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Restimulated memory Tc cells have a higher apparent avidity of interaction with targets than primary virus-immune Tc cells as indicated by anti-CD8 blocking

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Summary Previous experiments have shown that whereas a secondary *in vitro* Kunjin-immune cytotoxic T (Tc) cell population lysed equally well targets infected with either native flavivirus or a recombinant vaccinia virus expressing the immunodominant determinant, primary *in vivo* Kunjin-immune Tc cells were able to lyse only the recombinant vaccinia virus-infected targets. Using CD8 blockade to assess the avidity of T cell–target interaction, recombinant-infected targets express antigen more efficiently than native flavivirus infected targets and secondary *in vitro* Kunjin-immune Tc cells have a higher avidity for targets than do primary *in vivo* Kunjin-immune Tc cells. Secondary *in vivo* influenza-immune Tc cells are also of higher avidity than primary *in vivo* influenza-immune Tc cells. Thus, a restimulated memory Tc cell population interacts with targets with greater avidity than does a recently activated naive population.

Introduction

The murine cytotoxic T (Tc) cell response to the flavivirus Kunjin has been investigated using recombinant vaccinia viruses (VV) encoding various Kunjin proteins to infect targets cells and identify the immunogenic determinants.[1,2] Kunjin-immune Tc cells also lysed targets infected with the closely related flavivirus, West Nile (WNV). During these studies it was interesting to compare the primary *in vivo* with the secondary *in vitro* anti-Kunjin response. It was found that, in CBA/H mice, both populations of Tc cells lysed only targets which expressed both K^k and a Kunjin determinant in a 99 amino acid polypeptide derived from NS3 or NS4A (expressed by the recombinant VV vKV-1023). None of the rest of a panel of recombinant vaccinias which expressed all other Kunjin proteins was recognized nor were targets which expressed only D^k. Thus a major part of the Tc cell response to Kunjin was confined to an immunodominant K^k-restricted determinant(s) expressed by vKV-1023. However, whereas the secondary *in vitro* Kunjin-immune population efficiently lysed targets infected either with vKV-1023 or with the native flaviviruses Kunjin or WNV, the primary *in vivo* Tc cell population lysed only the vKV-1023-infected targets. This suggested that the recombinant vaccinia-infected targets presented more Kunjin antigen to Tc cells than the native flavivirus-infected targets and also that the secondary *in vitro* Tc cell population had a higher avidity for targets than the primary *in vivo* Tc cell population. In this paper an investigation of the avidity of interaction with targets of pri-

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mary and restimulated memory Tc cells is reported using both the flavivirus and influenza systems.

No means exist to measure directly the avidity of Tc cell–target interaction, but the dependence of the interaction on CD8 has been used as an indirect assessment. The murine CD8 molecule Lyt-2 is expressed on all major histocompatibility complex (MHC) class I-restricted T cells. CD8 is thought to interact with the class I molecule during a T cell receptor (TCR)/antigen interaction, strengthening the overall avidity of the cell–cell interaction.[3] Other contributors to the avidity of this interaction include the intrinsic affinity of the (TCR) for the MHC/peptide complex and the density of MHC/peptide complexes, TCR and perhaps accessory molecules. Where the avidity of Tc cell–target interaction is low due to low MHC density, low peptide concentration or suboptimal peptide sequence, target cell lysis is readily inhibited by anti-CD8 antibody, whereas high avidity interactions are relatively resistant to such inhibition.[4,5] Thus, the titre of anti-CD8 antibody needed to inhibit target cell lysis may be taken as an indication of the overall avidity of Tc cell–target interaction, at least when comparing lysis of different targets by the same Tc cell population. Comparison of different Tc cell populations is subject to the assumption that the threshold of interaction required to trigger Tc cell lytic function is constant in different Tc cell populations.

Materials and methods

Mice

Specific pathogen free CBA/H and C3H.OH mice were supplied by the Animal Breeding Establishment at the John Curtin School of Medical Research. The mice were immunized with virus at age 6 weeks or older.

