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# Branched α-(1,4) Glucans from *Lentinula edodes* (L10) in Combination with Radiation Enhance Cytotoxic Effect on Human Lung Adenocarcinoma through the Toll-like Receptor 4 Mediated Induction of THP-1 Differentiation/Activation

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**ABSTRACT:** This work investigated the role of structure in the binding of polysaccharides from 10 regionally different strains of *Lentinula edodes* to Toll-like receptor 4 (TLR-4) on monocytes (THP-1) and the potential effect of this interaction on tumor cell viability. Principal component analysis and multiple linear regression identified arabinose, glucose 1→4 linkage, and molecular weights about 2700 and 534 kDa as the significant determinant factors associated with TLR-4 binding activity. The branched α-(1,4)-glucan (L10) had the strongest ability to bind to TLR-4 and induce THP-1 cell differentiation. L10 induction of the THP-1 cell differentiation, superoxide production, and cytokine production followed the TLR-4/MyD88/IKK/NFκB pathway. Coculture of irradiated human lung adenocarcinoma A549 cells with L10-activated THP-1 cells resulted in significantly decreased percentage of viable A549 cells from 66 to 37% ($p = 0.018$), increased levels of superoxide, interleukin-8, and RANTES, and decreased levels of angiogenin and vascular endothelial growth factor. The results indicate that L10-activated monocytes have the potential to boost the antitumor immune response and antitumor activity of radiotherapy.

**KEYWORDS:** polysaccharide, multiple linear regression analysis, macrophage differentiation/activation, immunotherapy, radiation therapy

## INTRODUCTION

In the past 10 years, polysaccharides isolated from numerous sources (mushrooms, algae, lichens, and higher plants) have attracted great attention because of their many therapeutic properties and relatively low toxicity.[1] The polysaccharides modulate and stimulate innate immunity (more specifically, macrophage-dependent responses) and bind to a variety of immune cell surface receptors, such as Toll-like receptor 4 (TLR-4). The molecular basis of TLR functions has been extensively investigated.[2,3] Most TLRs interact with myeloid differentiation primary response protein 88 (MyD88) and tumor necrosis factor (TNF) receptor-associated factor 6 to activate nuclear factor κB (NFκB) and mitogen-activated protein kinases, which then promote transcription of more than 100 different genes.[1,3] However, the interactions of TLRs and polysaccharides with structural features that affect TLR-4-induced monocyte differentiation and macrophage activation are not well understood.

The antitumor efficacy of some strategies combining radiotherapy (RT) with modifiers of the immune response have been tested in the clinic. Because RT to the primary tumor can increase tumor in situ immunogenicity and homing of effector immune cells to the tumor site, combined treatment with RT and immunomodulators, such as oligosaccharides, has been proposed.[4]

In our previous study, we reported the monosaccharide composition, molecular weights, and structural linkages for polysaccharides extracted from 10 regional *Lentinula edodes* mushroom strains (Table 1).[5,6] The molecular weights of these polysaccharides ranged from $1 \times 10^4$ to $3 \times 10^6$ kDa. The polysaccharides were composed primarily of a backbone of (1→4)-glucan with side chains of (1→6)-glucan, but also contained arabinose (1→4) linkages and mannose (1→2) linkages. The composition analysis revealed the presence of heterogeneous monosaccharides (glucose, mannose, xylose, galactose, fucose, rhamnose, and arabinose). The differences in monosaccharide composition led to variation in backbone size and side-chain formations. Our results suggested that structural variation affected the level of RAW 264.7 macrophage cell stimulation.[5] We proposed that certain structural features of polysaccharides govern their interactions with TLR-4 on monocytes or macrophages. Our data showed that of the 10 polysaccharides evaluated, L10 had the strongest TLR-4 binding activity and induced TLR-4-mediated signal transduction. This molecular understanding of the effect of L10 on the induction of monocyte

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**Table 1. Structural and Bioactivity Data of 10 Polysaccharides from Different Strains of *L. edodes***

| | polysaccharide | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | L1 | L4 | L6 | L10 | L11 | L15 | L21 | L23 | L24 | L25 |
| monosaccharide composition | | | | | Molar Ratio | | | | | |
| arabinose | 0.457 | 0.786 | 0.490 | 0.571 | 0.594 | 0.586 | 0.500 | 0.542 | 0.273 | 0.416 |
| xylose | 0.365 | 0.748 | 0.350 | 0.410 | 0.458 | 0.415 | 0.349 | 0.386 | 0.139 | 0.293 |
| mannose | 2.448 | 3.457 | 1.891 | 1.976 | 3.186 | 2.169 | 1.989 | 1.876 | 0.892 | 1.995 |
| galactose | 0.109 | 0.358 | 0.154 | 0.262 | 0.378 | 0.154 | 0.221 | 0.224 | 0.004 | 0.121 |
| glucose | 4.203 | 1.622 | 3.027 | 4.130 | 4.144 | 7.467 | 2.813 | 3.956 | 10.950 | 3.588 |
| rhamnose | 0.000 | 0.016 | 0.014 | 0.000 | 0.012 | 0.030 | 0.033 | 0.023 | 0.056 | 0.026 |
| fucose | 0.000 | 0.037 | 0.014 | 0.000 | 0.021 | 0.012 | 0.033 | 0.013 | 0.015 | 0.016 |
| glycosyl linkage | | | | | Abundance $\times 10^{-6}$ | | | | | |
| 2,3,6-Me3-Glcp | 3.350 | 3.100 | 3.900 | 5.700 | 4.900 | 5.800 | 4.400 | 4.600 | 7.700 | 3.200 |
| 2,3,4-Me3-Glcp | 0.900 | 1.700 | 1.400 | 1.700 | 1.700 | 1.500 | 1.400 | 2.200 | 0.700 | 1.050 |
| 2,3-Me3-Glcp | 0.900 | 0.900 | 1.700 | 1.900 | 1.700 | 2.400 | 1.400 | 1.400 | 3.600 | 1.100 |
| 3,4,6-Me3-Manp | 1.400 | 3.500 | 1.700 | 2.200 | 2.500 | 1.700 | 1.700 | 1.300 | 0.100 | 0.100 |
| 2,3-Me3-Arap | 2.600 | 0.500 | 0.900 | 0.500 | 1.200 | 2.900 | 1.100 | 0.700 | 0.300 | 0.700 |
| molecular weight | | | | | Ratio | | | | | |
| A > 2,754,000 | 0.500 | 2.500 | 2.100 | 3.400 | 1.700 | 3.400 | 2.100 | 2.500 | 1.800 | 3.200 |
| B 2,754,000 | 0.000 | 0.400 | 0.400 | 0.800 | 0.000 | 0.400 | 0.400 | 0.000 | 0.600 | 0.700 |
| C 534,000 | 1.300 | 0.000 | 0.000 | 0.000 | 1.800 | 0.000 | 0.000 | 0.600 | 0.000 | 0.000 |
| D 11,700 | 1.700 | 5.000 | 1.900 | 2.200 | 0.300 | 0.900 | 0.700 | 0.700 | 5.500 | 2.800 |
| bioactivity | | | | | | | | | | |
| THP-1 differentiation ($OD_{570}$) | 0.263 | 0.285 | 0.272 | 0.310 | 0.250 | 0.292 | 0.306 | 0.301 | 0.302 | 0.282 |
| TLR-4 binding activity[a] | 2.000 | 2.400 | 1.950 | 2.800 | 2.500 | 1.900 | 2.150 | 1.700 | 2.300 | 1.750 |

