Secretion of Tumor-Specific Antigen by Myeloma Cells Is Required for Cancer Immunosurveillance by CD4+ T Cells

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Abstract

Tumor-specific CD4+ T cells orchestrate the adaptive immune responses against cancer. We have previously shown that CD4+ T cells recognize MHC class II–negative myeloma cells indirectly by collaborating with tumor-infiltrating macrophages. We, here, hypothesize that this critical step may be dependent on secretion of tumor-specific antigens by cancer cells. This was investigated using T-cell receptor–transgenic mice, in which CD4+ T cells mediate rejection of syngeneic MOPC315 myeloma cells. We analyzed the immune response against myeloma cell variants, which either secrete or retain intracellularly a tumor-specific idiotypic (Id) antigen. Our results reveal that CD4+ T cells helped by macrophages are capable of detecting nonsecreted tumor antigens from MHC class II–negative cancer cells. However, Id secretion was required for successful myeloma immunosurveillance. Antigen secretion resulted in stronger priming of naive myeloma-specific CD4+ T cells in tumor-draining lymph nodes. Secretion of antigen by at least some cancer cells within a tumor was shown to facilitate immunosurveillance. Treatment by local injection of purified tumor-specific antigen successfully enhanced immunity against nonsecreting myeloma cells. Collectively, the data indicate that antigen concentration within the tumor extracellular matrix must reach a certain threshold to allow successful cancer immunosurveillance by CD4+ T cells. [Cancer Res 2009;69(14):5901–7]

Introduction

Genetic mutations in cancer cells generate alterations of protein sequences that are used by T cells for specific immunologic recognition (1). Whole-genome studies have recently shown the abundance of genetic modification in protein-coding genes of human tumors. An average of 50 to 70 mutations per tumor was reported for breast, colorectal, pancreatic, and brain cancers (2–4). One can anticipate that sequencing of all protein-coding genes in human tumors. An average of 50 to 70 mutations per tumor was reported for breast, colorectal, pancreatic, and brain cancers (2–4). One can anticipate that sequencing of all protein-coding genes in human tumors will represent a powerful method to select suitable tumor antigens for personalized cancer immunotherapy (5).

Five recent studies have reported the first characterization of a successful primary antitumor immune response initiated by naive CD4+ T cells (22). In brief, we could show that s.c. injected MOPC315 myeloma cells were surrounded within 3 days by macrophages that captured myeloma protein antigens. Within 6 days, naive Id-specific CD4+ T cells became activated in draining lymph nodes (LN) and subsequently migrated to the incipient tumor site. Upon recognition of tumor-derived Id peptides presented on MHC class II by
macrophages, Id-specific CD4+ T cells were shown to secrete IFN-γ, which in turn activated macrophages in close proximity to the myeloma cells. Such locally activated macrophages inhibited tumor growth by a yet undefined molecular mechanism (22).

Because myeloma recognition by Id-specific CD4+ T cells seems to be dependent on tumor-infiltrating APCs (20–22), we hypothesized that this critical step may require secretion of tumor-specific antigens by cancer cells. To investigate this issue, we took advantage of four variants of the MOPC315 myeloma that differ in their secretion pattern of the myeloma protein. Our results reveal the importance of antigen secretion for optimal myeloma eradication mediated by Id-specific CD4+ T cells.

Materials and Methods

Mice, cell lines, and injection of tumor cells. Id-specific TCR-transgenic SCID mice or SCID littermates on a BALB/c background (17, 23) were bred in a (nontransgenic SCID × heterozygous TCR-transgenic SCID) fashion. MOPC315 (IgA, λ2315) is a transplantable BALB/c plasmacytoma (24) obtained from American Type Culture Collection (ATCC) and propagated as in vitro growing cells. The three variants of MOPC315 (clones 26, 36, and 37; ref. 25) were kindly provided by Dr. Alexander Marks, University of Toronto. F9 is a λ2315-transfectant of the A20 B2L/c-derived B-lymphoma cell line (26). Adult mice were injected s.c. in the interscapular region or in the flank with 1.6-7.5 × 10^6 tumor cells suspended either in 100 μL PBS (Life Technologies) or in 250 μL growth factor–reduced Matrigel (BD Biosciences). Tumor growth was followed over time by palpation. Mice with tumor diameter of ≥10 mm were euthanized. Comparison of data between the groups was performed by log-rank test (for tumor challenge survival curves) and Mann-Whitney test (for tumor onset day) using GraphPad Prism 4 software (GraphPad Software). The study was approved by National Committee for Animal Experiments.

