

New battlefields for costimulation

Natalia Martin-Orozco and Chen Dong

Costimulation regulates the activation of naive T cells as they first encounter antigens in the secondary lymphoid organs. But recently characterized costimulatory molecules of the B7 family appear to have roles beyond initial T cell activation. New evidence shows that negative costimulators expressed by tumors and normal tissues afford local protection from T cell-mediated attack.

The outcome of a naive T cell's encounter with an antigen-carrying dendritic cell (DC) in secondary lymphoid organs depends on the maturation and activation status of the DC, in particular on the costimulatory molecules expressed by DCs. There are both positive and negative costimulatory molecules, which may individually or collectively regulate T cell activation thresholds. If the positive signals are strong, T cells will be activated and differentiate into effector cells. If the negative signals dominate, T cells may be deleted or inactivated. The critical positive costimulatory receptors on T cells are the immunoglobulin superfamily molecules CD28 and ICOS (inducible costimulator), which bind the B7 family ligands B7-1/B7-2 and B7h, respectively. The first negative costimulatory receptor on T cells to be characterized was cytotoxic T lymphocyte antigen (CTLA)-4, which also binds to B7-1 and B7-2. Recently several novel costimulatory receptors have been shown to negatively regulate T cell activation, including the CD28 family molecules programmed death (PD)-1 (1), which binds PD-1 ligand (PD-L1; also known as B7-H1) and PD-L2 (also known as B7-DC), and B and T lymphocyte attenuator (2), which binds herpesvirus entry mediator (3). In addition, the new B7 family ligands B7-H3 (4, 5) and B7-H4 (also known as

B7S1 or B7x) (6–8) have been shown to inhibit T cell activation, but their receptors on T cells are not yet known.

The expression of B7-1 and B7-2 is restricted to professional antigen-presenting cells. The newer B7 family members, however, are widely expressed in many different tissues by both hematopoietic and nonhematopoietic cells, suggesting that these molecules may regulate T cells in settings beyond initial priming in lymph nodes. For instance, it has been shown that PD-L1 is expressed in the maternal part of the placenta during gestation; blockade of PD-L1 increased the spontaneous abortion rate, which indicates that PD-L1 is at the front line of fetomaternal tolerance (9).

First new battlefield: preventing autoimmune tissue destruction

PD-1 ligands have different tissue expression patterns, which suggest that they have different roles in regulating immune responses (1). PD-L1 is expressed by many tissues and various cancer types. In mouse hematopoietic cells, PD-L1 is expressed on T cells, B cells, macrophages, and DCs. In contrast, PD-L2 is only expressed on macrophages and DCs. Thus, both PD-L1 and PD-L2 may regulate T cells in the lymph nodes, whereas PD-L1 may have additional roles in regulating T cells in tissues or tumors.

The role of PD-1 ligands in naive T cell activation has been controversial. Results provided by several groups using different experimental systems have indicated that under some conditions PD-1 ligands can be activators of T cells, whereas in other conditions they can be inhibitors (1). In the case of

type 1 diabetes in nonobese diabetic (NOD) mice, blocking PD-L1 using antibodies was shown to rapidly precipitate the disease, indicating that PD-L1 inhibits autoreactive T cells (PD-L2 blockade had no effect) (10).

PD-L1 mRNA is constitutively expressed in several tissues such as heart, skeletal muscle, lung, kidney, and liver. The protein is found in macrophage-derived cells, such as Kupffer cells in liver, macrophages in lung, and histiocytes in the paracortical region of tonsil (11). The promoter region of PD-L1 contains several interferon (IFN)- γ -responsive elements (12), suggesting that PD-L1 protein expression may be induced as a result of a localized immune response. Indeed, PD-L1 protein is found in multiple human carcinomas (lung ovarian, renal, colon, melanoma, head and neck, and breast cancer) and can be induced in tumor cell lines with IFN- γ (11). Also, PD-L1 expression in tumors facilitates immune evasion by dampening T cell immunity, inducing apoptosis of tumor-reactive T lymphocytes (11), and conferring resistance to cytolysis by tumor-specific CD8 T cells (13). Moreover, blocking PD-L1 improves antitumor immunity in several experimental tumor models (13).