[a] The highest fluorescence intensity ($\times 10^2$) of the sample peak.

differentiation into macrophages provides a rationale for evaluating the combination of L10 with RT as treatment for lung cancer (A549 cells).

## MATERIALS AND METHODS

***L. edodes* Strains, Polysaccharide Isolation, and Polysaccharide Structure.** Ten isolates of *L. edodes* were used in this study: Tainung No. 1 "white cap" (L1) and "red cap" (L4) from Taiwan; Japanese 271 (L11, L15), Jongxing 5 (L6), Jongxing 8 (L10), Hey-King-Gang (L21), and Jong-Wen 600 (L23) from Japan; and No. 135 (L24) and No. 939 (L25) from China. Three distinct clusters were revealed by amplified fragment length polymorphism analysis (AFLP): (1) L24 and L25 isolates from China; (2) L1 and L4 isolates from Taiwan; (3) L6, L10, L11, L15, L21, and L23 isolates from Japan. The culture broth of the mycelia of the isolates was submerged and fermented in medium (pH 4.5) containing 2% oats, 0.5% yeast extract, 0.1% $KH_2PO_4$, 0.05% $MgSO_4$, and 0.15% $CaCO_3$ with reciprocal shaking (150 rpm $min^{-1}$) for 14 days at 26 °C.

The polysaccharides were extracted from the culture broth filtrates (CBFs) of different *L. edodes* strains. The polysaccharide in each CBF was precipitated by the addition of 3 volumes of 95% ethanol and stored at 4 °C overnight. The precipitate was collected by centrifugation, washed with 75% ethanol, and then freeze-dried. The polysaccharides were boiled (100 °C), and samples (2 mg/mL) were filtered (0.22 $\mu$m; Millipore, Bedford, MA) before assay of bioactivity.

The analysis of monosaccharide composition, glycosyl linkage composition, and molecular weight for different polysaccharides has been previously described in detail.[5,6] All polysaccharide samples lacked carboxylic acids, and derivatives such as esters and amides were determined by nuclear magnetic resonance or Fourier transform infrared spectroscopy (data not shown).

**NBT Reduction Assay.** The production of superoxide ($O_2^-$) by differentiating monocytes (THP-1 cells, a human monocyte cell line) was measured using a modified nitroblue tetrazolium (NBT, Sigma) reduction assay. THP-1 cells were cultured in RPMI 1640 medium (Gibco) containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT), plated into 24-well microtiter plates ($3 \times 10^5$ per well), treated with polysaccharide or vehicle for 48 h at 37 °C, harvested, washed in phosphate-buffered saline (PBS), and then covered with 500 $\mu$L of 2 mg $mL^{-1}$ of NBT for 4 h at 37 °C. The reduced formazan within cells was solubilized in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany). Optical density was measured using an ELISA reader at 570 nm.[7,8] The percent NBT reduced was estimated using the following equation: (sample average/control average) × 100.

**MTT Assay.** Normal cell line (MRC-5) of human embryonal lung and human lung adenocarcinoma epithelial cell line (A549) with densities of 3000 cells per well were planted into wells of a 96-well microtiter plate and treated with 20 $\mu$L of L10 polysaccharide with the final concentration of 2 mg/mL or vehicle. The microtiter plate was incubated for 72 h at 37 °C. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was prepared in PBS at 5 mg/mL concentration. To each well was added 20 $\mu$L of MTT solution, and the plate was further incubated for 4 h at 37 °C. Thereafter, the supernatant of each well was aspirated carefully, and the formazan crystals formed were dissolved in DMSO (Merck, Darmstadt, Germany). Optical density was measured using an ELISA reader at 540 nm. The percentage of cell viability was calculated in comparison to the control.[5,6]

**TLR-4 Binding Activity Assay.** The THP-1 monocytes ($3 \times 10^5$ cells/mL) were plated into 6-well plates, treated with different polysaccharides from the 10 strains of *L. edodes* or LPS for 48 h, harvested, washed in PBS, fixed in formalin solution (4%) for 30 min, washed three times, and incubated with saturating amounts of anti-TLR4 goat