Analysis of cells by flow cytometry. Matrigel plugs were treated with 1 mg/mL collagenase and 0.3 mg/mL DNase (both from Sigma) at 37°C for 10 min and squeezed through a stainless steel sieve (Silmo) to release the cells. Single-cell suspensions from draining axillary LN were made by squeezing LN through a stainless steel sieve. Unspecific binding was blocked by incubation with heat-inactivated (56°C, 30 min) 30% normal rat serum in PBS and 100 μg/mL anti-FcγR/II/III monoclonal antibody (mAb; clone 2.4G2) before staining with specific mAbs. Biotinylated mAbs were conjugated with FITC or biotin in our laboratory: antitransgenic-TCR (RM4.5 or GK1.5), CD11b (M1/70), MHC class II I-A/I-E (M5/114.15.2), conjugated with FITC or biotin in our laboratory: antitransgenic-TCR (RM4.5 or GK1.5), CD11b (M1/70), MHC class II I-A/I-E (M5/114.15.2), CD8 (25-1.1; BD Biosciences), CD4 (RPA-T4; BD Biosciences), and anti-CD25 (MPC11; BD Biosciences). Quadruple-stained cells were analyzed on a FACSCalibur detemed with streptavidin conjugated to peridinin chlorophyll protein (BD Biosciences). Quadruple-stained cells were analyzed on a FACSCalibur detemed with streptavidin conjugated to peridinin chlorophyll protein (BD Biosciences). Quadruple-stained cells were analyzed on a FACSCalibur detemed with streptavidin conjugated to peridinin chlorophyll protein (BD Biosciences). Quadruple-stained cells were analyzed on a FACSCalibur detemed with streptavidin conjugated to peridinin chlorophyll protein (BD Biosciences). Quadruple-stained cells were analyzed on a FACSCalibur detemed with streptavidin conjugated to peridinin chlorophyll protein (BD Biosciences).

Antibodies. The following commercially available mAbs were used, conjugated with either FITC, phycoerythrin, allophycocyanin, or biotin: CD4 (BM4.5 or GK1.5), CD11b (M1/70), MHC class II I-A/I-E (M5/114.15.2), TRC4 (H57-597; BD Biosciences); CD11b (3A33), CD69 (H1.2F3; Southern Biotechnologies), and anti-CD8 (25-1.1; BD Biosciences). The following mAbs were affinity-purified and, if needed, conjugated with FITC or biotin in our laboratory: antitransgenic-TCR clonotype (GB113), anti-FcγR/II/III (2.4G2; ATCC), M315 (MOPC315; ATCC), M460 (MOPC460; ATCC), anti-Vα1/Vβ2 (9B8; ref. 27), and anti-Cx2 (2B6; ref. 27).

Results

Antigen secretion by myeloma cells is required for cancer immunosurveillance mediated by CD4+ T cells. Our experiments are based on four myeloma variants that have been generated previously by in vitro mutagenesis (25). The original MOPC315 myeloma cells secrete monoclonal IgA with λ2315 L-chains that harbor the tumor-specific Id peptide in the CDR3 region (24). The antigen loss MOPC315.36 variant produces neither L-chain nor immunoglobulin heavy chain (25). The MOPC315.26 variant secretes free λ2315 L-chains, but not immunoglobulin heavy chains (25). The nonsecreting MOPC315.37 variant produces the λ2315 L-chain but retains it intracellularly (25). The absence of λ2315 secretion by MOPC315.37 has been shown to be due to a single Gly→Arg amino acid substitution at position 15 of λ2315 in the variable (V) region (28). This mutation is distant from the Id T-cell epitope (positions 94–96) and does not affect recognition by Id-specific CD4+ T cells, as shown in previous transfection experiments (26).

We first measured the levels of λ2315 Id antigen in supernatants and lysates from each of the four myeloma variants by ELISA (Supplementary Table S1). In accordance with published work (25), the λ2315 antigen could only be detected in the supernatants from the two secreting clones (MOPC315 and MOPC315.26), but not from nonsecreting MOPC315.37 cells. The analysis of cell lysates confirmed that the λ2315 antigen is produced by MOPC315, MOPC315.26, and MOPC315.37 (which retains λ2315 intracellularly), but not by the antigen loss MOPC315.36 variant (Supplementary Table S1).