In this issue, Keir et al. (p. 883) analyzed *PD-L1*^{-/-}, *PD-L2*^{-/-}, and doubly deficient mice (14). They found that the absence of both molecules in the BALB/c strain led to a greater increase in the expression of the cytokines interleukin (IL)-2 and interferon (IFN)- γ by T cell receptor transgenic T cells activated in vitro compared with cells from mice lacking just one of the PD-1 ligands. This indicates that PD-L1 and PD-L2 are negative regulators of T cell activation in this setting and they may synergistically inhibit T cell activation. The authors further analyzed the function of PD-1 ligands in autoimmune diabetes. Double-deficient (*PD-L1/PD-L2*^{-/-}) NOD mice, similar

N.M.-O. and C.D. are at Department of Immunology, MD Anderson Cancer Center, Houston, TX 77030.

CORRESPONDENCE

N.M.-O.: nmorozco@mdanderson.org
OR
C.D.: cdong@mdanderson.org

to *PD-1*^{-/-} animals on the same background (15), developed diabetes by 7 weeks of age with 100% penetrance in males and females. In wild-type NOD mice, in contrast, diabetes appeared after week 20 and occurred predominantly in females. Furthermore, mice deficient in PD-L1, but not PD-L2, developed diabetes with full penetrance in males and females by week 10, indicating that PD-L1 is required for resistance to autoimmune attack in the male mice. Infiltration of cells into the islets (insulinitis) also occurred earlier in *PD-L1/PD-L2*^{-/-} animals than in wild-type NOD mice, with more T cells proliferating in the pancreatic lymph node, suggesting that a negative signal through PD-1 occurs early during T cell priming.

Furthermore, the authors described an additional layer of T cell regulation by PD-L1. They reported that PD-L1 expressed on pancreatic islets may prevent diabetes by providing a negative costimulatory signal to infiltrating autoreactive CD4⁺ T cells (Fig. 1). In these experiments, the authors generated bone marrow chimeric mice and found that, surprisingly, expression of PD-L1 and PD-L2 by antigen-presenting cells, such as DCs, was not sufficient to prevent accelerated diabetes in *PD-L1/PD-L2*^{-/-} mice. *PD-L1/PD-L2*^{-/-} mice that received wild-type bone marrow cells reached the 100% incidence of diabetes only slightly slower than the mice that received *PD-L1/PD-L2*^{-/-} cells. This suggests that PD-L1 expressed by parenchyma cells may play a more important role than PD-L1 expressed on DCs. In support of this idea, the authors found that wild-type islets were more protected than *PD-L1/PD-L2*^{-/-} islets from autoimmune destruction when they were transplanted into diabetic mice. Thus, PD-L1 appears to have a strong inhibitory influence on autoreactive T cells in the islets, possibly by preventing cytokine secretion at the tissue site. Further research is required to detail whether PD-L1 in the islets also promotes T cells deletion or anergy, or whether it simply stops T cell proliferation and cytokine expression.

Ansari et al. had shown earlier that PD-L1 but not PD-L2 was the main ligand of PD-1 restricting autoimmune

diabetes in NOD mice (10). In addition, Liang et al. had reported that PD-L1 protein expression is up-regulated in infiltrated islet cells from 9-week-old NOD mice (16). So it was not surprising to find that PD-L1 is the main factor preventing autoimmune attack at the islet level. It is possible that islet β cells, similar to tumors, sense IFN- γ produced by early infiltrating autoreactive T cells and further up-regulate PD-L1 to protect them from the autoimmune destruction. However, earlier work from another group showed that transgenic expression of PD-L1 in islet β cells in the C57BL/6 mouse strain provoked spontaneous diabetes in some mice (17), implying that PD-L1 can promote autoreactive T cell activation. It is not clear why such sharply contrasting functions of PD-L1 in islets have been found. Genetic background is one possible explanation. It is also possible that the two studies analyzed nonoverlapping autoimmune reactions, in which one is dominated by CD4⁺ and the other by CD8⁺ T cells.

Based on the existing literature, it is tempting to suggest that PD-L1 is an attractive target for the treatment of autoimmune diseases. However, other genetic modifiers in humans that may influence the function of PD-1 and its ligands need to be considered in order to predict the outcome of a treatment. Polymorphisms in the PD-1 gene are associated with susceptibility to lupus, arthritis, and type I diabetes, but so far there are no reports of polymorphisms in the genes encoding PD-1 ligands (18). PD-1 deficiency in different mouse strains results in different autoimmune phenotypes—cardiomyopathy in BALB/c, lupus nephritis in C57BL/6, and accelerated diabetes in NOD—suggesting that there is a genetic aspect to the regulation of PD-1 function in different tissues.