Viruses

The flaviviruses WNV (Sarafend strain), Kunjin (strain MRM 16), were provided by Dr I. D. Marshall and passaged in suckling mouse brains. Supernatant was obtained by centrifugation of a sonicated preparation of mouse brain diluted 1 in 10 in gelatin-buffered saline, aliquoted and stored at −70°C for routine use. Virus activity was assayed by titration of plaque forming units (p.f.u.) on Vero cells, as previously described.[6] The titres of virus stocks used were: WNV: 10^9–10^{10} p.f.u./mL, Kunjin: $1–2 \times 10^8$ p.f.u./mL. Influenza virus, strains A/WSN and A/PC, was grown in eggs as previously described,[7] allantoic fluid was used as virus stock. Viral titre was assessed by haemagglutination.[7]

Kunjin–VV recombinants

The construction of the recombinant VV used in these experiments has been described in detail.[1] The recombinant vKV-1023 expresses the Kunjin proteins NS3, as well as flanking regions of the Kunjin polypeptide from NS2B and NS4A. A VV generated in the same manner but without new gene expression (VV-TK$^-$) was used as a control. To prepare virus stocks CV1 cells were infected for 48 h, lysed and sonicated, and this crude cell lysate was used to infect targets. Stocks were titrated on 143B cells; titres varied from 10^8 to 10^{10} p.f.u./mL.

Generation of Tc cells

Primary Kunjin-immune Tc cells were generated by the method described by Kesson *et al.*[8] for WNV. Adult mice were injected intravenously with 10^6 p.f.u. of Kunjin. Five days later the animals were killed and a single cell suspension of splenocytes prepared, which was centrifuged over Ficoll-Hypaque. The cells recovered from the interface were then used as primary Kunjin-immune effectors in cytotoxic assays. Secondary Kunjin-immune Tc cells were generated as described by Kesson *et al.*[9] Adult mice were injected intravenously with 10^6 p.f.u. of Kunjin. Six to 10 days later (occasionally up to 4 months later) the animals were killed and a single cell suspension of splenocytes prepared. One-tenth of the spleen cells were incubated with 2×10^7 p.f.u. Kunjin per spleen for 1 h, then added back to the rest of the splenocytes. After 5 days culture at 37°C these cells were used as secondary immune effectors in cytotoxic assays. To generate influenza-immune Tc cells, C3H.OH mice were injected intraperitoneally with 10^3

HAU influenza A/PC. Secondary *in vivo* effectors were generated by a second injection after a period of more than 4 weeks of influenza A/WSN (10^3 HAU). For routine experiments spleens were harvested 3 or 6 days after the last virus dose and a single cell suspension of splenocytes used directly in the cytotoxicity assay. To generate secondary *in vitro* effectors, mice were primed as above and the spleens harvested after a period of more than 4 weeks; 10^7 cells were infected with more than 400 HAU A/WSN for 1 h, washed, added back to the rest of the splenocytes and cultured for 5 days.

Target cells for cytoxicity assays

Peritoneal exudate cells obtained 5 days after intraperitoneal injection of mice with 1 mL of thioglycollate fluid broth (DIFCO) prepared at 59.6 g/L were used as targets for Kunjin-immune Tc cells. These cells were more than 95% activated macrophages as determined by morphology. Macrophage targets were incubated with 20–50 p.f.u./cell VV simultaneously with ^{51}Cr for 1 h, and washed twice before use in the assay. For flavivirus-infected targets, macrophages were incubated with 100–300 p.f.u./cell WNV, or 25–100 p.f.u./cell Kunjin in 1 mL medium for 1 h, then gently rotated in a centrifuge tube filled with medium at 37°C overnight, before labelling with ^{51}Cr as above. This prolonged incubation of target cells after flavivirus infection was essential for them to become susceptible to lysis by flavivirus-immune Tc cells.[8,9]

Targets for influenza-immune Tc cells were P815 mouse mastocytoma cells; 10^6 P815 cells were infected with 10^3 HAU A/WSN and labelled with ^{51}Cr simultaneously for 1 h.

Cytotoxicity assays

A standard ^{51}Cr release assay was used. Effector cells generated as described above were diluted to the required concentration, usually 10^7 cells/mL, and titrated in a round-bottomed 96 well plate to give triplicates of four three-fold dilutions; 2×10^4 targets were added per well. Medium without effector cells was added to target cells to give spontaneous ^{51}Cr release control values, and 1% Triton X was added to determine maximum releasable ^{51}Cr. The assay was run at 37°C in 5% CO_2 for 4–6 h, the plates spun, 100 mL supernatant harvested from each well, and γ irradiation counted. Per cent specific lysis (%SL) was calculated as 100× (ct/min experimental − ct/min spontaneous ^{51}Cr release) divided by (ct/min maximum − ct/min spontaneous ^{51}Cr release).

CD8 blocking studies

The hybridoma cell line 53.6.7 which secretes immunoglobulin (Ig)G antibody recognizing the murine CD8 molecule Lyt-2 was a gift from Dr R. Ceredig. 53.6.7 cells were cultured at 10^6 cells/mL until the medium was exhausted, the supernatant harvested, filtered and stored at 20°C for use in blocking studies.