To assess antigen production in vivo, SCID mice were injected s.c. with each myeloma variant in Matrigel and λ2315 was quantified at day +10 (Supplementary Table S2). Note that total numbers of myeloma cells at day +10 may differ for each variant due to varying growth rates. High antigen levels were detected in Matrigel and serum of mice inoculated with the secreting MOPC315 and MOPC315.26 variants, confirming that Id is secreted in vivo. As expected, λ2315 was undetectable in serum when nonsecreting MOPC315.37 was injected. Interestingly, low antigen levels (0.048 μg/mL) were detected in the cell-free (supernatant) fraction from MOPC315.37-containing Matrigel suggesting antigen release by some necrotic tumor cells. Considerably less antigen was found in the day +10 Matrigel lysates from MOPC315.37 compared with MOPC315 and MOPC315.26, most likely due to a reduced growth rate for MOPC315.37. However, it should be emphasized that the amount of intracellular antigen detected in MOPC315.37 cells in vivo (0.5 μg/Matrigel lysate) is likely to be rather high compared with most intracellular tumor antigens.

The four myeloma variants were used for tumor challenge experiments in Id-specific TCR-transgenic SCID mice and control nontransgenic SCID mice. As previously reported (17), TCR-transgenic SCID mice were protected against s.c. challenge with MOPC315 cells, which secretes a complete IgA with the λ2315 antigen (Fig. 1A). Similarly, TCR-transgenic SCID mice resisted a challenge with MOPC315.26 cells, which secrete free λ2315 L-chains (Fig. 1B). The protection was antigen specific because the transgenic mice failed to reject the antigen loss variant MOPC315.36 (Fig. 1D). Importantly, TCR-transgenic SCID mice were poorly protected against MOPC315.37 cells, which do not secrete λ2315 antigen but retain it intracellularly (Fig. 1C). The growth of MOPC315.37 tumors was significantly delayed by ~10 days in TCR-transgenic SCID mice compared with nontransgenic SCID mice, but ultimately, all mice succumbed to cancer (Fig. 1C). The delayed tumor growth observed in Fig. 1C suggests the existence of an antitumor response against the nonsecreting MOPC315.37. However, this response seems to be too weak to protect mice from tumor development.

MOPC315 variants are not directly recognized nor killed by Id-specific CD4+ T cells. An important characteristic of MOPC315 is that it does not possess MHC class II molecules and, therefore, cannot be directly recognized by Id-specific CD4+ T cells (29). In order not to introduce a bias in our studies, it was important to check that MHC class II was not produced by any of the three mutagenesis variants of MOPC315. Using flow cytometry, we could...
not detect MHC class II molecules on the surface of any of the four myeloma clones (data not shown). We then performed two functional assays to test MHC class II–restricted antigen presentation in vitro. We did first a T-cell proliferation assay for which MHC class II–positive, Id-expressing F9 lymphoma cells (26) were used as a positive control. As previously reported (26), F9 cells could present Id antigen directly and sustain the proliferation of Id-specific CD4+ T cells (Supplementary Fig. S1 A). In contrast, the four myeloma clones were unable to do so. The addition of IFN-γ, which is secreted by Id-specific CD4+ T cells (22) and up-regulates MHC class II on some other tumor cells (30), had no influence on the capacity of MOPC315 variants to present Id peptides to CD4+ T cells (Supplementary Fig. S1 B).

We also tested whether the MOPC315 variants could be killed by Id-specific Th1 cells in a thymidine-release (JAM) assay. As shown previously (31), F9 cells were efficiently recognized and killed by Id-specific Th1 cells. In contrast, none of the four myeloma variants could be killed in vitro by Id-specific Th1 cells, even in the presence of IFN-γ (Supplementary Fig. S1B). We can thus conclude that MOPC315 and its variants are all MHC class II–negative and can neither be directly recognized nor killed by Id-specific CD4+ T cells. Furthermore, IFN-γ does not induce expression of MHC class II molecules on MOPC315 variants.