Second new battlefield: tumor tolerance

Blocking negative costimulatory molecules to boost antitumor T cell responses in cancer patients is an attractive new strategy in cancer immunotherapy. Antibodies that block CTLA-4 on T cells have proven effective in treating melanoma patients (19). Although the precise mechanism and location of

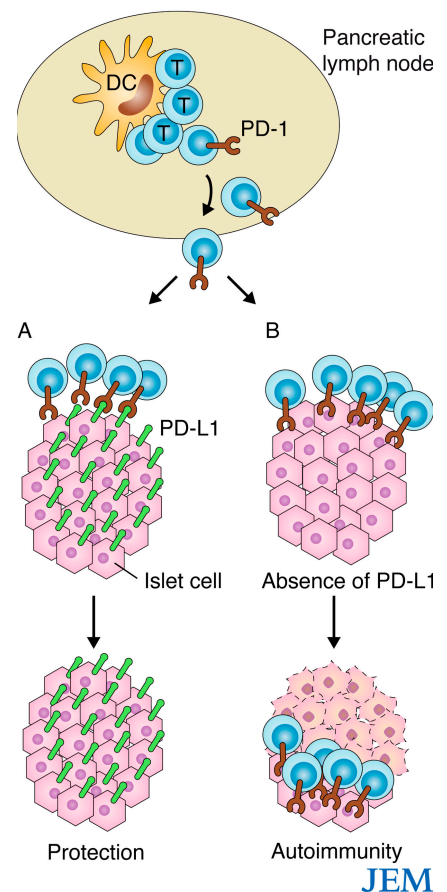


Figure 1. PD-L1 mediated protection from autoimmune attack. Autoreactive T cells recognize islet-derived antigen presented by DCs in the pancreatic lymph nodes. After activation and expansion, the diabetogenic clones migrate to the islets in the pancreas. (A) Islet cells protect themselves from the autoimmune attack by expressing PD-L1. (B) Absence of PD-L1 in the islets cells as described by Keir et al. (14) allows the autoreactive T cells to invade and destroy the islet cells, initiating the autoimmune process.

anti-CTLA-4's effects are not fully understood, the efficacy of these antibodies at least suggests that tumor-specific T cells can be activated in patients bearing tumors.

When tumors escape immune surveillance and progress to become a solid cell mass, immune tolerance mechanisms are established in the tumor microenvironment that strongly inhibit infiltrating T lymphocyte function (20). For example, prostaglandin E₂ secreted by monocytes and tumor-associated macrophages (TAMs) inhibits the proliferation of T cells and promotes

T helper (Th)2 responses, which down-regulate the antitumor Th1 response (21). Moreover, prostaglandin E₂ promotes the secretion of IL-10 by macrophages, DCs, and tumors, which maintains the immunosuppressive environment in the tumor. Macrophages from tumor stroma also produce the immunosuppressive cytokine TGF- β , which inhibits the antitumor activities of infiltrating cytotoxic T lymphocytes, natural killer cells, neutrophils, and macrophages. In addition, transforming growth factor- β induces the production of IL-10 by the tumor cells and down-regulates the expression of the activating receptor NKG2D—an important regulator of antitumor immune surveillance—on CD8⁺ T cells and natural killer cells (21). In this issue, Kryczek et al. (p. 871) show that another mechanism of tumor immune evasion involves TAMs that suppress T cell activation through B7-H4 (22).

B7-H4 is the newest addition to the B7 family that has been shown to negatively regulate T cell activation (6–8). The expression of B7-H4 in multiple nonlymphoid tissues suggests that it might mediate tolerance at the tissue level. In addition, human breast and ovarian cancers have been found to express B7-H4 (23–25). Interestingly, Kryczek et al. report the intracellular expression of B7-H4 protein in recently isolated human ovarian tumor cells from ascites fluid or tumor mass, and from established ovarian tumor cell lines (22). In contrast, cell surface expression of B7-H4 was found on the majority of freshly isolated TAMs from tumor ascites and in the ovarian epithelial carcinoma cells. This indicates that localization of B7-H4 protein is regulated, albeit by an unknown mechanism. The cytokines IL-6 and IL-10 present in the ascites were shown to induce expression of B7-H4 on normal blood monocytes. However, tumor ascites had no direct effect on intracellular or cell surface expression of B7-H4 in the tumor cells. The authors also found that the cytokines IL-4 and granulocyte/macrophage colony-stimulating factor (GM-CSF) suppressed IL-10-induced B7-H4 expression. Thus, the down-

regulation of B7-H4 may contribute to the potent adjuvant effects of GM-CSF in cancer immunotherapy.