For the blocking studies, 50 μL aliquots of 53.6.7 supernatant were titrated in two-fold dilutions across a 96 well plate. Effector Tc cells at the desired concentration in a 50 μL volume were added to each well containing antibody and to wells containing medium without antibody (for control lysis) and the plates incubated at 37°C for 30 min; 2×10^4 ^{51}Cr labelled target cells in 100 μL volumes were then added to each well, and the plates incubated for a further 4–6 h before harvesting supernatant as described above. Quadruplicate assays were performed for each antibody concentration. Spontaneous ^{51}Cr release and maximum releasable ^{51}Cr were determined, and %SL and s.e.m. for each quadruplicate calculated as described above. The per cent lysis of control was determined as the ratio of %SL in the presence of 53.6.7 to %SL in the absence of 53.6.7. Standard effector to target ratio (E : T) titrations were performed for each effector-target combination, and the results of the blocking study were only considered interpretable if the E : T ratio used in the blocking study was below that causing maximum lysis.

Results

Comparison of the primary in vivo *and the secondary* in vitro *Kunjin-immune Tc cell response*

Primary *in vivo* and secondary *in vitro* Kunjin-immune Tc cells generated as described above

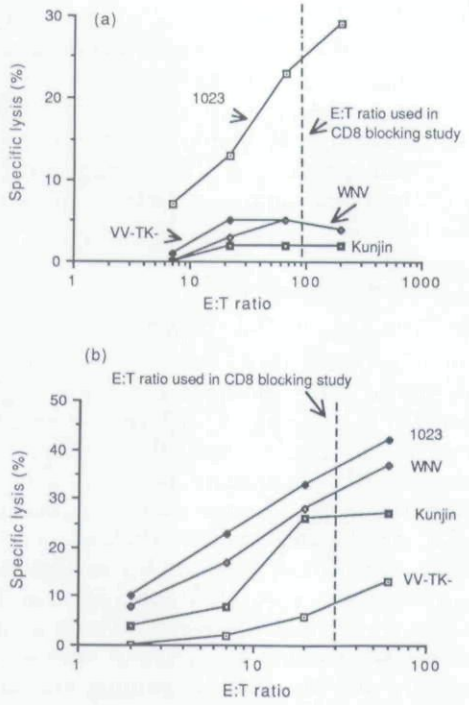

Fig. 1. (a) Primary *in vivo* Kunjin-immune Tc cell assay. (b) Secondary *in vitro* Kunjin-immune Tc cell assay. Per cent specific lysis by CBA/H Kunjin-immune Tc cell population of CBA/H macrophage targets infected with various viruses shown. Standard error of the mean was always less than 3%, and spontaneous ^{51}Cr release less than 25%.

were compared for their ability to lyse flavivirus- or recombinant-infected targets. Thioglycollate-induced macrophage target cells were either infected overnight with Kunjin or infected for 1 h prior to the assay with VV (the control VV-TK$^-$ virus or the Kunjin–vaccinia recombinant vKV-1023). The results are shown in Fig. 1. Secondary *in vitro* Kunjin-immune Tc cells lysed targets infected with WNV or vKV-1023 equally well and lysed targets infected with Kunjin slightly less well at higher killer to target ratios, presumably reflecting poorer target infection by this virus. In contrast, primary *in vivo* Kunjin-immune Tc cells lysed vKV-1023-infected targets, but failed to lyse WNV- or Kunjin-infected targets.

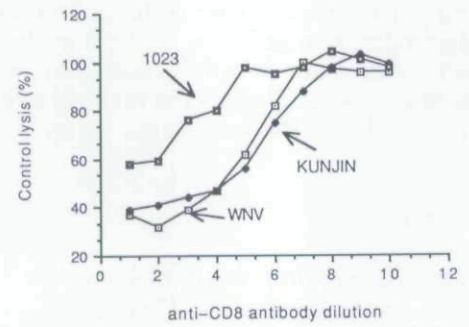

Fig. 2. Anti-CD8 antibody blocking of lysis by secondary *in vitro* Kunjin immune Tc cells of targets infected with Kunjin, WNV or vKV-1023.