MOPC315 variants can all be killed by tumoricidal macrophages. Tumor-specific CD4+ T cells eliminate MOPC315 by rendering tumor-infiltrating macrophages tumoricidal (22). Therefore, all four variants were tested for susceptibility to killing by activated macrophages. Tumoricidal macrophages, purified from MOPC315-injected TCR-transgenic SCID mice (22), were tested in vitro for tumor cell growth suppression. The highest effector-to-target ratio (20:1) caused efficient suppression of all four myeloma variants (Fig. 2). Thus, failure of TCR-transgenic mice to reject MOPC315.37 (Fig. 1C) is not caused by this variant being intrinsically resistant to killing by macrophages.

Naive CD4+ T cells in draining LN are poorly activated by nonsecreted tumor antigens. We analyzed in vivo the cellular immune response against secreting versus nonsecreting myeloma cells. TCR-transgenic SCID mice were injected s.c. with Matrigel containing each one of the four MOPC315 variants. Ten days later, draining LN and Matrigel plugs were analyzed by flow cytometry. Inoculation of antigen-secreting MOPC315 or MOPC315.26 cells resulted in a strong activation of Id-specific CD4+ T cells in draining LN, as measured by the up-regulation of the CD69 (Fig. 3A) and CD44 (Supplementary Fig. S2 A) activation markers. This activation was antigen-specific because it was absent in mice injected with the antigen loss variant MOPC315.36. Strikingly, only very few activated (CD69+ or CD44high) Id-specific CD4+ T cells could be
detected in draining LN of mice injected with nonsecreting MOPC315.37 cells (Fig. 3A, arrow; Supplementary Fig. S2A). Thus, the intracellular antigen retention of MOPC315.37 results in severely decreased activation of naive CD4+ T cells in draining LN compared with antigen-secreting myeloma cells.

**CD4+ T cells are capable of detecting nonsecreted tumor antigens.** We have reported that macrophages were the main APCs responsible for local presentation of Id antigens from MOPC315 to CD4+ T cells (22). Similarly, s.c. injection with all variants resulted in recruitment of large numbers of CD11b+ macrophages (Fig. 3B; Supplementary Fig. S3A). In contrast, we did not find a CD11chigh population of dendritic cells (DC) in the Matrigel plugs. However, some Matrigel-infiltrating CD11b+ cells expressed low to intermediate levels of CD11c, indicating monocyte-derived DCs (Supplementary Fig. S2B). Thus, in numbers, macrophages seem to represent the main APC at the injection site regardless of the secretory status of the myeloma cells, but a contribution of DCs cannot be excluded.

We then analyzed the local activation of macrophages at the incipient tumor site. We have previously shown that Matrigel-infiltrating macrophages up-regulate the activation marker MHC class II in response to IFN-γ, which is locally produced by Id-specific Th1 cells upon antigen recognition (22). Similarly, a large population of activated MHC class IIhigh macrophages was observed in Matrigel containing any of the λ2315-producing myeloma variants, i.e., MOPC315, MOPC315.26, and MOPC315.37 (Fig. 3B; Supplementary Fig. S3B). Macrophage activation was antigen-specific because MHC class II expression was not up-regulated on macrophages that had infiltrated Matrigel plugs containing antigen loss MOPC315.36 cells. Interestingly, there was no indication of reduced macrophage activation in mice injected with MOPC315.37 cells, which retain λ2315 intracellularly, compared with secreting myeloma variants (Fig. 3B; Supplementary Fig. S3B). Furthermore, macrophage activation in mice injected with MOPC315, MOPC315.26, or MOPC315.37 was associated with the presence in Matrigel of a small but distinct population of activated (CD69+) Id-specific T cells (Fig. 3C; Supplementary Fig. S3C and D). In accordance with our previous findings that activation in draining LN is a prerequisite for CD4+ T-cell migration to the incipient tumor site (22), no Matrigel-infiltrating T cells could be detected in mice injected with the antigen loss MOPC315.36 variant (Fig. 3C; Supplementary Fig. S3C). Thus, the antigen-dependent activation of tumor-specific CD4+ T cells and macrophages at the incipient tumor site was similar for secreting versus nonsecreting tumor cells.