Expression of B7-H4 by TAMs inhibited the *in vitro* proliferation and effector function of CD8⁺ T cells specific for the tumor antigen Her-2/neu. Using morpholino antisense oligonucleotides, the authors showed that the immunosuppression was mediated specifically by B7-H4 and not other immunosuppressive effectors known to be expressed by macrophages, such as PD-L1, arginase, or inducible nitric oxide synthase (21). The B7-H4-dependent immunosuppression by macrophages at the tumor site was then confirmed in a mouse ovarian tumor model. Macrophages treated with oligonucleotides to block B7-H4 expression reduced the growth of tumors implanted in lymphocyte-deficient mice that received T cells specific for tumor antigens. In contrast, macrophages treated with control oligonucleotides did not influence the tumor growth. This further indicates that B7-H4 is the critical immunosuppressive molecule expressed by TAMs.

A possible scenario that might explain these findings is that ovarian cancer cells secrete IL-6 and IL-10 to induce the expression of B7-H4 on infiltrating macrophages, which would then inhibit the proliferation of and cytokine production by infiltrating T cells via B7-H4. IL-10 produced by macrophages may also suppress T cell function directly (Fig. 2). The data presented by Kryczek et al. suggests that blockade of B7-H4 enhances the activity of tumor-infiltrating T cells, shifting the balance of immunity and immune evasion in favor of tumor destruction (22). It remains unclear what type of T cells (for example, Th cells, regulatory T cells, or cytotoxic T cells) are targeted by the suppressive macrophages. Moreover, the B7-H4 receptor on T cells is not known. It will be important to identify this receptor, determine which cells express it, and investigate its regulation.

These findings add another level of regulation of host responses to tumors by macrophages, which are already known to promote angiogenesis and tumor invasion of tissues by a crosstalk with tu-

mor cells (26). TAMs found in the basement membrane of solid tumors secrete matrix metalloproteinases that promote the escape of the tumor cells to surrounding stromal area where the tumor can continue growing. Macrophages also secrete epidermal growth factor, which promotes chemotaxis of tumor cells to blood vessels, allowing them to escape into the circulation. In addition, carcinoma cells secrete colony stimulator factor (CSF)1 to attract circulating macrophages into the tumor. Additionally, in hypoxic areas of tumors, macrophages are induced to secrete proangiogenic factors, such as vascular endothelial growth factor, CXCL8, angiopoietin, and cyclooxygenase-2, which promote the development new vessels to supply the tumors with blood (27).

Future considerations

The two papers in this issue have revealed crucial roles of negative costimulators in preventing tissue and tumor attack by infiltrating T cells (14, 22). Enhancing

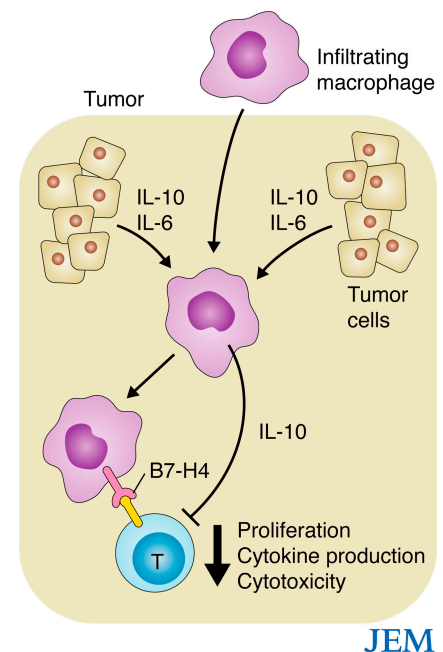


Figure 2. Tumor immune evasion assisted by macrophages. Cells of solid tumors, such as ovarian cancer described by Kryczek et al. (22), secrete IL-10 and IL-6 to induce the expression of B7-H4 in infiltrating macrophages, which in turn inhibit infiltrating T cell functions within the tumor environment. IL-10 secreted by macrophages may also inhibit T cell function.

the function of these molecules may be of benefit in the treatment of autoimmune diseases, whereas inhibition of their function might be an effective therapeutic strategy to boost antitumor immunity.