Comparison of the avidity of flavivirus-infected targets with vKV-1023-infected targets in their interaction with effector cells by anti-CD8 antibody blocking

The observation that primary *in vivo* Tc cells could lyse vKV-1023-infected targets but not native flavivirus-infected targets suggested that the vKv-1023-infected targets interacted with the Tc effectors with a higher avidity than the flavivirus-infected targets. We tested this assumption by comparing the titre of anti-CD8 monoclonal antibody (MoAb) required to block the lysis by secondary *in vitro* Kunjin-immune Tc cells of WNV-, Kunjin- and vKV-1023-infected targets. The effector population and targets used in this experiment were those used for the experiments shown in Fig. 1. The dotted line in Fig. 1 shows the E : T ratio used for the CD8-blockade experiments. Figure 2 shows that the anti-CD8 MoAB was able to inhibit less of the lysis of vKV-1023- than of WNV- or Kunjin-infected targets (40 versus 60%), and that a four- to eight-fold higher concentration of anti-CD8 antibody was required to inhibit the lysis of vKV-1023-infected targets to an equivalent degree to the flavivirus-infected targets.

Anti-CD8 antibody blockage of lysis of vKV-1023-infected cells by primary in vivo *or secondary* in vitro *Kunjin-immune Tc cells*

A second assumption in our explanation for the lysis of flavivirus-infected cells by secondary *in vitro* but not primary *in vivo* Kunjin-

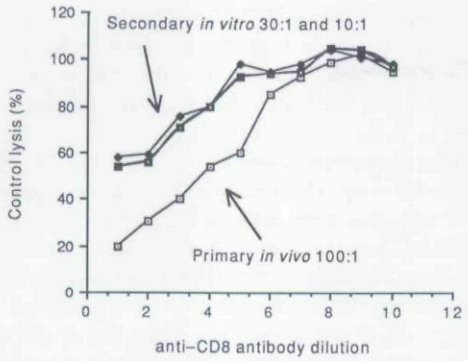

Fig. 3. Primary *in vivo* and secondary *in vitro* Kunjin-immune Tc cells: blocking of lysis of vKV-1023-infected targets by anti-CD8 antibody.

immune Tc cells is that the primary *in vivo* population interacted with targets with lesser avidity than the secondary *in vitro* population. The avidity of interaction was compared with vKV-1023-infected targets of these two Tc cell populations by assessing their inhibitibility by anti-CD8 MoAb. Figure 3 shows the results of the assay. The primary *in vivo* population was inhibited to a greater degree and at a lower concentration of antibody than the secondary *in vitro* population.

Differential susceptibility to CD8 blockade of primary and secondary influenza-immune Tc cells

In order to separate the effects of second antigen exposure from those due to the *in vitro* environment we utilized influenza virus, where immunization with serologically non-cross-reacting influenza strains has been shown to elicit a secondary *in vivo* Tc cell response.[10] Primary *in vivo*, secondary *in vivo* and secondary *in vitro* influenza-immune Tc cells were generated as described above. Figure 4 demonstrates that the secondary *in vivo* Tc cell response shows the characteristic early rise, higher peak and sustained activity of a secondary immune response. The avidity of interaction of the primary and secondary populations with targets was assessed by the titre of anti-CD8 MoAb required to inhibit lysis, as described above. The results are shown in Figure 5. Some of the increase in avidity in the secondary *in vitro* population could be attributed to *in vitro* conditions, as it was in excess of that seen in the secondary *in vivo* population. However, the secondary *in vivo* population showed a significant increase in avidity over the primary *in vivo* population.

Discussion

The CD8 blocking studies shown in Fig. 2 imply that vKV-1023-infected targets interact

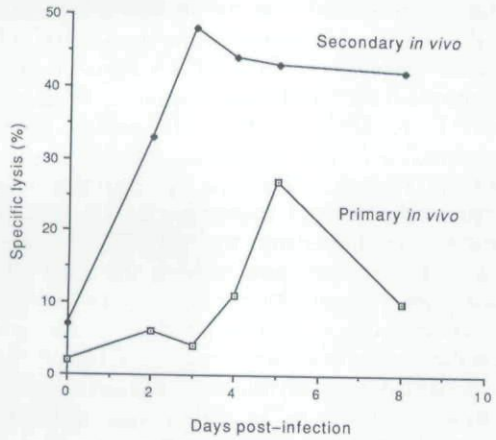

Fig. 4. Kinetics of primary and secondary influenza-immune Tc cell responses. Targets were P815 cells infected with A/WSN influenza virus. Standard error of the mean was always less than 3% and spontaneous ^{51}Cr release less than 10%.