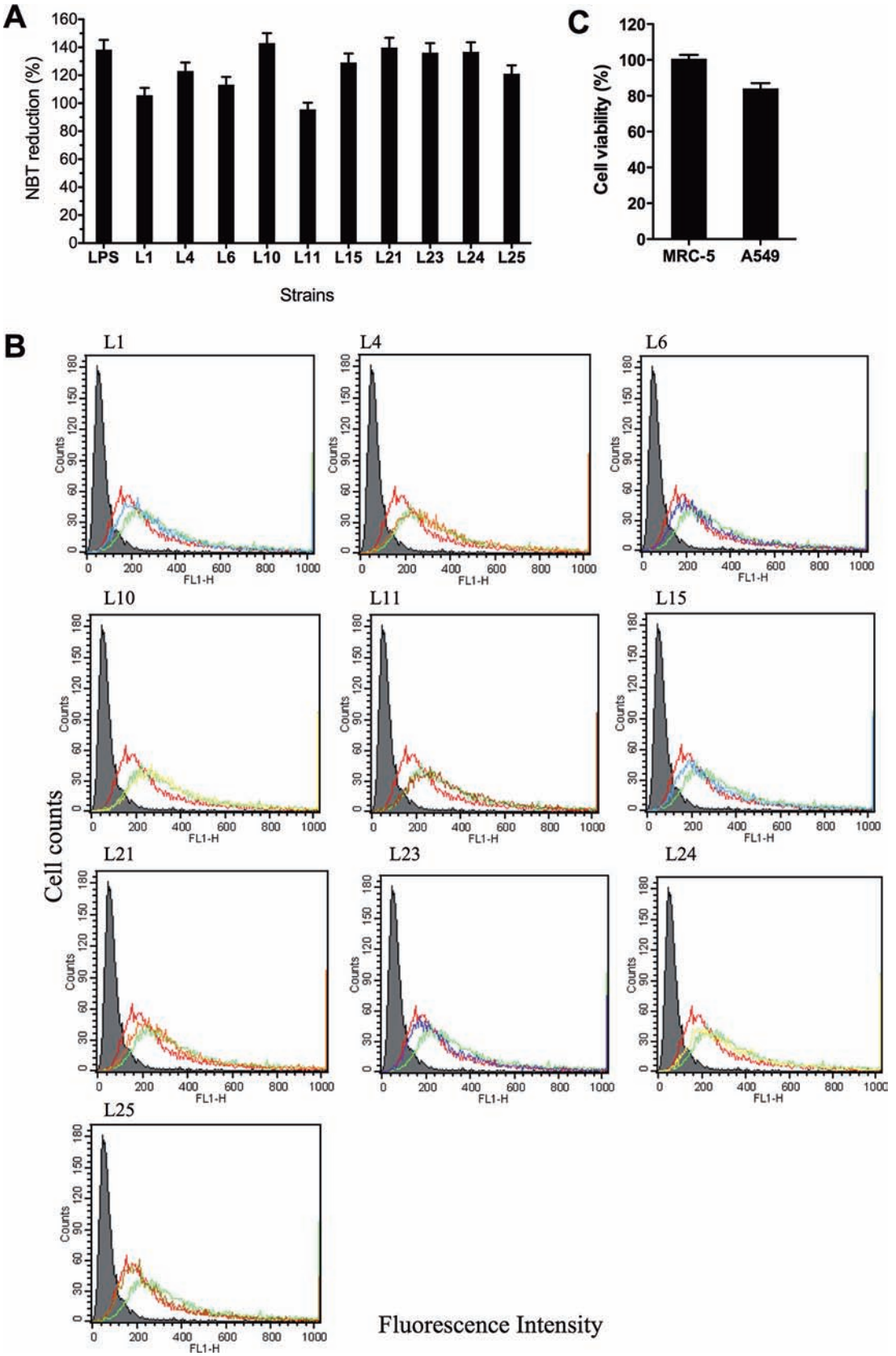


**Figure 1.** (A) THP-1 monocyte differentiation assay (% NBT reduction) using polysaccharides from 10 different strains of *L. edodes*. (B) TLR-4 binding abilities of 10 polysaccharides determined by flow cytometry analysis. The polysaccharide treatment on THP-1 cells was compared with untreated negative control (red) and with LPS positive control (yellow). (C) MTT cytotoxic assay (% viability) using the L10 polysaccharide (2 mg/mL).

polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h and then with Alexa Fluor 488-conjugated rabbit anti-goat secondary reagent for 30 min (Invitrogen, Carlsbad, CA). The fluorescence of the stained cells was compared with that of the unstained control.[9] Flow cytometric measurements were performed using a four-color Partec CyFlow Counter (Partec GmbH, Munster, Germany) and Modfit LT software.

**Western Blotting Analysis.** Briefly, L10-treated and vehicle-treated THP-1 cells ($1 \times 10^6$ cells) were washed with PBS twice. Cell pellets were solubilized in lysis buffer containing complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Lysates were centrifuged at 14000 rpm for 10 min at 4 °C. Protein concentration was determined with Bradford reagent (Bio-Rad, Hercules, CA), and samples with the same amount of total protein were analyzed on a 10% SDS-PAGE gel. The separated proteins were transferred to polyvinylidene difluoride membranes, which were blocked with 5% nonfat milk in Tris-buffered saline Tween 20 (TBST) containing 0.05% Tween 20 (Sigma) at room temperature, probed with primary antibodies overnight at 4 °C, and then incubated with secondary antibodies. The antibodies used in this study were anti-human TLR-4, CD14, MyD88, IKK, and NFκB (Santa Cruz Biotechnology). Membranes were washed and developed using an enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. β-Actin was used as a loading control.[10] Gel images were captured using a Biospectrum AC imaging system (Upland, CA), and band analysis was performed on Vision Works LS Acquisition and Analysis software (Upland, CA).

**Principal Component Analysis (PCA).** PCA was used to transform the number of potentially correlated variables (descriptors) into a smaller number of relatively independent variables that could be ranked on the basis of their contribution to the variation of the whole data set.[11,12] Thus, the original high-dimensional data could be mapped onto a lower dimensional space to substantially reduce the complexity of high-dimensional pattern classification.[13] In our present study, the data matrix consisting of normalized structure compositions (including 7 monosaccharide components, 5 glycosyl linkage, and 4 molecular weight fractions, Table 1) from 10 strains of *L. edodes* and pattern recognition based on PCA were performed using SPSS version 10.0.[11,12,14]

**Multiple Linear Regression Analysis (MLRA).** MLRA was conducted as previously reported with minor modifications.[15] In the multiple regression model, a generalized straight-line equation (eq 1) was used to describe the relationship:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \ldots + \beta_p X_p + e \quad (1)$$

$Y$ denotes the response variable (TLR-4 binding activity), $\beta_0$ is a constant (intercept), $X_1$, $X_2$, ..., $X_p$ are vectors of $p$ predictor variables (structure composition values), and $\beta_1$, $\beta_2$, ..., $\beta_p$ are vectors of $p$ regression coefficients. Each predictor variable has its own coefficient, and the outcome variable is predicted from a combination of all the variables multiplied by their respective coefficients plus an error term. The relationship was determined using the least-squares fitting technique. A residual analysis was used to detect model violations.[14] MLRA was performed using SPSS and S-PLUS. A $p$ value of <0.05 was considered to be statistically significant.

