulation has been associated with the acquisition of cytotoxic effector function (26, 27). We do not dispute this assertion, but the fact that gB-specific cytotoxicity was found in the spleen in the absence of CD25 on specific T cells suggests that its continued expression is not required for maintenance of effector function. Moreover, $CD25^+$ lymphoid cells are also known to contain already stimulated HSV-1-specific CTL precursors that are capable of dividing in the absence of further encounter with Ag (26). Recent studies have shown that T cells only require a very brief exposure to Ag to fully progress along their maturation pathway in the total absence of further stimulation (28, 29). Thus, once the activation program has been set in motion in the draining lymph nodes, $CD8^+$ T cells can continue to proliferate after they leave this site in the absence of any further stimulation. Consistent with this extra-lymph node expansion, if one takes into account the cellularity in each organ then the maximum value of 5% at the day 5 peak translates to around 5×10^4 gB-specific T cells per lymph node while levels of nearly 2.5×10^6 cells were found in the spleen at the peak time of day 7 after infection. An analogous splenic expansion of CTL has been seen in another example of a localized infection involving pulmonary influenza infection (30).

Finally, gB-specific CTL did not re-enter lymph nodes once they were activated and had migrated to the spleen, since they were not seen to accumulate in the nonresponding lymph nodes. This is consistent with the down-regulation of CD62L that is required for naive lymphocyte movement through the high endothelial venules (1, 2). It further reinforces the notion that those gB-specific T cells found early in the draining popliteal lymph nodes were there because they were either in the process of being stimulated or had only recently encountered Ag and had yet to leave this site. Once they emerged, they then appeared within the spleen most likely enroute to sites of localized infection. Overall, this study shows that the activation, arming, and expansion of HSV-1-specific CTL

occurs very quickly after infection, followed by the rapid dissemination of the resultant migratory effector pool which is responsible for limiting the extent of local viral replication.

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