Tumor immunity and autoimmunity are alike to some extent. Tumor reactivity and autoreactivity exist in our T cell receptor repertoire but are restricted by many inhibitory mechanisms such as peripheral tolerance and the action of regulatory T cells. The studies discussed in this article, along with others, suggest that further checkpoints exist in tissues and tumors that are mediated by negative costimulatory molecules such as PD-L1 and B7-H4. These studies also suggest that cytokines (pro- or antiinflammatory) are crucial regulators of the expression of these inhibitory molecules.

A lot still needs to be learned about negative costimulation in tissues and tumors and its role in regulating T cell activity. For instance, is positive costimulation in tissues or tumors required for the function of self- or tumor-reactive T cells? And what are the differences in the function and regulation of the various negative costimulators? From our experience in studying costimulation in the context of the regulation of naive T cell activation, we may expect to observe combinations of various positive and negative costimulatory factors that regulate T cell activities in tissues and tumors. Thus, it may eventually be possible to design effective immunotherapies that specifically perturb autoimmune responses without impinging on immunity to infection or that break tumor tolerance without provoking autoimmune diseases.

We thank our colleagues for discussion.

C. Dong was supported by the National Institutes of Health, and is a Cancer Research Institute Investigator and an MD Anderson Cancer Center Trust Fellow.