**Colony-Formation Assay.** The THP-1 cells were induced to differentiate by treatment with L10 polysaccharide at 37 °C for 24 h. The cells were collected, centrifuged (1500 rpm for 5 min), and resuspended in RPMI 1640 medium with 10% FBS. A human lung adenocarcinoma cell line (A549; $1 \times 10^3$ cells/well) was cultured in 6-well plates, incubated in DMEM with 10% FBS overnight at 37 °C, and irradiated (3 Gy). Differentiated THP-1 cells were added to each well at a ratio of 1:8 (A549/THP-1), with L10 polysaccharide or vehicle. After 7 days, cells were washed with PBS to remove the THP-1 and dead A549 cells and stained with crystal violet/MeOH. The number of colonies with >50 cells was counted.[10]

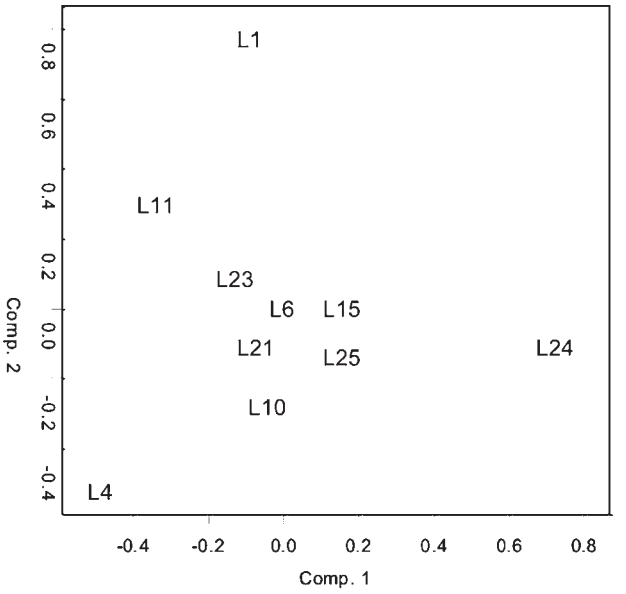


**Figure 2.** Principal component analysis for the structural data: relationship among different strains: plots of components (Comp.) 1 and 2.

**ELISA.** ELISAs for human TNF-α, interleukin (IL)-8, regulated upon activation, normal T-cell expressed, and secreted (RANTES), angiogenin, and vascular endothelial growth factor (VEGF) were performed using the Quantikine Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.[16] The culture supernatants were collected by centrifugation. Color intensity was measured using a DTX 880 Multimode microplate reader (Beckman Coulter, Fullerton, CA), and the data were analyzed using multimode analysis software.

**Statistical Analysis.** Student's $t$ test was used for statistical analyses of continuous variables, and a two-sided $p$ value of <0.05 was considered to be significant.

## RESULTS

**Bioactivities and Cytotoxicities of Polysaccharides from Different Strains of *L. edodes*.** Recognition of polysaccharides by TLR-4 and/or TLR-2 is thought to mediate monocyte differentiation and macrophage activation.[17,18] Activation of these receptors triggers intracellular signaling cascades resulting in transcriptional activation and the production of pro-inflammatory cytokines.

In this study, the effects of our polysaccharides on THP-1 monocyte differentiation, TLR-4 binding activity, and NFκB expression were investigated. Of all the tested polysaccharides, Jongxing 8 (L10) polysaccharide (2 mg/mL) was the most potent stimulator of THP-1 differentiation (NBT assay; Table 1 and Figure 1A) and had the greatest binding ability to TLR-4 (flow cytometry; Table 1 and Figure 1B). However, Japanese 271 (L11) and Hey-King-Gang (L21) polysaccharides (2 mg/mL) were the most potent stimulators of NFκB expression (data not shown).

The cytotoxic effect of L10 polysaccharide on normal lung cell line (MRC-5) and lung adenocarcinoma cell line (A549) with 2 mg/mL was evaluated by MTT assay. The average cell viabilities after 72 h of treatment with L10 for MRC-5 and A549 were 101 and 86%, respectively.