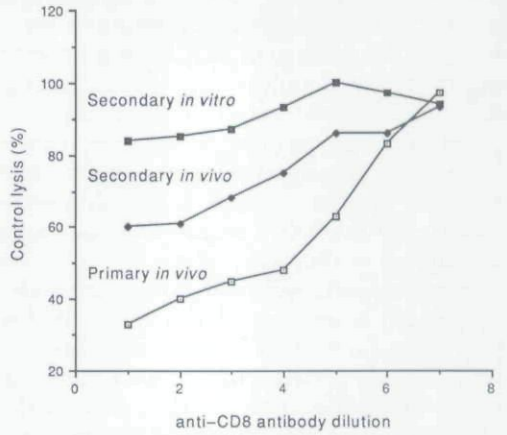

Fig. 5. Anti-CD8 blocking of lysis by primary *in vivo*, secondary *in vivo* and secondary *in vitro* influenza-immune Tc cells. Targets were P815 cells infected with A/WSN influenza virus as in Fig. 4.

with Tc cells with higher avidity than Kunjin- or WNV-infected cells, presumably because of a higher K^k + peptide concentration on the cell surface. This was unexpected, because VV is known to shut down host protein synthesis and decrease the level of MHC expression, whereas flavivirus infection leads to increased cell surface MHC expression.[11,12] We assume that under the strong VV p7.5 promoter, the recombinant VV-infected cells produce more of the Kunjin protein than the Kunjin-infected cells thus leading to more MHC-Kunjin peptide complex formation, although we have been unable to quantify Kunjin protein synthesis in vKV-1023-infected cells to test this.

Having established that the recombinant-infected targets presented antigen more efficiently to Tc cells than the flavivirus-infected cells, we asked whether the inability of primary *in vivo* Kunjin-immune Tc cells to recognize the latter was due to their having lower avidity for antigen bearing cells than the secondary *in vitro* population. Lysis by the primary *in vivo* population was more CD8 dependent than was lysis by the secondary *in vitro* population. However, these experiments were made difficult to interpret by the fact that different E : T ratios had to be used for each population to achieve interpretable lysis. The observation that primary *in vivo* Tc cells were able to lyse vKV-1023-infected targets but not Kunjin-infected targets, combined with the demonstration that Kunjin-infected targets presented antigen less efficiently than vKV-1023-infected targets had already suggested that the primary *in vivo* population interacted with targets with less avidity than secondary *in vitro* population. The difference in degree of inhibition by anti-CD8 antibody lent support to this conclusion.

In order to strengthen the observation and to distinguish between an increase in avidity due to a real difference between memory and naive Tc cell populations and an increase in some way dependent on *in vitro* condition, the influenza system was utilized to generate secondary *in vivo* influenza-immune Tc cells. Mice exposed to influenza A and later exposed to influenza virus of a serologically non-cross-reactive subtype generate influenza-immune Tc cells with the classic pattern of a secondary immune response.[10] Although the secondary *in vitro* Tc cells had the highest avidity of interaction with targets, the secondary *in vivo* Tc cells had higher avidity than the primary *in vivo* Tc cells.

The apparent increase in avidity of the secondary *in vivo* population is of special interest, as it represents a more physiological memory T cell population than that generated *in vitro*. Memory Tc cell populations have a higher precursor frequency of clones of a given antigenic specificity than naive populations and respond to antigen with a brisker rise and higher peak of Tc cell activity.[13] Resting memory human T lymphocytes have a higher level of expression of the T cell adhesion molecules LFA-3, CD2 and LFA-1.[14] Data presented here are consistent with the idea that reactivated memory Tc cell populations interact with targets with a higher avidity than those having undergone only their primary activation. This suggests the possibility that immunoselection for higher affinity clones may take place *in vivo*, although we have not excluded the possibility that the observed increase in resistance to anti-CD8 blocking could be due to increased density of TCR or accessory molecules (including CD8 itself) or simply increased numbers of $CD8^+$ T cells in the bulk population tested. Cloned Tc cells would be needed for definitive answers to these possibilities. Clones could also determine whether single or multiple Tc cell determinants exist within the crucial 99 amino acids of the NS3-NS4A sequence responsible for K^k-restricted responses. If different TCR affinities for different epitopes occurred, selective expansion of the higher affinity Tc clones in secondary responses may explain the present observations. The possibility that hypermutation of TCR genes might augment the pool of high affinity TCR available to immunoselection could be readdressed, although thus far this mechanism has not been shown to operate in T cells.

A final possibility not excluded here is that reactivated memory Tc cells require a lower threshold of interaction with targets in order to trigger lysis. Regardless of which explanation is correct, restimulated memory Tc cells are likely to prove more potent in their ability to control a viral infection than are primary responders.

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