**Vaccination with antigen-secreting myeloma cells fails to provide protection against nonsecreting myeloma.** The results described above suggest that unsuccessful rejection of nonsecreting MOPC315.37 cells could be explained by insufficient activation of naive Id-specific CD4+ T cells in draining LN. We designed a system to test this hypothesis. Because rejection of antigen-secreting MOPC315 cells in TCR-transgenic SCID mice was associated with a robust T-cell activation in draining LN (see Fig. 3A), we reasoned that live MOPC315 cells could be used as a vaccine to boost the anti-MOPC315.37 immune response. In a first pilot experiment, TCR-transgenic SCID mice were injected s.c. with antigen-secreting
MOPC315 cells on the right flank and with nonsecreting MOPC315.37 cells on the left flank. One week later, mice were sacrificed and the activation of naive Id-specific CD4+ T cells in the LNs draining each flank was analyzed by flow cytometry. A strong CD4+ T-cell activation was observed in the LN draining the MOPC315 injection site (Fig. 4A; Supplementary Fig. S4). In contrast, activated CD69+ or CD44high T cells were hardly detectable in the contralateral LN draining the MOPC315.37 injection site. This experiment shows that (a) the activation of naive tumor-specific CD4+ T cells takes place locally in the LN draining the incipient tumor site and (b) it is possible to induce a strong activation of naive Id-specific CD4+ T cells in a mouse injected with MOPC315.37 at another location by using antigen-secreting MOPC315 cells as a vaccine.

In a second, larger experiment, TCR-transgenic SCID mice were vaccinated thrice by s.c. injections on the right flank with antigen-secreting MOPC315 cells at day −34, 0, and +14. Nonvaccinated TCR-transgenic SCID mice were used as a control. At day 0, mice in both groups were injected s.c. on the left flank with nonsecreting MOPC315.37 cells. Vaccinated mice did not show any improved protection against MOPC315.37 tumor growth (Fig. 4B). These data show that robust activation of naive tumor-specific CD4+ T cells in LN is not sufficient by itself to induce rejection of a nonsecreting tumor.

Local injection of tumor-specific antigen delays tumor formation by nonsecreting myeloma cells. We next tested whether injections of purified tumor-specific antigen into the tumor site could restore CD4+ T cell–mediated rejection of the nonsecreting myeloma variant. In a first experiment, Id-specific TCR-transgenic SCID mice were injected s.c. with either Id-containing IgA (M315) or Id-negative control IgA (M460). At day +8, a small, but distinct, population of activated (CD69+) Id-specific CD4+ T cells could be detected in draining LN upon Id-containing M315 injections (Fig. 5A, arrow).

We proceeded to examine whether protection against nonsecreting MOPC315.37 myeloma cells could be improved by local injections of purified tumor antigens. TCR-transgenic SCID mice were injected s.c. with nonsecreting MOPC315.37 cells. Mice were then treated thrice weekly by local s.c. injections with either M315 or M460 IgA in PBS. Treatment with Id-containing IgA resulted in significantly delayed tumor onset compared with control IgA (Fig. 5B). This experiment shows that increasing the local concentration of tumor-specific antigen by s.c. injections results in improved protection against nonsecreting myeloma cells.

Inoculation of mixed secreting and nonsecreting myeloma cells enhances immunosurveillance. To investigate the importance of continuous secretion of myeloma protein in the tumor, TCR-transgenic SCID mice were injected s.c. with a mixture of nonsecreting MOPC315.37 and secreting MOPC315 myeloma cells. Control mice received MOPC315.37 cells only. Cancer immunosurveillance was strongly improved by injecting secreting myeloma cells together with nonsecreting MOPC315.37 cells (Fig. 6). Thus, antigen secretion by at least some cancer cells within a tumor facilitates immunosurveillance. The data presented in the last two experiments (Figs. 5B and 6) strongly suggest that the mechanism by which antigen secretion enhances antitumor immunity is by increasing antigen availability locally at the incipient tumor site.

Discussion

Previous work in our laboratory has revealed that Id-specific CD4+ T cells recognize MHC class II–negative myeloma cells indirectly by collaborating with professional APCs (20–22). We hypothesized that this critical step may be dependent on secretion of the tumor-specific antigen by myeloma cells. Our data confirm this prediction and show that antigen secretion results in stronger priming of naive myeloma-specific CD4+ T cells in LN and is
required for successful immunosurveillance against the MOPC315 myeloma.