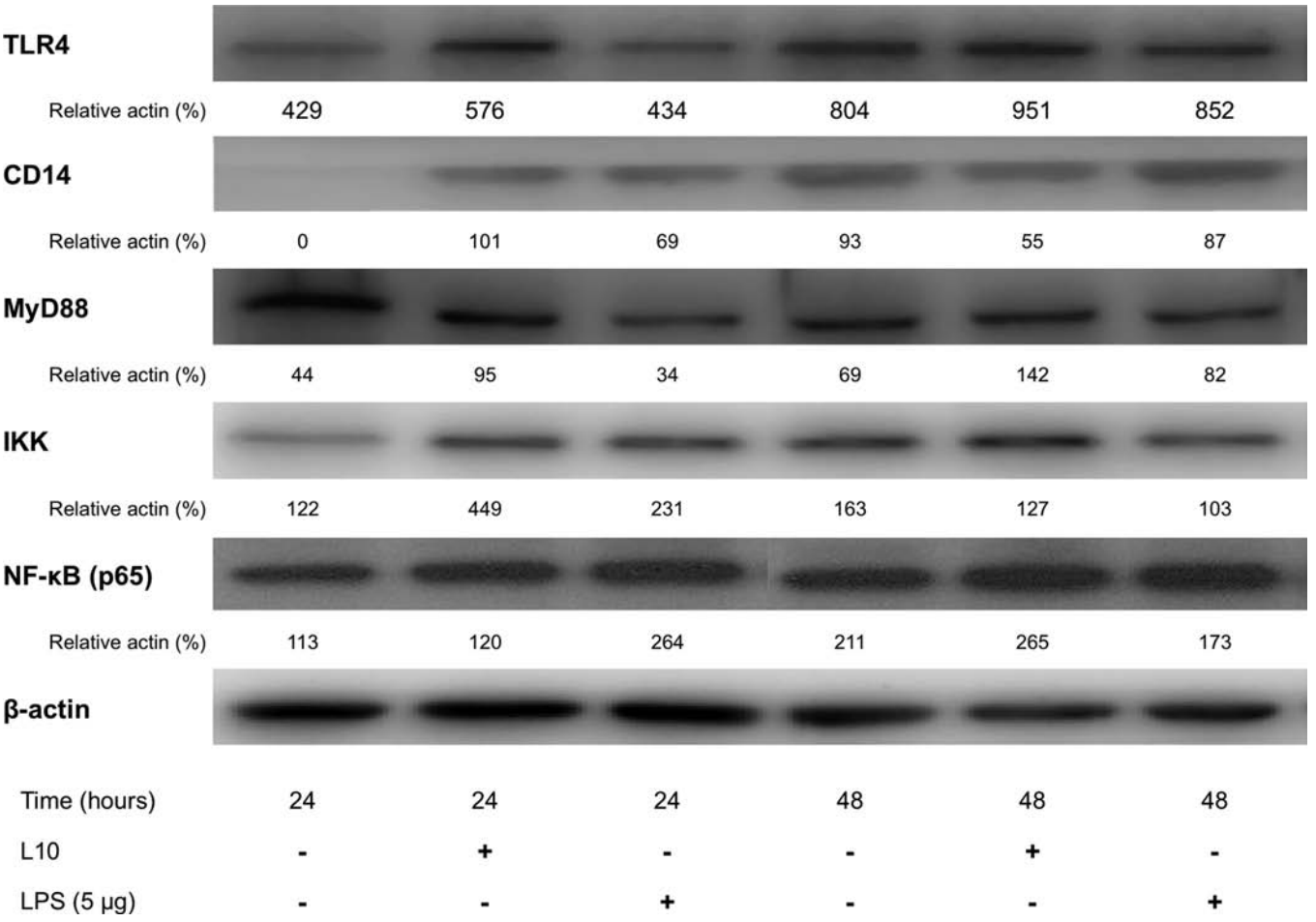


**Figure 3.** Western blotting analysis of TLR-4, CD 14, MyD88, IKK, and NF$\kappa$B in THP-1 cells treated with L10 or LPS-conditioned medium. The number below each lane represents the relative percentage of protein levels as compared to their $\beta$-actin as the loading control.

**Principal Component Analysis.** PCA was used to identify those components of high-dimensional data that were amenable to investigation by MLR analysis in the next step. The dominant factors in the first principal component (Comp. 1) were arabinose, xylose, mannose, glucose, galactose, and mannose 1→2 linkage (eigenvalue = 7.24), accounting for 45% of all polysaccharide structural properties; those in the second principal component (Comp. 2) were three-dimensional structures (eigenvalue = 3.02) such as molecular weight A (MWA, mw > 2750 kDa), molecular weight B (MWB, mw ~ 2700 kDa), and molecular weight C (MWC, mw ~ 534 kDa), accounting for 19% of polysaccharide structural properties, and that in the third (Comp. 3) was the glucose 1→4 linkage (eigenvalue = 1.65), accounting for 10% of polysaccharide structural properties. The PCA two-dimensional plot (i.e., Comp. 1 vs Comp. 2; Figure 2) revealed the separation of L1, L4, L11, and L24 from the other polysaccharides and the close proximity of L6, L10, L15, L21, and L23 (from Japan) to one another.

**Multiple Linear Regression Analysis.** The MLRA method predicted TLR-4 binding activity from the PCA method indicated the major characteristic components of polysaccharides. In multiple regression analysis with backward elimination, the $p$ values for linear regression were (a) 0.013 for arabinose, (b) 0.006 for glucose, (c) 0.003 for glucose 1→4 linkage, (d) 0.007 for mannose 1→2 linkage, (e) 0.005 for MWA, (f) 0.001 for MWB, and (g) 0.002 for MWC. For all other monosaccharides, the $p$ values were >0.1. The coefficient of determination ($R^2$), which measures the model fit, was 0.999. To predict the relationship between monosaccharide ratio and TLR-4 binding activity, eq 2 was obtained:

$$Y\ (\text{TLR-4 binding activity}) = -0.257 + 3.403X_{\text{Ara}} - 0.108X_{\text{Glc}} + 0.323X_{\text{Glc-4}} - 0.389X_{\text{MWA}} + 1.723X_{\text{MWB}} + 0.371X_{\text{MWC}} \quad (2)$$

Here, $X$ is defined as the concentration of monosaccharide measured in the extract. The positive coefficients for $X_{\text{Ara}}$, $X_{\text{Glc1-4}}$, $X_{\text{MWB}}$, and $X_{\text{MWC}}$ indicated that TLR-4 binding activity increases with increasing $X_{\text{Ara}}$. The negative coefficients for $X_{\text{Glc}}$ and $X_{\text{MWA}}$ indicated that TLR-4 binding activity decreases with the corresponding increase in $X_{\text{Glc}}$ and $X_{\text{MWA}}$.