REFERENCES

- Greenwald, R.J., G.J. Freeman, and A.H. Sharpe. 2005. The B7 family revisited. *Annu. Rev. Immunol.* 23:515–548.
- Watanabe, N., M. Gavrieli, J.R. Sedy, J. Yang, F. Fallarino, S.K. Loftin, M.A. Hurchla, N. Zimmermann, J. Sim, X. Zang, et al. 2003. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. *Nat. Immunol.* 4:670–679.
- Sedy, J.R., M. Gavrieli, K.G. Potter, M.A. Hurchla, R.C. Lindsley, K. Hildner, S. Scheu, K. Pfeffer, C.F. Ware, T.L. Murphy, and K.M. Murphy. 2005. B and T lymphocyte attenuator regulates T cell activation through interaction with herpesvirus entry mediator. *Nat. Immunol.* 6:90–98.
- Suh, W.K., B.U. Gajewska, H. Okada, M.A. Gronski, E.M. Bertram, W. Dawicki, G.S. Duncan, J. Bukczynski, S. Plyte, A. Elia, et al. 2003. The B7 family member B7-H3 preferentially down-regulates T helper type 1-mediated immune responses. *Nat. Immunol.* 4:899–906.
- Prasad, D.V.R., T. Nguyen, Z. Li, Y. Yang, J. Duong, Y. Wang, and C. Dong. 2004. Mouse B7-H3 is a negative regulator of T cells. *J. Immunol.* 173:2500–2506.
- Prasad, D.V., S. Richards, X.M. Mai, and C. Dong. 2003. B7S1, a novel B7 family member that negatively regulates T cell activation. *Immunity.* 18:863–873.
- Sica, G.L., I.H. Choi, G. Zhu, K. Tamada, S.D. Wang, H. Tamura, A.I. Chapoval, D.B. Flies, J. Bajorath, and L. Chen. 2003. B7-H4, a molecule of the B7 family, negatively regulates T cell immunity. *Immunity.* 18:849–861.
- Zang, X., P. Loke, J. Kim, K. Murphy, R. Waitz, and J.P. Allison. 2003. B7x: a widely expressed B7 family member that inhibits T cell activation. *Proc. Natl. Acad. Sci. USA.* 100:10388–10392.
- Guleria, I., A. Khosroshahi, M.J. Ansari, A. Habicht, M. Azuma, H. Yagita, R.J. Noelle, A. Coyle, A.L. Mellor, S.J. Khoury, and M.H. Sayegh. 2005. A critical role for the programmed death ligand 1 in fetomaternal tolerance. *J. Exp. Med.* 202:231–237.
- Ansari, M.J., A.D. Salama, T. Chitnis, R.N. Smith, H. Yagita, H. Akiba, T. Yamazaki, M. Azuma, H. Iwai, S.J. Khoury, et al. 2003. The programmed death-1 (PD-1) pathway regulates autoimmune diabetes in nonobese diabetic (NOD) mice. *J. Exp. Med.* 198:63–69.
- Dong, H., S.E. Strome, D.R. Salomao, H. Tamura, F. Hirano, D.B. Flies, P.C. Roche, J. Lu, G. Zhu, K. Tamada, et al. 2002. Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat. Med.* 8:793–800.
- Mazanet, M.M., and C.C. Hughes. 2002. B7-H1 is expressed by human endothelial cells and suppresses T cell cytokine synthesis. *J. Immunol.* 169:3581–3588.
- Hirano, F., K. Kaneko, H. Tamura, H. Dong, S. Wang, M. Ichikawa, C. Rietz, D.B. Flies, J.S. Lau, G. Zhu, et al. 2005. Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res.* 65:1089–1096.
- Keir, M.E., S.C. Liang, I. Guleria, Y.E. Latchman, A. Qipo, L.A. Albacker, M. Koulmanda, G.J. Freeman, M.H. Sayegh, and A.H. Sharpe. 2006. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J. Exp. Med.* 203:883–895.
- Wang, J., T. Yoshida, F. Nakaki, H. Hiai, T. Okazaki, and T. Honjo. 2005. Establishment of NOD-Pdcd1^{-/-} mice as an efficient animal model of type I diabetes. *Proc. Natl. Acad. Sci. USA.* 102:11823–11828.
- Liang, S.C., Y.E. Latchman, J.E. Buhlmann, M.F. Tomczak, B.H. Horwitz, G.J. Freeman, and A.H. Sharpe. 2003. Regulation of PD-1, PD-L1, and PD-L2 expression during normal and autoimmune responses. *Eur. J. Immunol.* 33:2706–2716.
- Subudhi, S.K., P. Zhou, L.M. Yerian, R.K. Chin, J.C. Lo, R.A. Anders, Y. Sun, L. Chen, Y. Wang, M.-L. Alegre, and Y.-X. Fu. 2004. Local expression of B7-H1 promotes organ-specific autoimmunity and transplant rejection. *J. Clin. Invest.* 113:694–700.
- Prokunina, L., C. Castillejo-Lopez, F. Oberg, I. Gunnarsson, L. Berg, V. Magnusson, A.J. Brookes, D. Tentler, H. Kristjansdottir, G. Grondal, et al. 2002. A regulatory polymorphism in PDCD1 is associated with susceptibility to systemic lupus erythematosus in humans. *Nat. Genet.* 32:666–669.
- Phan, G.Q., J.C. Yang, R.M. Sherry, P. Hwu, S.L. Topalian, D.J. Schwartzentruber, N.P. Restifo, L.R. Haworth, C.A. Seipp, L.J. Freezer, et al. 2003. Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. *Proc. Natl. Acad. Sci. USA.* 100:8372–8377.
- Dunn, G.P., L.J. Old, and R.D. Schreiber. 2004. The immunobiology of cancer immunosurveillance and immunoediting. *Immunity.* 21:137–148.
- Ben-Baruch, A. 2006. Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators. *Semin. Cancer Biol.* 16:38–52.
- Kryczek, I., L. Zou, P. Rodriguez, G. Zhu, S. Wei, P. Mottram, M. Brumlik, P. Chen, T. Curiel, L. Myers, A. Lackner, X. Alvarez, A. Ochoa, L. Chen, and W. Zou. B7-H4 expression identifies a novel suppressive macrophage population in human ovarian carcinoma. *J. Exp. Med.* 203:871–881.
- Tringler, B., W. Liu, L. Corral, K.C. Torkko, T. Enomoto, S. Davidson, M.S. Lucia, D.E. Heinz, J. Papkoff, and K.R. Shroyer. 2006. B7-H4 overexpression in ovarian tumors. *Gynecol. Oncol.* 100:44–52.
- Tringler, B., S. Zhuo, G. Pilkington, K.C. Torkko, M. Singh, M.S. Lucia, D.E. Heinz, J. Papkoff, and K.R. Shroyer. 2005. B7-H4 is highly expressed in ductal and lobular breast cancer. *Clin. Cancer Res.* 11:1842–1848.
- Salceda, S., T. Tang, M. Kmet, A. Munteanu, M. Ghosh, R. Macina, W. Liu, G. Pilkington, and J. Papkoff. 2005. The immunomodulatory protein B7-H4 is overexpressed in breast and ovarian cancers and promotes epithelial cell transformation. *Exp. Cell Res.* 306:128–141.
- Condeelis, J., and J.W. Pollard. 2006. Macrophages: obligate partners for tumor cell migration, invasion, and metastasis. *Cell.* 124:263–266.
- Lewis, C.E., and J.W. Pollard. 2006. Distinct role of macrophages in different tumor microenvironments. *Cancer Res.* 66:605–612.