A drawback in using myeloma variants generated by in vitro mutagenesis is the changes in immunogenicity or tumorigenicity that could arise irrespective of the Id antigen. We believe that this is not a major concern in our study. First, the issue of immunogenicity was dealt with using TCR-transgenic SCID mice. These mice have T cells that can only recognize Id and no other antigen. Second, the tumorigenicity of the variants seems to be similar, as shown by tumor growth in nontransgenic SCID mice (Fig. 1). Finally, all four variants were equally susceptible to macrophage killing (Fig. 2).

We performed several experiments to investigate the reasons why Id-specific TCR-transgenic mice were unable to completely eradicate myeloma cells that produce but do not secrete the Id antigen. Vaccination by s.c. injection of Id-secreting myeloma cells on one flank failed to provide protection against nonsecreting myeloma injected s.c. on the contralateral flank. This experiment revealed that robust activation of tumor-specific CD4 + T cells in LN is not sufficient by itself to induce rejection of a nonsecreting tumor. In contrast, immunity against nonsecreting myeloma could be partially restored by local injection of purified tumor-specific antigen. Vaccination by s.c. injection of Id-secreting myeloma cells is not sufficient by itself to induce rejection of a nonsecreting myeloma. Notably, the coinjection experiment was the most successful one in terms of survival, which indicates that a continuous supply of tumor-specific antigen is advantageous for protection. Collectively, the data indicate that successful cancer immunosurveillance mediated by CD4 + T cells requires (a) efficient activation of tumor-specific CD4 + T cells in LN and most importantly (b) a sufficient concentration of tumor-specific antigen within the tumor extracellular matrix. If these two conditions are not fulfilled, e.g., due to intracellular antigen retention in MOPC315.37 cells, cancer eradication will presumably fail.

A rather surprising finding at the incipient tumor site was that activation of macrophages, as measured by up-regulation of MHC class II, was similar for Id-secreting versus Id-nonsecreting myeloma cells. The activation of tumor-infiltrating macrophages is both T-cell dependent and antigen specific (22). This implies that, in mice injected with nonsecreting MOPC315.37 cells, some few Id-specific CD4 + T cells became activated in draining LN and migrated to the incipient tumor site where they, in turn, activated macrophages. In the MOPC315.37 tumor microenvironment with relatively lower antigen levels, macrophage activation was apparently not optimal because tumor growth was delayed but not abolished. However, these data show that naive tumor-specific CD4 + T cells helped by macrophages are capable, albeit inefficiently, of responding to nonsecreted tumor antigens produced by MHC class II–negative myeloma cells. It is not clear how tumor-infiltrating macrophages captured nonsecreted Id antigen for presentation to CD4 + T cells. A possible explanation is that tumorigenesis is associated with some degree of cancer cell death, which may contribute to the release of nonsecreted tumor antigens into the extracellular fluid. Additionally, necrotic and apoptotic cancer cells are efficiently phagocytosed by macrophages (32).
which may thereby process intracellular cancer antigens. The observation that CD4+ T cells are capable of detecting nonsecreted tumor-specific antigens is an important finding for cancer immunotherapy, in general, if such poor T-cell activation can be enhanced, because most mutated proteins in cancer cells are not likely to be secreted (2–4).

Our conclusion that antigen secretion facilitates immunosurveillance does not necessarily imply that secreted tumor antigens should always be the first choice for cancer immunotherapy. It is important to keep in mind that immunologic tolerance is a main issue for cancer immunotherapy. In fact, deletion of Id-specific CD4+ T cells has been observed in the thymus and secondary lymphoid organs of mice with established MOPC315 myeloma tumors, when serum myeloma protein levels exceeded 50 μg/mL (33, 34). Thus, secretion of tumor-specific antigen by cancer cells may represent a double-edged sword, which either stimulates cancer cell eradication or, in situations where immunosurveillance has failed, induces a stronger tolerance to the tumor.

In conclusion, our data indicate that antigen concentration within the tumor extracellular matrix must reach a certain threshold to allow successful cancer immunosurveillance mediated by CD4+ T cells. These observations provide experimental support to treatment strategies, such as combined immunotherapy and chemotherapy (35–37), which induce killing of some cancer cells, and thereby increase the availability of tumor-specific antigens for T cells within tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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