**L10 Polysaccharide Induces Macrophage-like Differentiation and/or Activation of THP-1 Cells via the TLR-4 Signaling Pathway and NF$\kappa$B Activation.** The effects of L10 polysaccharide on aspects of monocyte−macrophage differentiation including TLR-4 expression, CD14 expression, cell differentiation, MyD88/IKK/NF$\kappa$B signaling cascade, and secretion of cytokines were investigated. THP-1 cells were treated with 2 mg/mL L10 or vehicle for different durations. Western blot analysis (Figure 3) showed that TLR-4 levels after 24 or 48 h of treatment

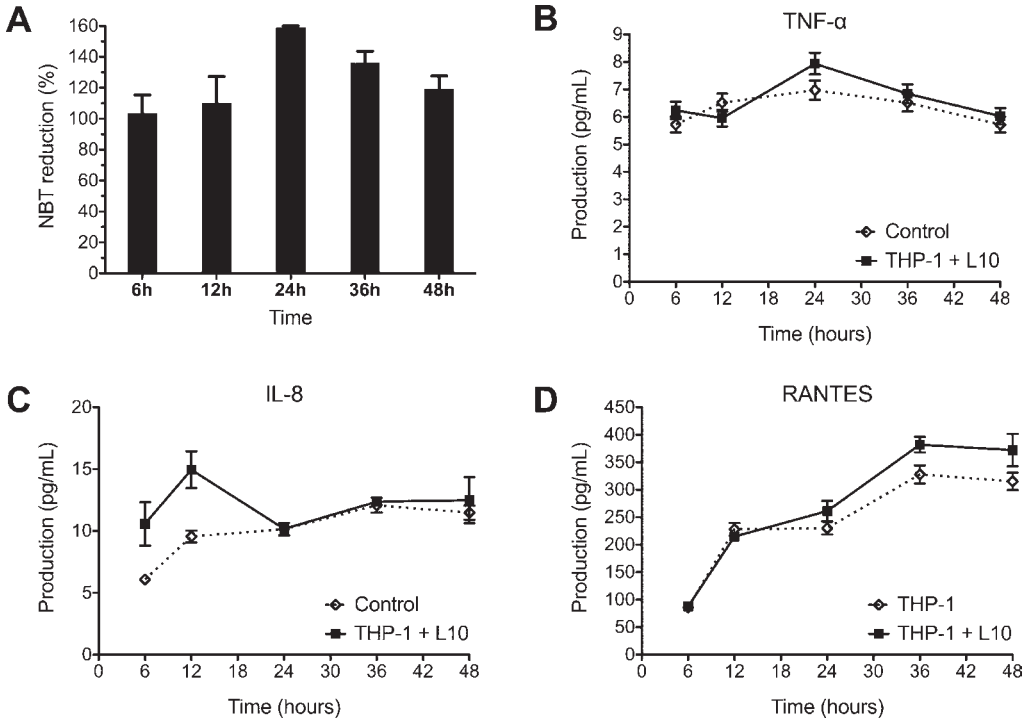


**Figure 4.** Potential of L10-stimulated THP-1 monocytes to differentiate into macrophages. THP-1 cells were treated with or without L10 for up to 48 h. (A) L10 treatment was able to increase NBT reduction significantly. L10 synergistically induced expressions of (B) TNF-$\alpha$, (C) IL-8, and (D) RANTES in THP-1 cells, as assessed by ELISA.

with L10 were higher than control levels. The expression of TLR-4 in THP-1 cells (as a percentage of $\beta$-actin expression) was 429% for the control at 24 h, 576% for cells treated with L10 at 24 h, 434% for cells treated with LPS at 24 h, 804% for the control at 48 h, 951% for cells treated with L10 at 48 h, and 852% for cells treated with LPS at 48 h. Treatment with polysaccharides has been reported to up-regulate expression of various surface markers on monocytes (e.g., CD11b, CD14, and CD68[19]). CD14 is a high-affinity receptor for microbial lipopolysaccharide and polysaccharides. We found that CD14 expression in L10-activated THP-1 cells was markedly elevated at 24 h.

The downstream signals of the TLR family are mediated by at least four adapter proteins: MyD88, MyD88 adapter-like/Toll interleukin (IL)-1 receptor-associated protein, TLR-associated activator of interferon, and TLR-associated molecule.[20] We found that MyD88 protein expressions (relative to $\beta$-actin expression) were 95 and 142% after stimulation with L10 for 24 and 48 h, respectively, and persistently activated after stimulation for 48 h (Figure 3).

After initiation of TLR-4/CD14/MyD88 upstream signaling, cells were evaluated to determine whether downstream TLR-4 signaling involved induction of IKK and NF$\kappa$B. Although NF$\kappa$B is activated via numerous signaling pathways, the IKK complex is activated via receptor-specific pathways. The L10-stimulated IKK expression (Figure 3) was highest (449%; 3.6-fold higher than control) after treatment for 24 h. Thus, L10 up-regulates NF$\kappa$B expression (Figure 3).

**L10 Induces the Production of Superoxide and Various Cytokines and Thereby the Differentiation/Activation of THP-1 Cells.** The polysaccharides have been shown to increase the cytotoxic activity of macrophages against tumor cells and microorganisms, to activate phagocytic activity, to increase reactive oxygen species and nitric oxide production, and to enhance secretion of cytokines and chemokines, such as TNF-$\alpha$, IL-1$\beta$, IL-6, IL-8, IL-12, interferon (IFN)-$\gamma$, and IFN-$\beta$2.[1] Moreover, L10 treatment was found to stimulate NF$\kappa$B secretion and thereby time-dependently promote secretion of pro-inflammatory cytokines, such as TNF-$\alpha$ (increasing from 7.2 pg/mL at 20 h to 7.9 pg/mL at 24 h; Figure 4B), IL-8 (increasing from 10.58 pg/mL at 6 h to 14.95 pg/mL at 12 h; Figure 4C), and RANTES (increasing from 250 pg/mL at 20 h to 381.94 pg/mL at 36 h; Figure 4D) in THP-1 monocyte culture supernatants. The increase in RANTES production was greater than the increases in TNF-$\alpha$ and IL-8 production. These results suggest that the L10-induced NF$\kappa$B expression seen in THP-1 cells was correlated with the induction of TNF-$\alpha$, IL-8, and RANTES expression.

**L10-Activated THP-1 Cells Enhance Radiosensitivity of Lung Adenocarcinoma (A549) Cells.** Figure 5A shows the percentage of A549 tumor cell colonies formed after RT (3 Gy) and then cocultured with L10-stimulated THP-1 cells for 7 days at 37 °C. The addition of L10-activated THP-1 cells significantly decreased the viability of irradiated A549 cells from 66 to 37% ($p$ = 0.018), increased the level of superoxides in the coculture supernatant (136.59% NBT reduction, Figure 5B), increased the IL-8 level (from 4536 to 6761 pg/mL; Figure 5C) and the RANTES level (Figure 5D), and decreased the levels of metastasis-related cytokines (angiogenin, Figure 5E; and VEGF, Figure 5F).

These results indicate that continuously stimulated monocytes can reduce tumor progression (i.e., as indicated by reduced angiogenin and VEGF levels) after RT. Thus, RT plus L10-activated THP-1 may enhance the antitumor immune response.

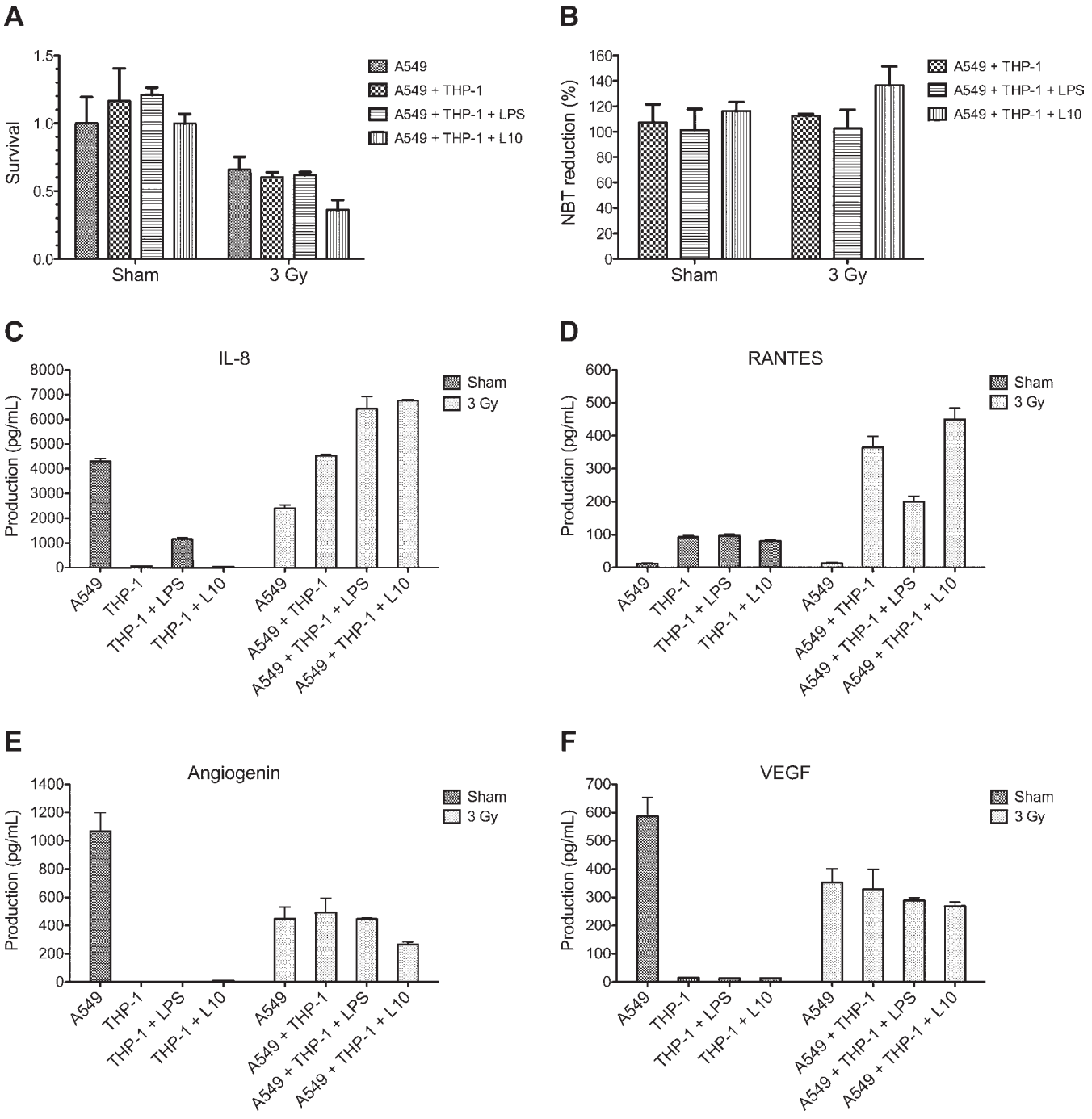


**Figure 5.** (A) The combination of radiation and L10-activated THP-1 decreased the survival of A549 cells by colony formation assay. The radiation group (3 Gy) was compared with a sham irradiated group. The A549 cells from different treatment groups were assessed for the secretion of (B) superoxide, (C) IL-8, (D) RANTES, (E) angiogenin, and (F) VEGF in THP-1 cells.

## DISCUSSION

Several studies have suggested that the biological activities of polysaccharides are determined by higher order structural features, molecular weight, backbone linkage, degree of branching, side-chain units, and monosaccharide composition.[21−23] Polysaccharides from botanical sources and microbes bind to similar surface receptors and induce similar immunomodulatory responses in macrophages, implying that diverse groups of organisms have pattern recognition receptors sensitive to evolutionarily conserved polysaccharide structural features.[1] Little is known about the polysaccharide structures that bind to these receptors or trigger various bioactivities. Thus, it is currently not possible, in most cases, to determine how specific polysaccharide structural features stimulate the immune functions of macrophages.[1] The present study showed, by two different statistical methods, that polysaccharide structural characteristics were both directly and indirectly related to receptor binding ability. Principal component analysis identified arabinose, xylose, mannose, galactose, and glucose as the main primary

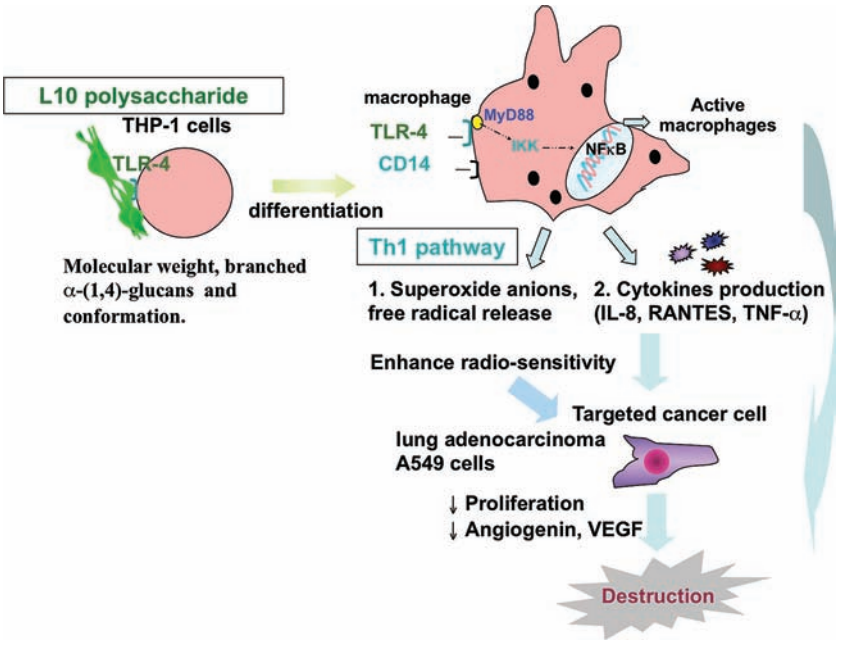


**Figure 6.** Schematic illustration of how L10 synergistically activates NFκB and the induction of IL-8, RANTES, and TNF-α in THP-1 cells, as well as the proposed model for the mechanism of combined therapy of radiation and L10-activated THP-1 cells to enhance the antitumor immune response.

structural characteristics, glucose 1→4 linkage and mannose 1→2 linkage as the main secondary structural characteristics, and molecular weight up to 500 kDa as the main tertiary structural characteristic (MWA, MWB, and MWC) of polysaccharides affecting their binding to receptors. Multiple linear regression analysis showed that levels of arabinose, glucose 1→4 linkage, MWB (~2700 kDa), and MWC (534 kDa) were positively correlated with TLR-4 binding activity (eq 2). The positive contributions of each factor in decreasing order of importance were arabinose (3.4) > MWB (1.72) > MWC (0.37) > glucose 1→4 linkage (0.32). Of all polysaccharides tested, L10 had the greatest TLR-4 binding activity and the following structural features: 7.77% arabinose, 0.8 ratio of MWB, absence of MWC, and 5.7 ratio of glucose 1→4 linkage.[5]

Polysaccharides can activate monocytes, macrophages, polymorphonuclear leukocytes, and natural killer cells. Polysaccharide-induced monocyte differentiation or macrophage activation is generally considered as the first step in the immune response. Glucan receptors help bind polysaccharides to monocytes or macrophages. Ando et al. demonstrated that safflower polysaccharides activate NFκB through TLR-4 and induce expression of TNF-α and NO by macrophages.[24] Saito et al. reported that soluble branched β-(1,4) glucans from *Acetobacter* readily induce IL-12 via the TLR-4 signaling pathway.[25] The functions of the main polysaccharide fraction of the Reishi mushroom (Reishi-F3) (i.e., induction of macrophage-like differentiation in THP-1 cells, activation of protein tyrosine kinase to induce IL-1 expression in macrophage, and promotion of Ig secretion) seem dependent on TLR-4 and TLR-2 signaling in B lymphocytes.[17,19,26,27] The effects of these botanical polysaccharides on classically activated macrophages (i.e., enhancement of reactive oxygen species, nitrogen oxide, TNF-α, IL-1β, IL-6, IL-8, and IFN-γ secretion to promote the type 1 T-helper cellular immune response) are well described in the literature. Our previous results showed that the polysaccharides from 10 different strains of *L. edodes* can activate the mouse macrophage cell line (RAW 264.7),[5] and the present study extends these findings to show that *L. edodes* polysaccharides also induce monocyte differentiation and enhance superoxide and cytokine production via the TLR-4/MyD88/IKK/NFκB pathway. Our results provide a molecular explanation for the effect of branched α-(1→4)-glucan (L10) on differentiation/activation of THP-1 cells and demonstrate that such cells when combined with RT enhance the cytotoxic effect of RT on the human lung adenocarcinoma cell line (A549).

Worldwide, lung cancer is a major cause of cancer death, accounting for 1.3 million deaths per year—more deaths than those from stomach, colorectal, liver, and breast cancer. Lung cancer is treated by surgery, chemotherapy, RT, and immunotherapy.[28] However, none of these therapies is highly effective, and new innovative therapies are still needed.[28] Recently, Roses et al. reported a novel immunotherapy capable of improving conventional treatment. Radiation may complement immunotherapies because they enhance tumor antigenicity and promote stromal targeting. Effects linked to RT (up-regulation of death receptors on tumor cells and TLR signaling on immune cells) have been demonstrated.[3,4,29,30] Moreover, the potential of immunotherapy combined with chemotherapy has been investigated preclinically.[3,31] RT is emerging as a more powerful therapeutic modality when combined with immunotherapy.[3] In this study, combining RT with L10-stimulated monocytes decreased the viability of A549 lung cancer cells by stimulating inflammatory and cytotoxic responses (Figure 6). In vivo studies are warranted to elucidate the effects of radiation plus L10 treatment on the mechanisms of antitumor immunity.

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## ■ ABBREVIATIONS USED

CBF, culture broth filtrate; CD, cluster of designation; ELISA, enzyme-linked immunosorbent assay; IFN, interferon; IKK, IκB kinase; IL, interleukin; MLRA, multiple linear regression analysis; MTT, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; MW, molecular weight; MyD88, myeloid differentiation primary response gene (88); NBT, nitroblue tetrazolium; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; PNS, phosphate-buffered saline; PCA, principal component analysis; RANTES, regulated upon activation, normal T-cell expressed, and secreted; RT, radiotherapy; TLR, Toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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